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A Glial Cell Line-Derived Neurotrophic Factor-Secreting Clone of the Schwann Cell Line SCTM41 Enhances Survival and Fiber Outgrowth from Embryonic Nigral Neurons Grafted to the Striatum and to the Lesioned Substantia Nigra

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We have developed a novel Schwann cell line, SCTM41, derived from postnatal sciatic nerve cultures and have stably transfected a clone with a rat glial cell line-derived neurotrophic factor (GDNF) construct. Coculture with this GDNF-secreting clone enhances *in vitro* survival and fiber growth of embryonic dopaminergic neurons. In the rat unilateral 6-OHDA lesion model of Parkinson's disease, we have therefore made cografts of these cells with embryonic day 14 ventral mesencephalic grafts and assayed for effects on dopaminergic cell survival and process outgrowth. We show that cografts of GDNF-secreting Schwann cell lines improve the survival of intrastriatal embryonic dopaminergic neuronal grafts and improve neurite outgrowth into the host neuropil but have no additional effect on amphetamine-induced rotation. We next looked to see whether bridge grafts of GDNF-secreting SCTM41 cells would promote the growth of axons to their striatal targets from dopaminergic neurons implanted orthotopically into the 6-OHDA-lesioned substantia nigra. We show that such bridge grafts increase the survival of implanted embryonic dopaminergic neurons and promote the growth of axons through the grafts to the striatum.

Key words: bridge graft; GDNF; Parkinson's disease; 6-OHDA; Schwann cells; SCTM41; substantia nigra; neuronal graft

The anatomical and behavioral repair mediated by intrastriatal embryonic nigral grafts in animal models of Parkinson's disease (Björklund and Stenevi 1979; Perlow et al., 1979; Dunnett, 1991; Freed et al., 1991; Annett et al., 1994), particularly the 6-hydroxydopamine (6-OHDA) model (Ungerstedt, 1968), has paved the way for human grafting trials. Although clinical benefits have been shown with nigral grafts implanted ectopically within the parkinsonian striatum (Lindvall et al., 1990; Kordower et al., 1995; Leroy et al., 1996; Olanow et al., 1996, Wenning et al., 1997), they do not provide a complete cure (Lindvall, 1997). There are at present two main limitations to striatally placed grafts. Survival of dopaminergic neurons after standard implantation procedures is only between 5 and 10% (Björklund, 1991), and only some of the symptoms of parkinsonism are alleviated, probably because with ectopic placement of dopaminergic tissue, the whole nigrostriatal circuitry cannot be restored (Dunnett et al., 1987).

Homotopic placement of grafted nigral tissue should, in principle, be much more effective, but only if connections between a graft in the ventral mesencephalon (VM) and distant striatal targets can be established. Homotopically placed nigral grafts do not grow their axons to the striatum through the inhibitory milieu of the adult brain (Björklund et al., 1983) but nevertheless have some restoratory effect because of connections made within the substantia nigra (Nikkhah et al., 1995). However, if provided with permissive substrates connecting them with their striatal target, nigral grafts can also make connections to the striatum. Such "bridges" have included peripheral nerve (Aguayo et al., 1984), embryonic striatal tissue (Dunnett et al., 1989), a Schwann cell line (Brecknell et al., 1996a,b), excitotoxic lesions within the neuropil (Zhou et al., 1996), and glial cell line-derived neurotrophic factor (GDNF) (Wang et al., 1996). The fiber growth achieved by these strategies is sparse, and intracerebral bridge grafts have only achieved a maximum of a few hundred ingrowing fibers. Even this small number of axons has achieved partial restoration of function (Dunnett et al., 1989; Brecknell et al., 1996a,b). The search for an optimal bridge tissue therefore continues.

An ideal bridge graft material should be able to improve the survival of grafted dopaminergic neurons, as well as promote growth of their axons. The most potent survival factors are members of the transforming growth factor-β family of molecules, particularly GDNF and neurturin. They have been shown to support the survival of embryonic nigral neurons *in vitro* (Lin et al., 1993), *in oculo* (Johansson et al., 1995), and intrastriatally (Rosenblad et al., 1996; Sinclair et al., 1996), and also to promote

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axon growth from dopaminergic neurons (Rosenblad et al., 1996; Sinclair et al., 1996; Wang et al., 1996). Physiologically, GDNF is expressed by the basal ganglia during development (Nosrat et al., 1996), whereas developing dopaminergic neurons express the GDNF receptor (GDNFRa-1) and the necessary coreceptor Ret (Nosrat et al., 1997).

Schwann cells provide the substrate for peripheral nerve regeneration and can also mediate regeneration of CNS axons, including those of the nigrostriatal tract (David and Aguayo, 1981; Gage et al., 1985; Kromer and Cornbrooks, 1985; Xu et al., 1995). This can be attributed to their expression of axonotrophic molecules within their membranes (Ard et al., 1987; Bixby et al., 1988), extracellular matrix (Ide et al., 1983; Bunge et al., 1989; Martini, 1994), and their production of diffusible tropic factors (Politis et al., 1982). Moreover, a variety of trophic molecules are known to be produced by Schwann cells, including in some circumstances GDNF (Lindholm et al., 1987; Meyer et al., 1992; Rufer et al., 1994; Lee et al., 1995; Hammarberg et al., 1996). In this study, we have combined the neurotrophic and neurotropic effects of GDNF and Schwann cells to produce bridge grafts and cografts that increase the survival of dopaminergic grafts and promote nigrostriatal connectivity.

MATERIALS AND METHODS

Generation of Schwann cell lines

The cell line SCTM41 was derived from neonatal rat sciatic nerve cultures purified from Schwann cells. All cells were cultured in DMEM (Life Technologies, Paisley, Scotland) supplemented with penicillin–streptomycin (100 U/ml; Life Technologies) and 10% fetal calf serum (FCS). Nerves were obtained from postnatal day 2 (P2) sciatic nerve from CD rats (Charles River Laboratories, Margate, UK), placed in L-15 medium, and cleared of blood vessels, musculature, and their epineurial sheaths. Thereafter, they were placed into a 35-mm-diameter plastic dish containing trypsin (0.1%; Sigma, St. Louis, MO) and collagenase (0.03%; Sigma), cut very finely using dissection scissors, and placed in an incubator at 37°C and 10% CO_2 for 30 min. After this incubation, an equal volume of triturating solution [300 mg of BSA, 1 mg of DNase, 50 mg of trypsin inhibitor, and 100 ml of HBSS (all per ml of HBSS) (all from Sigma)] was added, and the whole mixture was gently triturated using a flame-polished glass Pasteur pipette. Having spun down the cells into a pellet by centrifugation at 1000 rpm for 3–5 min, the cells were then resuspended in DMEM with 10% FCS and plated on poly-L-lysine (0.01%; Sigma) at a density of 5000 cells/mm^2. On the following day, cells were treated with cytosine arabinoside (Ara-C) (1×10^{-5} M; Sigma) for 3 d. After 2 d in normal untreated FCS-containing medium, the Ara-C was again applied for an additional 3 d. The few remaining fibroblast contaminants were then killed via complement-mediated lysis using rabbit serum (a gift from R. Oldroyd, Cambridge University, Cambridge, UK) and the IgM class anti-Thy1.1 (1:1000; Serotec, Oxford, UK). Subsequently, the Schwann cells (>98% pure) were maintained in FCS supplemented with bovine pituitary extract (10 mg/ml; Sigma) and forskolin (2 mM; Sigma).

The SCTM41 cell line is based on the constitutive expression of a synthetic ligand-gated proto-oncogene, in this instance, tamoxifen-regulated human c-Myc. The retrovirus pBpuro MycMERG525R, which carries both a puromycin-resistance gene and the DNA encoding the c-myc/G525R (a mutant murine oestrogen receptor) (Danielian et al., 1993) fusion protein (Littlewood et al., 1995), was used for the transformation process. Supernatant from producer cell lines (a generous gift from Trevor Littlewood, Imperial Cancer Research Fund Laboratories, London, UK) was collected every 2 d, passed through a 0.45 mm Millipore (Bedford, MA) filter, and used as the source of retrovirus.

Confluent cultures of purified Schwann cells (see above) were incubated with retrovirus-containing supernatant in the presence of 8 μg/ml Polybrene. After 4 hr, the supernatant was removed and replaced with DMEM and 10% FCS and maintained at 37°C. After 3 d, 5 mg/ml puromycin and 100 nM 4-hydroxytamoxifen (Semat) was included in the culture medium (CM) to select for virus-infected cells. After an additional 10 d, puromycin-resistant cells were replated in a 96-well plate at a density of 0.5 cells per well (50 ml volume) and maintained in the tamoxifen-containing medium. Ten days later, colonies were observed in a proportion of the wells. One of these, SCTM41, was selected on the basis of morphology, expression of Schwann cell markers, and promotion of axon growth (see below).

Transfection with the rat GDNF construct

The relevant portion of GDNF cDNA, including the signal sequence, was cut out of Bluescript vector (Stratagene, La Jolla, CA) with the restriction enzymes *Xba*I and *Kpn*I and inserted into the equivalent site of the polylinker in the mammalian expression vector pcDNA 3.1 (Invitrogen, San Diego, CA). In this vector, transcription of the inserted gene is under the control of the cytomegalovirus promoter, which has been shown to drive high levels of gene expression in a wide variety of cell types (Pasleau et al., 1985).

The transfection procedure was as follows. One 75 ml flask containing SCTM41 cells at ~50% confluence was incubated with 15 μg of plasmid mixed with lipofectamine in 5 ml of Optimem, a serum-free CM (Life Technologies). After 5 hr, incubation of an equal volume of medium containing 20% FCS was added, and the cells were incubated overnight. The following day, the medium was removed and replaced with "normal" SCTM41 medium (DMEM with 10% FCS, 100 nM tamoxifen, and 0.4% penicillin–streptomycin). After an additional 72 hr, the cells were selected with G418 at a concentration of 800 μg/ml.

After two weeks of selection, the G418 was removed. Clones were then generated by plating cells into 96-well plates at densities of one, two, or four cells per well. Individual clones were then expanded and analyzed for GDNF secretion using an ELISA assay. The analysis was conducted by collecting medium from 25 ml flasks while the cells were dividing unhindered by confluence.

Schwann cell-specific marker expression by SCTM41

The novel SCTM41 cell line was screened for the Schwann cell markers low-affinity nerve growth factor receptor p75 (LNGFR), the calcium binding protein S100, laminin, and growth associated protein 43 (GAP-43). The nonSchwann cell-specific marker Thy1.1 (a characteristic surface protein of fibroblasts) was also tested. Protein expression was assessed using indirect immunofluorescent labeling. SCTM41 cells were plated onto poly-L-lysine-coated sterile 13 mm coverslips at a density of 10^5 cells per coverslip. The antibodies to S100 (Dako, Glostrup, Denmark), LNGFR (Boehringer Mannheim, Mannheim, Germany), and GAP-43 (a generous gift from G. Wilkin, Department of Biochemistry, Imperial College, London, UK) were all used at a dilution of 1:2000, whereas the anti-mouse Thy1.1 was used at 1:1000. The tissue was fixed in 4% paraformaldehyde for 30 min, blocked with either PBS–Triton X-100 (0.2%) and 5% NGS (in the case of GAP-43 and S100) or the same solution lacking Triton X-100 (LNGFR and Thy1.1), and then incubated for 45 min (at room temperature) with the primary antibody (in a 1% NGS solution). Secondary antibodies were either anti-mouse or anti-rabbit biotinylated conjugates (1:200 in 1% NGS; Boehringer Mannheim). Streptavidin conjugated rhodamine tertiaries allowed visualization (1:200 in 1% NGS; Boehringer Mannheim).

Preparation of embryonic ventral mesencephalic cultures

Ten to fourteen embryos of a single litter [embryonic day 14 (E14); crown-rump length, 11 mm) were removed by hysterotomy and kept in HBSS (Life Technologies) while the VM was dissected free. After removal of the mesencephalic flexure, tissue pieces were incubated for 10 min in 0.01% trypsin (Worthington, Freehold, NJ), followed by trituration in triturating solution [(in mg/ml HBSS): 10 bovine serum albumen, 0.5 trypsin inhibitor, and 0.01 DNase (all from Sigma)] and finally in CM [10% FCS (Sera-Lab, Sussex, UK), 1% penicillin, streptomycin, and fungizone in DMEM (all from Life Technologies)]. Tissue was then triturated with ~12 strokes of a flame-polished Pasteur pipette in 1 ml of fresh CM.

Cocultures of GDNF-secreting Schwann cells with embryonic VM

SCTM41 or SCTM41 GDNF cells were plated onto poly-L-lysine-coated sterile 13 mm coverslips at a density of 10^5 cells per coverslip and were allowed to grow to confluence in normal tamoxifen-containing medium (see above). Embryonic VM was plated out at a density of 10^5 cells per coverslip on either monolayers of SCTM41 ($n = 11$) or SCTM41 GDNF ($n = 11$). Poly-L-lysine-coated coverslips ($n = 12$) served as control. From the moment of plating VM onto the substrates, tamoxifen-free CM was

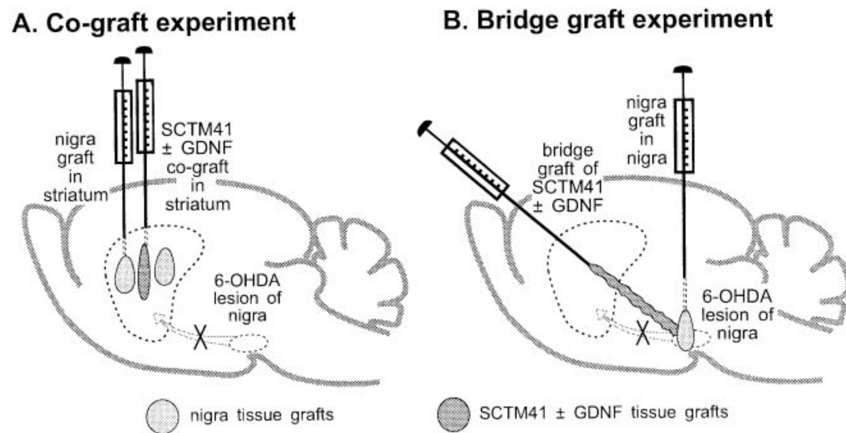


Figure 1. The types of transplant performed in these experiments. *A*, In the cograft experiment, the nigrostriatal pathway was lesioned with 6-OHDA, and then after checking for the completeness of the lesions using amphetamine-induced rotation, grafts of SCTM cells and dissociated nigra were made to the striatum. *B*, In the bridge graft experiment, the nigra was lesioned with 6-OHDA, the animals were rotated, a bridge graft of SCTM cells was inserted between nigra and striatum, and then 2 d later a nigral graft was placed in the nigra.

used. After 24 hr, coverslips were fixed in 4% paraformaldehyde and assessed for tyrosine hydroxylase (TH) expression. Nonspecific staining was blocked with 5% NGS, and then the cells were labeled with primary antibodies to TH (1:400; Boehringer Mannheim) overnight, followed by biotinylated goat anti-mouse Ig (1:200; Boehringer Mannheim) for 1 hr and sulforhodamine 101-labeled streptavidin (1:200; Boehringer Mannheim) for 1 hr (all antibodies in PBS, pH 7.4, 1% NGS, and 0.2% Triton X-100, followed by three washes in PBS). Stained coverslips were mounted onto glass slides with glycerol/PBS (1:1).

The number of TH-positive cells was determined under a fluorescent microscope by counting the number of immunoreactive cells in two orthogonal 0.5-mm-wide strips across the coverslip. The maximum fiber lengths (distance from the cell body to tip of its longest process) of ~50 TH-positive neurons per substrate were measured using a calibrated graticule.

Cografts of embryonic nigra with a GDNF-secreting Schwann cell line within the dopamine-depleted striatum

Animals. Thirty six young adult female rats of the CD strain (Charles River Laboratories) were housed in groups of six under a natural light/dark cycle, with food and water available *ad libitum*. Graft tissues were obtained from embryos of the same outbred strain.

6-OHDA lesion surgery. All rats received unilateral nigrostriatal lesions with 6-OHDA under halothane–nitrous oxide anesthesia. A 5 mg/ml solution of 6-OHDA HBr (free base 3.3 mg/ml in 0.02% ascorbate saline) was infused at a rate of 1 ml/min over 4 min via a stereotaxically positioned 30 gauge stainless steel cannula at the following coordinates: anterior (A) from bregma, −4.4 mm; lateral (L) to the midline, 1.0 mm; and vertical (V) below dura, 7.7 mm; with the nosebar set 2.3 mm below the interaural line. The cannula was left *in situ* for diffusion for 1 min before withdrawal.

Rotation. The rats were assessed for amphetamine-induced rotation in a bank of automated rotometer bowls. Rats were given five 90 min tests, each after intraperitoneal injection of 5 mg/kg D-amphetamine sulfate, conducted 1 and 2 weeks after initial lesion surgery and at 2, 4, and 6 weeks after grafting. All rats included in the experiments achieved an initial screening criterion of more than seven turns per minute on at least one of the first two tests after the nigrostriatal lesion.

Grafting. The 36 animals with nigrostriatal lesions were divided into three groups matched on the basis of rotation scores on the first two tests. One group received intrastriatal grafts of SCTM41 cells (GDNF-transfected), one received nontransfected SCTM41 cells, and one received sham surgery and injection of vehicle. Each of these groups was further subdivided into two, such that of each set of 12, six also received nigral grafts; the others received sham surgery and the injection of vehicle only. There was one SCTM graft in each animal and two nigral grafts placed nearby (Fig. 1). Table 1 displays this design.

Schwann cell line graft surgery. Three weeks after the 6-OHDA lesion surgery, cell suspensions were made from cultures of both GDNF-secreting and -nonsecreting SCTM41 cells. The procedure was as follows. Culture flasks were washed in PBS containing 0.02% EDTA. Excess saline was removed, and PBS containing 0.1% trypsin (Sigma) was added. After 5 min incubation at 37°C, triturating solution [(in mg/ml HBSS): 300 BSA, 1 DNase, and 50 trypsin inhibitor (all from Sigma)] was added, and the whole mixture was gently triturated using a flame-polished glass Pasteur pipette. The cells were spun on a centrifuge and resuspended in grafting medium (0.002% DNase in L-15 medium; Life Technologies) at a final density of ~$7.0 \times 10^5/\mu l$, with a viability of >90%.

Table 1. Experimental design for nigral–Schwann cell cograft experiment

Group	Schwann cell graft	Nigral graft
1	SCTM41 GDNF	Yes
2	SCTM41 GDNF	Sham
3	SCTM41	Yes
4	SCTM41	Sham
5	Sham	Yes
6	Sham	Sham

Experimental animals were reanesthetized with halothane and nitrous oxide and placed in a stereotactic apparatus. An incision was made in the scalp, and a burr hole was made in the skull to expose dura mater. A small volume of either one of the cell suspensions or the buffer vehicle was drawn up into a 10 μl grafting syringe (Scientific Glass Engineering), and 2.5 μl of this was deposited via its 23 gauge needle over 2.5 min at stereotactic coordinates from bregma–dura: A, 0.9 mm; L, 2.9 mm; V, 4.5 mm. The needle was left in place for 2 min after the implantation, before removal and closure of the wound. Rats from the three groups underwent surgery in counter-balanced order. Animals were immunosuppressed from this point on with daily intraperitoneal injections of 2 mg of cyclosporin A (Sandoz, Basel, Switzerland) in 0.2 ml of normal saline solution to prevent graft rejection.

Nigral graft surgery. The following day, nigral cell suspensions were prepared according to the same procedure as described for tissue culture, with one VM being suspended in 5 μl. Cell viability and density counts of aliquots of the suspension in a cytometer, using the trypan blue dye exclusion method, indicated a cell density of ~6.8×10^5 cells/μl and a viability >95%.

Half of the rats (the "nigral graft" group; $n = 18$) received two 2.5 μl intrastriatal deposits of graft suspension by direct injection via the 23 gauge needle of the same 10 μl glass syringe (Scientific Glass Engineering) positioned at the following stereotaxic coordinates: A, 1.6 mm; L, 2.4 mm; V, 4.5 mm; and A, 0.2 mm; L, 3.4 mm; V, 4.5 mm. Each graft deposit was infused over 2.5 min, with an additional 2 min allowed for diffusion before retraction of the needle. The remaining rats (the "sham graft" group; $n = 18$) received identical injections of buffered glucose saline alone. All rats received three additional rotation tests 2, 4, and 6 weeks after graft surgery.

TH immunohistochemistry. After the last rotation test, all animals were perfused with 150 ml of PBS, followed by 250 ml of 10% formalin in PBS under terminal sodium pentobarbitone anesthesia (Euthatal; May and Baker). The brains were removed, fixed overnight in 10% formalin, and then transferred to 30% sucrose for cryoprotection. Coronal sections (50 mm) were cut from the anterior margin of the corpus callosum to the pons on a freezing sledge microtome, and free-floating sections were

collected in 0.1 M TRIS-buffered saline, pH 7.4. Every sixth section was stained immunohistochemically as described by Torres et al. (1993), using a primary antibody against TH (1:10,000; Institut Jacques Boy), followed by a streptavidin–biotin complex kit (Dako). Specific antibody binding was revealed by horseradish peroxidase acting on diaminobenzidine.

Cell counts and analysis of fiber outgrowth from the grafts. Dopamine neuronal survival in the grafts was analyzed by counting all TH-positive neurons in the one-in-six series through the grafts, corrected as described by Abercrombie (1946). Counts were the aggregate of both deposits. The extent of dopaminergic fiber outgrowth was estimated as follows. For each of the two grafts in each brain, the section nearest the midpoint of each graft was taken, and the footprint of the graft was divided vertically into four quartiles. With the assistance of a computer-driven image-analysis system (Cast-Grid; Olympus, Albertslund, Denmark), three "ladders" (width of 55 μm, rungs at 80 μm intervals) were aligned perpendicularly to the edge of the graft at the three interquartile points, and the numbers of fibers crossing each rung were counted. Values for the three ladders on each side of each graft were pooled. In this way, values of fiber density versus distance from the graft, proximal and distal to the GDNF infusion cannula, were obtained.

To obtain estimates of the volumes of TH-immunoreactive cell bodies in the grafts, the stereological "nucleator" technique was used (Gundersen and Jensen, 1987). Twenty cells in which the nucleus was visible, indicating that the plane of section ran through the middle of the cell, were randomly selected from each medial and lateral graft deposit in each animal and measured, and their volume was calculated. The results were pooled to give a mean cell volume for each animal.

Bridge grafts of GDNF-secreting Schwann cells combined with homotopic nigral graft placement

A second batch of 36 animals all found to satisfy the post-6-OHDA lesion criterion of more than seven turns per minute ipsilaterally after an amphetamine challenge (as above) were used as subjects for the bridge graft experiment. Animals were divided into three groups of 12 animals. Three different bridge graft substrates were tested: the parent Schwann cell line (SCTM41), the GDNF-secreting variant of this line (SCTM41 GDNF), and a control bridge (grafting medium; vehicle).

Bridge graft surgery. All 36 animals received bridge grafts of one of the three above cell types using a technique modified from that of Dunnett et al. (1989) (Fig. 1). Rats were mounted in a stereotaxic apparatus (Kopf), with the incisor bar set at 10.0 mm above the level of the ear bars. A burr hole was drilled through the frontal bone ipsilateral to the previous 6-OHDA lesion, and subsequently through the pterygoid bone, to allow free access of a blunt-ended 23 gauge grafting needle to the nigrostriatal pathway. This needle, attached to a 10 ml glass syringe (Scientific Glass Engineering), was mounted at an angle of 44° to the vertical and lowered to the following coordinates (relative to the midaural point): A, −0.5 mm; L, 2.4 mm; V, 4.8 mm. A total of 8 ml of graft suspension (containing either SCTM41 cells or SCTM41 GDNF cells at a density of 5×10^5 cells/μl in grafting medium; 0.002% DNase in L-15) or vehicle (grafting medium alone) was injected in deposits of 0.5 μl spaced at 0.5 mm intervals along the needle track. At each of the 16 locations, the deposit was gently extruded, and the needle was left in place for 30 sec before withdrawing it by 0.5 mm. Animals were immunosuppressed from this point on with daily intraperitoneal injections of 2 mg of cyclosporin A (Sandoz) in 0.2 ml of normal saline solution to prevent graft rejection.

Homotopic nigral graft surgery. Two days after bridge grafting, all animals received two grafts of dissociated embryonic ventral mesencephalic tissue (see above preparation techniques) (Fig. 1). One ventral mesencephalic fragment was suspended in 5 μl. Cell viability and density counts of aliquots of the suspension in a cytometer, using the trypan blue dye exclusion method, indicated a cell density of ~6.9×10^5 cells/μl and a viability of >92%. All rats were reanesthetized and placed in a stereotaxic apparatus, with the incisor bar set 2.3 mm inferior to the interaural line. They received two 2.5 μl of intrastriatal deposits of graft suspension by direct injection via the 23 gauge needle of the same 10 μl glass syringe (Scientific Glass Engineering) positioned at stereotaxic coordinates within substantia nigra: A, −5.0 mm from bregma; L, −2.9 and −1.9 mm from bregma; V, 7.2 and 7.8 mm from dura mater. Each graft deposit was infused over 2.5 min, with an additional 2 min allowed for diffusion before retraction of the needle.

Fluorogold injections. All animals received tracer injections of fluorogold into their striata. Two days before each animal was to be killed, it was reanesthetized and placed once again into a stereotaxic frame, with the incisor bar 2.3 mm below the interaural line. To assess the proportion of implanted nigral neurons whose neurites had reached striatum, 0.4 μl of a 2% solution of fluorogold (Schmued and Fallon, 1986) in distilled water was injected over 1 min at the following striatal coordinates: A, 0.6 mm; L, 2.4 mm (from bregma); V, −4.5 mm (from dura mater). The needle of the 1 ml glass syringe (Hamilton) was left in place for 5 min before gentle withdrawal. The experimental design is depicted in Table 2.

Table 2. Experimental design for the bridge graft experiment

Time–bridge material	Vehicle	SCTM41	SCTM41 GDNF
No. of animals receiving FG 3 d after nigral implant	4	3*	4
No. of animals receiving FG 8 d after nigral implant	4	4	4
No. of animals receiving FG 13 d after nigral implant	4	4	4

Bridge grafts were implanted 2 d before nigral implantation. Animals were killed 2 d after fluorogold (FG) injection. * indicates loss of an animal during the surgical procedure.

Tissue processing. After either 5, 10, or 15 d after nigral graft, animals were perfused, and their brains were processed as described for the cograft experiment. Immunohistochemistry was first performed on free-floating 50 μm parasagittal sections (one in four) for TH, using a rabbit polyclonal antibody (Institut Jacques Boy) amplified by biotin anti-rabbit (Dako) and streptavidin peroxidase (Vector Laboratories, Burlingame, CA) and revealed by the diaminobenzidine–peroxidase method. Total counts of TH-positive neurons within both nigral grafts were made, and fiber density within the bridge graft track was calculated at a distance of 1.5 mm from the anterior margin of each nigral graft; the total number of fibers crossing a line perpendicular to the axis of the bridge was obtained and divided by the width of the graft at that point and again by 50 mm. An additional series of sections (one in four) were stained using a mouse monoclonal antibody against TH (Boehringer Mannheim), followed by biotinylated anti-mouse antibodies and streptavidin conjugated to rhodamine (Dako) for fluorescence analysis of the proportion of TH neurons that also contained fluorogold, retrogradely transported from the striatal injection site.

Statistical treatment

All data were analyzed by multifactorial ANOVA using the Genstat 5.3 statistical package (AFRC Experimental Research Station, Rothampsted, UK) and SigmaStat (Jandel Scientific, San Rafael, CA), using the appropriate *post hoc* tests for multiple comparisons to confirm the locus of significant mean effects and interactions.

Animal procedures and care were in accordance with the United Kingdom Animal (scientific procedures) Act of 1986.

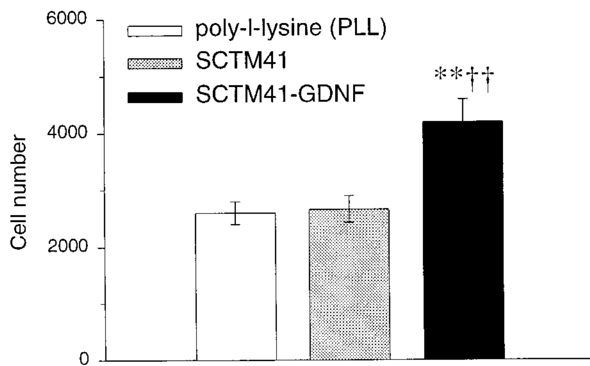
RESULTS

GDNF production by SCTM41 and transfected clones

Individual clones were expanded and analyzed for GDNF secretion using an ELISA assay. The analysis was conducted by collecting medium from 25 cm^2 flasks while the cells were dividing unhindered by confluence. Four milliliters of medium were added to each flask and removed after 48 hr incubation. Medium from clone 2 was found, by ELISA assay, to contain 46 ± 4.6 ng/ml GDNF. Each flask contained ~10^6 cells; the secretion rate of GDNF by this clone is therefore ~92 ng/10^6 cells/d. The parent cell line SCTM41 was also assayed and found to secrete no detectable levels of GDNF.

Expression of Schwann cell markers by SCTM41

SCTM41 were stained for the expression of LNGFR, GAP-43, S100 laminin, and Thy1.1. Of these markers, SCTM41 were found to strongly express S100, GAP-43, and laminin. LNGFR expression was marginal, only slighter stronger than background staining. SCTM41 do not express Thy1.1. This expression pattern is consistent with a Schwann cell-like phenotype.



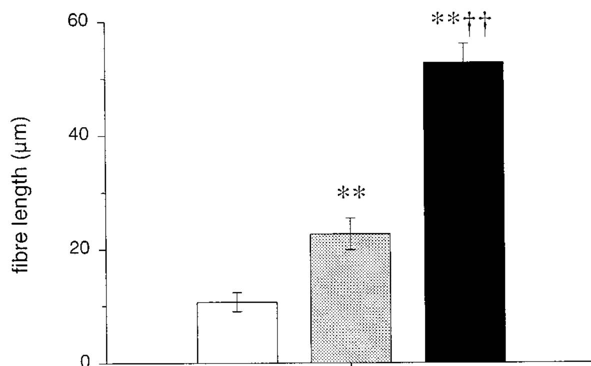


Figure 2. The effects on TH-immunoreactive neuronal survival (*A*) and neurite length (*B*) of plating a suspension of E14 ventral mesencephalic cells on poly-L-lysine, SCTM41 cells, and SCTM41 GDNF cells. ***p* < 0.01, significantly different from poly-L-lysine group; ††*p* < 0.01, significantly different from untransfected SCTM41 group.

Coculture experiments of dopaminergic neurons with the Schwann cell lines

To assess the survival and growth potential of dopaminergic neurons in contact with the GDNF-transfected cell line, an *in vitro* monolayer assay was used. E14 VM was plated onto three different substrate types: poly-L-lysine, SCTM41, or SCTM41 GDNF cells. The ~10^5 SCTM41 GDNF cells in each well will have secreted ~9 ng of GDNF in 24 hr into a medium volume of 0.25 ml, giving an eventual concentration of ~36 ng/ml. The medium will very rapidly, therefore, have attained the ED_{50} dose for GDNF on nigral dopaminergic neurons of 20 pg/ml (Fawcett et al., 1995; Pong et al., 1997). After 1 d in culture, cells were fixed and stained for TH expression. TH-positive neuron counts were obtained for a total of 34 coverslips performed over three separate determinations. The largest number of TH-positive neurons was found with the GDNF-producing variant of SCTM41 (4162 ± 390) compared with both the non-GDNF-producing parent and control substrates (2628 ± 251 and 2597 ± 225, respectively) (Fig. 2*A*). To determine the precise effect of each substrate on TH-positive cell numbers, ANOVA was performed on the data. This revealed a strong effect of substrate on the likelihood of observing a TH-positive neuronal phenotype within the culture ($F_{(2,32)} = 9.02$; $p < 0.001$). A *post hoc* Newman–Keuls test revealed that GDNF-secreting Schwann cells significantly increase the likelihood of observing TH-positive neurons within the culture compared with either the parent cell monolayer or the control substrate ($p < 0.01$).

Process lengths of dopaminergic neurons were measured in a random selection of cells. TH-positive fibers extended up to five times as far on the GDNF-secreting cells (52.80 ± 3.54 mm) compared with control (10.73 ± 1.81 mm). The parent cell line allowed 22.69 ± 2.82 mm average fiber extension (Fig. 2*B*). A one-factor ANOVA revealed a very strong effect of substrate on fiber length ($F_{(2,144)} = 64.25$; $p < 0.001$). A *post hoc* Newman–Keuls test revealed that the parent (non-GDNF-secreting) Schwann cell line had a significant growth-promoting effect compared with control ($p < 0.01$). However, the GDNF-secreting Schwann cells were found to be even more growth supportive than the nonsecreting cells ($p < 0.01$).

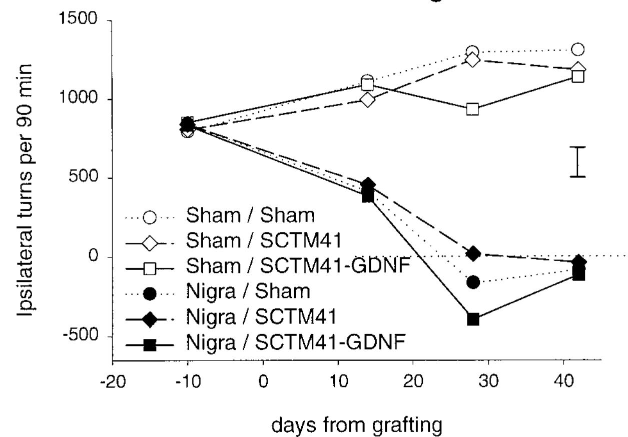


Figure 3. Amphetamine-induced rotation of animals with various combinations of embryonic mesencephalic and cograft to the striatum. Animals with sham mesencephalic grafts show no improvement, even in the presence of GDNF. All the animals with mesencephalic grafts show a steady improvement over 30 d, and there were no significant differences between these three groups. Error bar indicates SEM.

Cograft experiment (adjacent mesencephalic and Schwann cell grafts to the striatum)

Rotation

All groups of animals exhibited similar high rates of amphetamine-induced rotation before transplantation. Those that received no nigral component of the cograft remained highly asymmetrical over the 6 weeks after graft surgery. The animals in receipt of nigral grafts all exhibited complete restoration of symmetry and subsequent "overcompensation" as assessed by amphetamine-induced rotation (Fig. 3). These three groups differed significantly over time from the "sham nigra" groups (tests × group, $F_{(3,90)} = 7.71$; $p < 0.001$). All experimental groups with nigral grafts displayed the same rate of recovery, regardless of either GDNF production or the presence of Schwann cells (tests × group × cells, $F_{(6,90)} = 0.11$; tests × cells, $F_{(6,90)} = 0.97$; both not significant). The control grafts contained sufficient surviving dopaminergic neurons to fully correct amphetamine-induced rotation, so no extra effect from the additional surviving cells in the GDNF group would be expected.

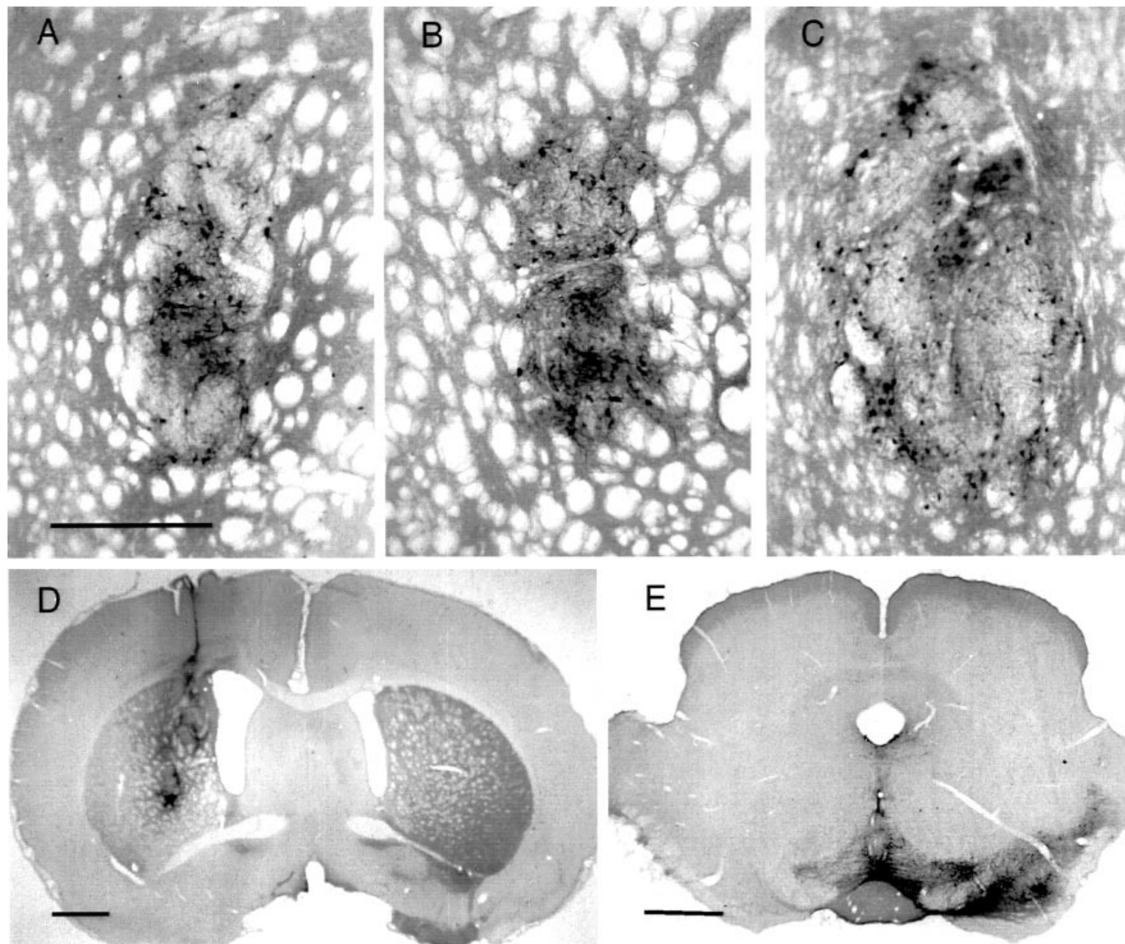


Figure 4. Grafts of E14 VM to the 6-OHDA-lesioned animals 6 weeks after transplantation, immunostained for TH. A sham graft was transplanted nearby in *A*, SCTM41 cells in *B*, and SCTM41 GDNF cells in *C*. *D* is a low-power shot of an SCTM41 GDNF cotransplanted mesencephalic graft. *E* shows that all the TH-immunoreactive neurons in the substantia nigra on one side are removed by the 6-OHDA lesion. Scale bars, 500 μm.

TH histology

In accordance with the pretransplantation and posttransplantation rotation data, all animals not in receipt of a nigral graft showed a virtually complete absence of TH-positive neurons in substantia nigra ipsilateral to the 6-OHDA lesion, accompanied by an absence of TH staining in the ipsilateral striatum, irrespective of any Schwann cell graft. In all animals that received nigral transplants, well defined healthy grafts were visible. These neurons were observed to have put out neurites into the host striatum, which had grown for up to 2 mm from the graft–host interface (Fig. 4). There was great variation in the size of grafts; those that were cografted with GDNF-secreting Schwann cells were larger and contained more dopaminergic neurons than the other groups. In some cases, within this group, the two grafts were so large that they both appeared in the same coronal section, despite their oblique placement.

Cell counts

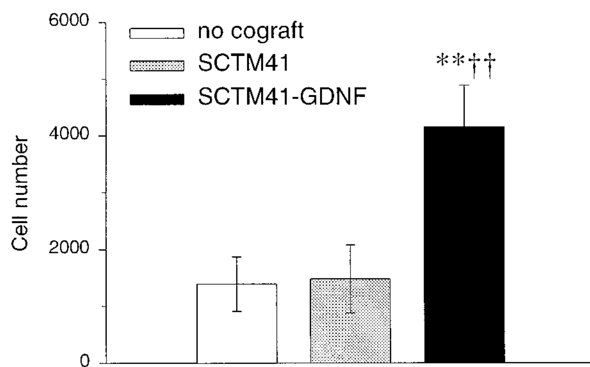
The number of TH-positive neurons surviving in the grafts was approximately three times higher in the presence of the GDNF-secreting Schwann cell line than in the presence of the control Schwann cells or in control animals with sham grafts (cells, $F_{(2,15)} = 6.68$; $p < 0.01$) (Fig. 5*A*). Mean total TH-positive cell number in both deposits was 1390 for control grafts, 1482 for cografts with SCTM cells, and 4158 for the GDNF-transfected cells. Because one mesencephalic fragment was suspended in 5 μl and each of the two grafts contained 2.5 μl of suspension, a total of one mesencephalon was injected into each host. Because each mesencephalon would contain ~47,000 dopaminergic neurons, overall survival of TH neurons was 3% in the control group, 3.2% in the cografts with untransfected cells, and 8.9% in cografts with GDNF transfected cells.

Cell body volume

Cotransplantation with Schwann cells also affected the size of the somata of the surviving TH cells (Fig. 5*B*). The effect of cografted cells on cell body volume was highly significant (cells, $F_{(2,15)} = 7.35$; $p = 0.006$).

Fiber outgrowth

Estimates of fiber density at different distances (as at subsequent rungs of ladders) from the graft–host border are shown in Figure 6. The data are segregated according to graft site (medial and lateral) and according to direction of outgrowth relative to the Schwann cell cograft (proximal and distal). In all groups of animals, fiber density declined with distance from the graft. None of the interactions of direction with any other factor was significant, suggesting that there were no direction-specific effects on



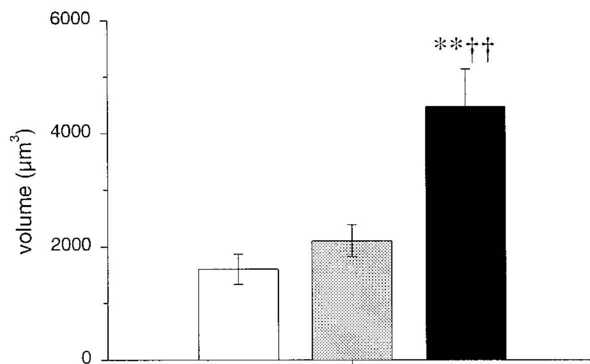


Figure 5. *A*, The numbers of surviving TH-immunoreactive neurons in embryonic mesencephalic grafts to the striatum, with a nearby sham, SCTM41, or SCTM41 GDNF graft. The SCTM41 cells have no survival-promoting effect, but the GDNF cells increase survival approximately threefold. *B*, Estimated cell body volume of surviving TH-immunoreactive neurons. The presence of GDNF-secreting cells more than doubles the cell volume. Error bars indicate SEM. $^{**}p < 0.01$, significantly different from no cograft group; $^{\dagger\dagger}p < 0.01$, significantly different from untransfected SCTM41 cograft group.

the grafts. There were, however, two significant effects of Schwann cell cograft on fiber density. The first was an effect of the type of cograft on the overall density of fiber outgrowth. On examination of Figure 6, it is apparent that the GDNF-secreting cells greatly increased fiber density over both the nonsecreting Schwann cells and the control grafts. At the graft–host border, the nontransfected cograft group does not differ from control values (as revealed by *post hoc* analysis); the GDNF-transfected group does differ significantly, however ($p < 0.01$). Moreover, this density increase, by a factor of approximately three, is the same as the factor by which cell numbers were increased by the presence of GDNF cells. GDNF did not, therefore, increase the number of neurites per neuron. The second effect of the Schwann cell cografts was on the gradient of the decline in density with distance from the graft; both cell lines caused a reduction in the gradient, indicating that the cografts promoted the outgrowth of fibers from the graft. Outgrowth was no greater on the side of the nigral grafts adjacent to the SCTM41 or SCTM41 GDNF cells, suggesting that neither SCTM41 nor GDNF has a chemoattractant effect on dopaminergic neurons.

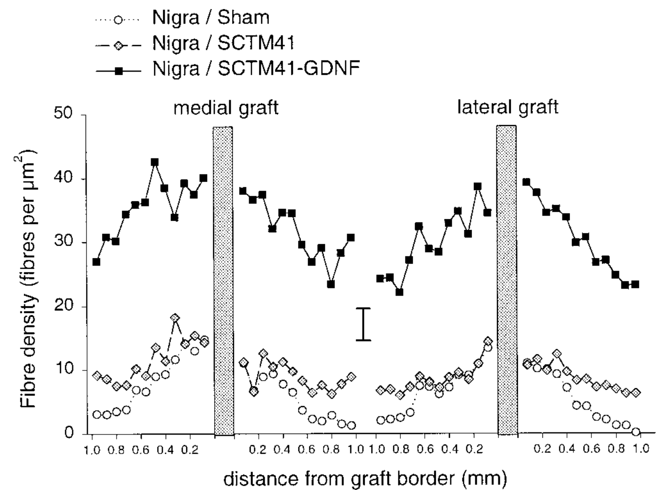


Figure 6. TH-immunoreactive fiber density measured at various distances from the border of embryonic mesencephalic grafts. Both medial and lateral grafts are shown. In between these was a sham graft, or a graft of SCTM41 or SCTM41 GDNF cells. Fiber density declines with distance from the graft in all cases, but the fiber density is much higher in grafts with access to GDNF. There is no significant difference in the fiber densities or the rate of decline of fiber density on the sides of the grafts adjacent to the Schwann cell grafts relative to the other sides of the grafts, so there is no evidence that GDNF is having a chemoattractive effect.

Bridge graft experiment (orthotopic mesencephalic graft with Schwann bridge to the striatum)

This experiment was designed to assess the effectiveness of SCTM41 cells as a bridge graft promoting axon growth from homotopically placed nigral grafts toward the striatum and the effects of GDNF secretion by the bridge graft cells. A bridge of SCTM cells was placed along a line from nigra to striatum, and 2 d later, a graft of embryonic nigra was placed within the lesioned nigra, adjacent to the caudal end of the bridge graft.

Histology of the dopaminergic grafts

To assess survival of the intranigral grafts, TH–diaminobenzidine histology was performed on 50 mm frozen parasagittal sections (frequency; 1:4). Grafts appeared large and healthy in most animals, containing many TH-positive neurons that could be seen to extend fibers a short distance into the adjacent host neuropil. Easily distinguishable from the grafted neurons by their size, morphology, and position (Fig. 7), very few intrinsic dopaminergic neurons spared by the 6-OHDA lesions could be seen in some cases lying rostral and dorsal to the graft.

Cell counts: TH-positive cells within the grafts

An Abercrombie (1946) corrected mean determination of TH-positive neurons present within the grafted site for each subgroup is presented graphically in Figure 8. A two-factor ANOVA was performed on these data and revealed a strong effect of survival time on cell number ($F_{(2,25)} = 7.09; p < 0.01$) and no overall effect of bridge graft material ($F_{(2,25)} = 2.51; p > 0.05$), but there was an interaction between time and bridge material ($F_{(4,25)} = 3.57; p < 0.05$). The basis for these effects was revealed by *a posteriori* analysis to be the increased survival of nigral grafts with SCTM41 GDNF cells at 15 d survival time. This group was significantly different from the GDNF bridge groups at 5 and 10 d survival ($p < 0.01$ for both cases). Moreover, the GDNF bridge

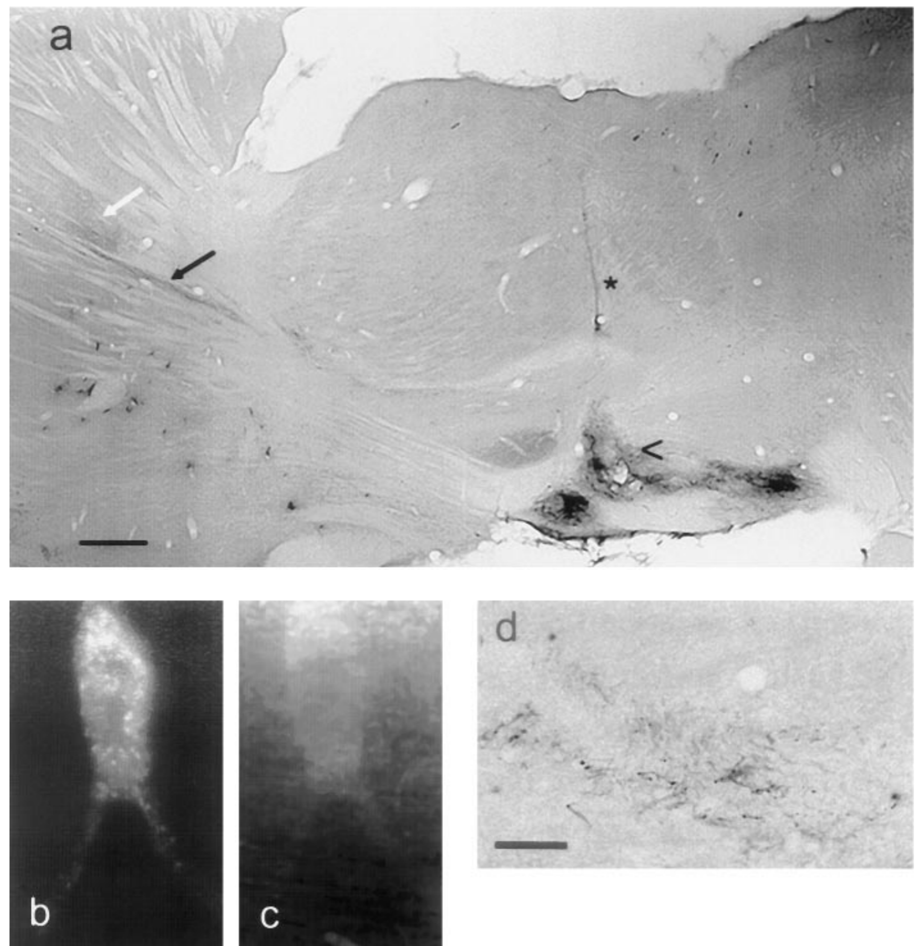


Figure 7. *a* shows a TH immunostained section from an animal that received a graft of embryonic mesencephalon to the region of a 6-OHDA-lesioned substantia nigra 15 d before fixation, together with a bridge graft of SCTM41 GDNF cells. The *less than* sign indicates the graft of embryonic dopaminergic neurons, and the *asterisk* indicates the needle track through which it was introduced. The bridge graft, which contains some dopaminergic axons, comes into the plane of the section just caudal to the striatum and is marked with a *black arrow*. The *white arrow* marks a puff of dopaminergic terminals where an axon has left the bridge graft to innervate the striatum. Scale bar, 500 μm. *b* and *c* show, respectively, fluorogold and TH fluorescence in a neuron within the mesencephalic graft. *d* shows a high-power shot of TH-immunoreactive axons growing in the environment of an SCTM41 GDNF bridge graft. Scale bar, 50 μm.

group was significantly different from the other bridge groups at the 15 d survival time ($p < 0.01$). At 15 d, the overall survival of TH neurons through the grafting procedure was 4.9% in the sham graft control group, 4.2% in the group with untransfected SCTM41 cells, and 10.4% in the cografts with GDNF-transfected cells.

Cell counts: TH-positive cells of intrinsic nigra

To assess the effects of either GDNF or time on the expression of TH by intrinsic nigra that had survived the 6-OHDA insult, these cells were counted. There were no significant differences between any group at any of the time points.

Dopaminergic neurite outgrowth

TH immunostaining with diaminobenzidine as the chromogen was used to assess fiber growth. TH-positive fibers were seen to leave the nigral graft site and were clearly visible within the constraints of the bridge graft coursing their way toward the striatum (Fig. 7). The number of fibers crossing an arbitrary line running perpendicular to the axis of the bridge graft were counted at a distance of 1.5 mm from the nigral graft. This value was then converted into a measure of fiber density (axons/100 μm^2) following an estimation of bridge graft diameter. A two-factor ANOVA on these data revealed a strong effect of bridge graft material on fiber density within the bridge ($F_{(2,25)} = 23.908$; $p < 0.001$) but no effect of time and no interaction between time and substrate. Therefore, results were pooled across time and are graphically expressed in Figure 9*A*. *Post hoc* Newman–Keuls tests revealed significant differences between all bridge graft types: SCTM41 GDNF > SCTM41 > vehicle ($p < 0.01$ in all cases).

Cresyl violet-stained sections were examined to obtain actual bridge graft diameter values, which were subsequently used to generate values of total TH-positive fibers within the bridge (assuming the bridge graft to be a uniform cylinder) (Brecknell et al., 1996a,b). Again, two-factor ANOVA revealed an effect of bridge graft material on the number of generated fibers ($F_{(2,25)} = 6.08$; $p < 0.01$) but no effect of time and no interactions. Results were therefore pooled across time and are depicted graphically in Figure 9*B*. *Post hoc* Newman–Keuls tests revealed that both SCTM41 and SCTM41 GDNF gave rise to greater fiber numbers than vehicle ($p < 0.05$ and 0.01, respectively) but did not differ significantly from each other.

Fluorogold and TH double-labeled cell counts

To assess the growth of axons from the nigral grafts to their striatal target, fluorogold was injected into the striatum 2 d before the termination of the experiment. Double-labeled cells with fluorogold incorporation and TH immunoreactivity were counted within the graft site (Fig. 7*b,c*). Few unlesioned intrinsic nigral neurons were identifiable within sections, anatomically distinctive from extrinsic neurons and therefore not counted. The Abercrombie (1946) corrected determinations of mean numbers of double-labeled grafted cells are presented graphically in Figure 10. Two-way ANOVA revealed an effect of time on the number of double-labeled neurons within the graft ($F_{(2,26)} = 4.27$; $p < 0.05$) and a significant interaction between time and the nature of the

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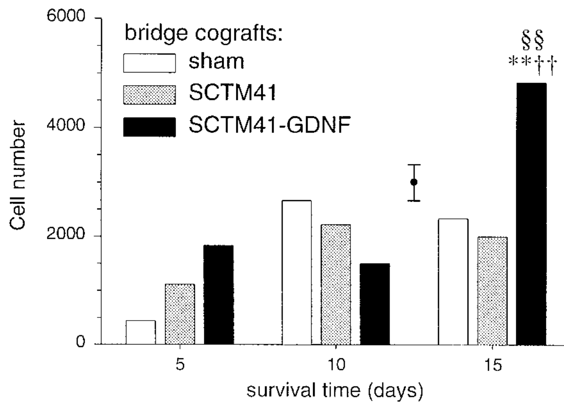


Figure 8. The effects of a bridge graft on the survival of TH-immunoreactive neurons in transplants placed in the region of the substantia nigra. The presence of GDNF had a strong survival-promoting effect but only when assayed at 15 d. Error bar indicates SEM. $^{**}p < 0.01$, significantly different from sham cograft group; ††$p < 0.01$, significantly different from untransfected SCTM41 cograft group; §§$p < 0.01$, significantly different from the same group at earlier survival times.

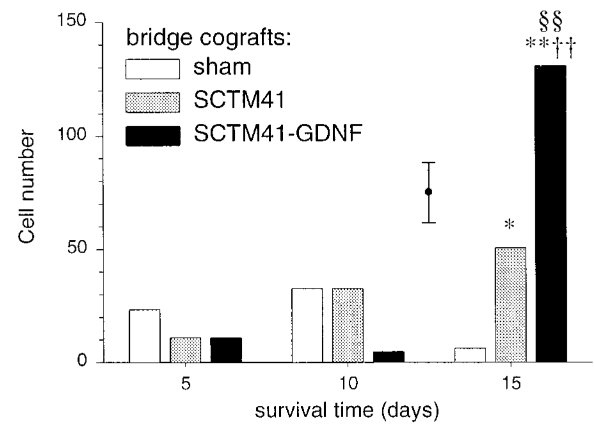
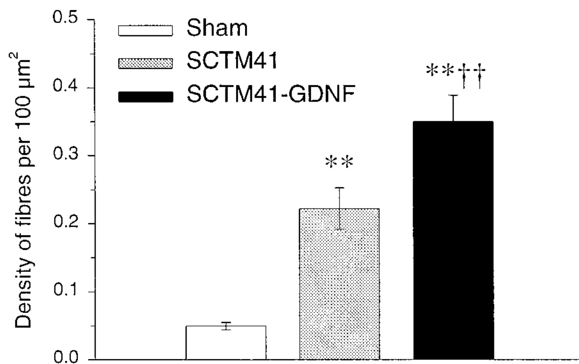


Figure 10. Retrograde labeling from the striatum of TH-immunoreactive neurons in grafts of E14 mesencephalic tissue placed in the substantia nigra, with a bridge graft between the nigral graft and the striatum. There was no significant difference between the groups, except that at 15 d greater connectivity with the striatum could be seen in grafts whose axons were growing through GDNF-secreting cells. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from sham group; ††$p < 0.01$, significantly different from SCTM41 bridge graft group; §§$p < 0.01$, significantly different from the same group at earlier survival times.



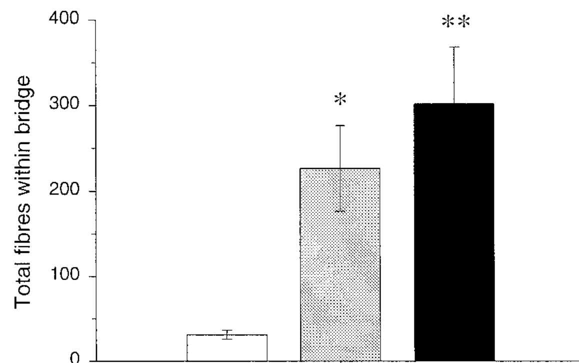


Figure 9. The density (*A*) and overall number (*B*) of TH-immunoreactive axons in bridge grafts, measured 15 d after operation. SCTM41 cells promoted axon growth relative to a sham graft, and SCTM41 GDNF cells were significantly better than the parent cell line. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from sham graft group; ††$p < 0.01$, significantly different from SCTM41 bridge graft group.

bridge graft substrate ($F_{(4,26)} = 4.24$; $p < 0.01$). *Post hoc* Newman–Keuls tests revealed significant differences between the number of double-labeled cells within GDNF-secreting bridge-grafted animals at 15 d compared with the other bridge substrates [SCTM41 GDNF > SCTM41 ($p < 0.01$) > vehicle ($p < 0.05$)]. Animals within this group were also significantly different from GDNF-secreting bridge-grafted animals at earlier survival times ($p < 0.01$ for both cases).

DISCUSSION

The two major problems in the use of embryonic mesencephalic grafts for the treatment of Parkinson's disease are the poor survival of grafted neurons and their inability to grow axons through the host CNS to their appropriate targets. We show here that the combination of Schwann cells and GDNF goes some way toward solving these problems. When dissociated embryonic mesencephalon was plated *in vitro* onto the SCTM41 Schwann cell line in untransfected and GDNF-transfected forms, dopaminergic neuronal survival was greater on GDNF cells than on the parent line or poly-L-lysine. Axon growth from the dopaminergic neurons was greater on SCTM41 cells than on poly-L-lysine and greater still on GDNF-transfected cells (Fig. 2). To assay for survival-promoting and axon growth-promoting effects *in vivo*, SCTM41 cells were grafted adjacent to deposits of fetal mesencephalon in the striatum of animals with 6-OHDA lesions of the substantia nigra. Nontransfected SCTM41 cells had no more effect on survival than sham surgery, but GDNF-transfected cells greatly increased dopaminergic neuronal survival, cell body size, and axon growth; however, this increased axon growth was not directed preferentially toward the GDNF-secreting cells (Figs. 4–6). Nontransfected and GDNF-transfected SCTM41 cells were then assessed for their ability to provide a bridge of axon growth-permissive material between fetal mesencephalic grafts placed orthotopically in the 6-OHDA-lesioned substantia nigra and their target in the striatum. A significant number of axons

grew through untransfected SCTM41 cells to the striatum, but the density of growth and the number of graft dopaminergic neurons that could be back labeled from the striatum was increased in GDNF-transfected cells (Figs. 7, 9, 10). The presence of a bridge graft of GDNF-transfected cells also increased the number of surviving dopaminergic neurons in the grafts (Fig. 8).

Secretion of trophic factors by Schwann and SCTM41 cells

Schwann cells are reported to secrete several trophic factors under some circumstances. Little trophic factor secretion is seen except in degenerating and regenerating peripheral nerves, and in Schwann cells in culture that are not in contact with axons. Under these circumstances, Schwann cells may produce NGF, BDNF, CNTF, GDNF, and other factors (Heumann et al., 1987; Meyer et al., 1992; Henderson et al., 1994; Lee et al., 1995). Untransfected SCTM41 cells do not produce quantities of GDNF, BDNF, or NT3 that are detectable by Western blot or two-site ELISA. Production of trophic factors by Schwann cells or peripheral nerve grafts implanted into the CNS has not been studied, but grafted cells downregulate c-jun and GAP-43 (Vaudano et al., 1995, 1996), two molecules associated with activation, which in turn is also associated with trophic factor production. Thus, it is possible that grafted cells produce little trophic factor. It is therefore likely that, like the SCTM41 line, primary Schwann cells will have to be modified if they are to secrete trophic factors when implanted into the CNS. In other respects, the SCTM41 line also closely resembles primary Schwann cells in that they express S100, GAP-43, laminin, and low levels of LNGFR.

Effects of SCTM41 and SCTM41 GDNF cells on dopaminergic neuronal survival

Untransfected SCTM41 cells had no effect on dopaminergic neuronal survival *in vitro*, and deposits of these cells implanted near embryonic mesencephalic grafts had no effect on dopaminergic neuronal survival *in vivo*. This is consistent with the absence of trophic factor production in the parent cell line. The GDNF-producing line, however, increased dopaminergic neuronal survival both *in vitro* and *in vivo*. When cocultured with GDNF-secreting cells, dopaminergic neuronal survival was increased by 158% over the first 24 hr, whereas grafts of GDNF-secreting cells placed adjacent to grafts of embryonic mesencephalic grafts increased dopaminergic survival approximately three times after 6 weeks in one experiment and approximately two times after 15 d in the other. Effects of GDNF on the survival of both embryonic and adult mesencephalic dopaminergic neurons have been reported by several previous authors (Engele and Franke, 1996; Rosenblad et al., 1996; Sinclair et al., 1996; Wang et al., 1996; Winkler et al., 1996; Tseng et al., 1997). An unexpected feature of our experiments was that grafted dopaminergic neuronal survival was only increased relative to controls at time points longer that 10 d. We saw an effect on survival *in vitro* within 24 hr, and much of the cell death in grafted dopaminergic neurons occurs within the first days after implantation, possibly because of damage sustained soon after transplantation (Barker et al., 1996). Thus, our cografting strategy probably did not deliver effective doses of GDNF sufficiently rapidly to prevent the first wave of cell death. It is likely, therefore, that the GDNF secreted by the cografts of SCTM GDNF cells was primarily effective in improving the survival and enhancing the expression of TH in neurons that had survived the first wave of cell death in the day or so immediately after implantation. The quantities of GDNF secreted by our grafts (~90 ng/day) were much lower that in previous experiments in which daily injections of up to 1000 ng were given. Although in those experiments 1000 ng/d had a greater effect on dopaminergic neuronal survival than 500 ng/d (Sinclair et al., 1996), these are massive doses compared with an ED_{50} of 20 pg/ml on dopaminergic neurons in culture(Pong et al., 1997), and the amount secreted by our grafts should have brought the nearby nigral grafts up to an effective dose sufficient for a maximal effect within a short time, particularly in view of the ability of substantia nigra neurons to retain and transport GDNF (Lapchak et al., 1997).

Axon growth from grafted dopaminergic neurons

In vitro, dopaminergic axonal growth was greater on SCTM cells than on poly-L-lysine and greater again on GDNF-transfected SCTM cells. We examined two types of axon growth *in vivo*: that from mesencephalic grafts placed in the striatum through host striatal tissue, and that from mesencephalic grafts place in the nigra through bridge grafts of SCTM cells. Growth into striatum from grafts in the striatum was affected by both the presence of SCTM cells and the presence of GDNF. The density of dopaminergic processes leaving the grafts and entering the striatum was unaffected relative to control by the presence of SCTM cells but was considerably increased by cografts of SCTM GDNF cells. However, the number of processes increased by a factor of three over controls, and the number of surviving cells also increased by a factor of three in the presence of GDNF-secreting cells, so there was no increase in the number of processes per neuron. The decline in process density over distance was decreased by the presence of both transfected and untransfected SCTM cells. We saw no evidence that neurites were growing preferentially toward SCTM or SCTM GDNF grafts, so the effect was trophic rather than tropic.

A major objective of this work was to enhance the connectivity with the striatum of mesencephalic grafts placed in the substantia nigra. No axons grow from rat embryonic mesencephalic grafts placed in the substantia nigra to the striatum unless some form of bridge is provided. In a previous study, we used the Schwannoma cell line RN22 as a bridge graft, which, because of its tumorogenicity, had to be irradiated (Brecknell et al., 1996a,b). RN22 cells attracted ~100 axons from the mesencephalic grafts. Most of these axons were able to leave the graft, enter the host striatum, and produce a reduction in amphetamine-induced rotation. To improve the bridge grafts, we have used a new Schwann cell line, SCTM41, which does not form tumors, and transfected it to secrete GDNF, which is the most potent trophic factor for mesencephalic dopaminergic neurons. Our expectation was that GDNF secretion would improve the survival of grafted neurons, as discussed above, and that axon growth would be enhanced. We found that the untransfected cell line supported the growth of axons from mesencephalic grafts to striatum in greater numbers than the RN22 line we have used before. Moreover, transfection with GDNF further increased the number and density of axons in the grafts. The mean number of axons in the GDNF grafts was ~300, the largest number being 899. This is still only a small proportion of the 3–5000 neurons surviving in our grafts, but sufficient to correct most of the behavioral deficits, which requires ~110–200 neuronal projections (Brundin et al., 1985; Brecknell et al., 1996a,b). Retrograde tracing with injections of fluorogold confirmed that the dopaminergic axons were derived from grafted neurons. These connections were not seen by retrograde tracing until 10 d after the neuronal and bridge grafts were

inserted. Clearly, there is still scope for improving axon recruitment into bridge grafts beyond what we have achieved in these experiments with GDNF transfection. This might be achieved by changes to the surface properties and molecules secreted by Schwann cells, by improving the geometry of the grafting operations, by increasing the axon growth potential of the dopaminergic neurons, and perhaps by adding other trophic factors and larger amounts of GDNF.

Grafts of embryonic mesencephalon have been used with some success to treat human patients with Parkinson's disease, but all these grafts have been placed in the caudate nucleus and putamen. In principle, it should be possible to use Schwann cell-derived bridge grafts to allow grafts placed in the substantia nigra to connect to these basal ganglia, but because of the large size of these structures in human brain, several tracks would be required to distribute the axons throughout the target structures.

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