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# Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury

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## Abstract

Neural stem cells (NSCs) offer the potential to replace lost tissue after nervous system injury. This study investigated whether grafts of NSCs (mouse clone C17.2) could also specifically support *host* axonal regeneration after spinal cord injury and sought to identify mechanisms underlying such growth. *In vitro*, prior to grafting, C17.2 NSCs were found for the first time to naturally constitutively secrete significant quantities of several neurotrophic factors by specific ELISA, including nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor. When grafted to cystic dorsal column lesions in the cervical spinal cord of adult rats, C17.2 NSCs supported extensive growth of host axons of known sensitivity to these growth factors when examined 2 weeks later. Quantitative real-time RT-PCR confirmed that grafted stem cells expressed neurotrophic factor genes *in vivo*. In addition, NSCs were genetically modified to produce neurotrophin-3, which significantly expanded NSC effects on host axons. Notably, overexpression of one growth factor had a reciprocal effect on expression of another factor. Thus, stem cells can promote host neural repair in part by secreting growth factors, and their regeneration-promoting activities can be modified by gene delivery.

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**Keywords:** Neural stem cells; Neurotrophins; Axonal growth; Spinal cord injury; Transplantation; Ex vivo gene therapy

## Introduction

The natural capacity of the central nervous system (CNS) to recover from injury is limited (Ramon y Cajal, 1928/1991), thus most research into spinal cord injury (SCI) focuses upon promoting axonal growth and reducing neuronal degeneration. Multipotent neural stem cells (NSCs) could enhance neural repair after SCI either by replacing host cells that have died or, perhaps more importantly, by promoting *host* axonal regeneration. Stem cells could promote axonal regeneration either by reconstituting a “bridge” through a lesion site capable of supporting axonal attachment and growth or by secreting diffusible molecules such as growth factors to attract injured axons. Previous studies

have reported the feasibility of transplantation of neural stem cells or fetal spinal cord cells into intact or injured spinal cords and reported limited stem cell differentiation (Cao et al., 2001; Chow et al., 2000; Hammang et al., 1997; Liu et al., 1999; McDonald et al., 1999; Onifer et al., 1997; Shihabuddin et al., 2000; Zompa et al., 1997; for review see Cao et al., 2002; Keirstead, 2001). Among these studies, McDonald et al. demonstrated that transplantation of embryonic stem cells promotes functional recovery after SCI, yet mechanisms underlying this beneficial effect have not been fully defined. Remyelination of host axons by neural stem cells may be one mechanism generating functional recovery (Liu et al., 2000), but whether stem cells also promote host axonal regeneration is unknown.

The murine C17.2 clone is a prototypical, stable, and extensively characterized NSC line. Originally derived from neonatal mouse cerebellum, it evinces the prototypical and defining features of a stem cell: multipotency, self-renewal,

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self-maintenance, expression of stem cell antigens, and responsiveness to various stem cell trophins (Ryder et al., 1990; Snyder et al., 1992). Importantly, as required of a stem cell, C17.2 NSCs contribute to the development of the organism throughout the neuraxis and across developmental time periods, from fetal to adult (Lacorazza et al., 1996; Snyder et al., 1992). In addition, it has been reported that grafting these cells to the nervous system corrects various abnormalities in models of abnormal CNS development or injury (Rosario et al., 1997; Snyder et al., 1995, 1997a, 1997b; Yandava et al., 1999).

The present study investigated whether grafts of NSCs (as modeled by clone C17.2) could support host axonal growth after SCI and sought to identify potential mechanisms underlying such growth. We examined the hypothesis that NSCs constitutively produce and secrete neurotrophic factors, which could promote the growth of host spinal axons after injury. In addition, we hypothesized that genetically modifying NSCs to produce augmented levels of growth factors, in this case neurotrophin-3 (NT-3), would enhance their growth, differentiation, and regeneration-promoting activities. Findings of this study indicate that grafts of neural stem cells to sites of spinal cord injury promote robust axonal growth, that intrinsic growth factor production by NSCs corresponds to observed patterns of axonal growth, and that augmentation of natural growth factor production by transduction with NT-3 extends the spectrum of host axon sensitivity to stem cell grafts.

## Materials and methods

### Cell culture

The C17.2 cell line was generated as previously described (Ryder et al., 1990; Snyder et al., 1992). Cells were grown in high-glucose DMEM with 10% fetal bovine serum (Sigma), 5% horse serum (Gibco), and 2 mM glutamine, on poly-L-lysine- or poly-L-ornithine-coated (10  $\mu$ g/ml, Sigma) tissue culture dishes in standard humidified 5% CO<sub>2</sub> at 37°C. Cells were maintained in culture either by splitting 1:10 into fresh medium or by feeding twice weekly with a 1:1 mixture of conditioned medium from confluent C17.2 cultures and fresh medium. Primary cultures of Fisher 344 rat fibroblasts (FF227, FF501) were generated from skin biopsies as previously described (Tuszynski et al., 1996a) and maintained in standard medium containing 10% fetal bovine serum.

### Transduction of C17.2 NSCs and rat primary fibroblasts with the green fluorescent protein (GFP) reporter gene

To track grafted cells in vivo, C17.2 NSCs and rat primary fibroblasts were transduced with the GFP reporter gene. The GFP cDNA was obtained from pHGFP-S65T (Clontech, Palo Alto, CA) by digestion with *Xba*I and

*Hind*III. The digested GFP plasmid termini were blunted with Klenow polymerase and ligated into the *Hpa*I-digested, dephosphorylated plasmid pLXSN vector (Clontech). Recombinant clones were identified by *Eco*RI and *Bam*HI double digestion and the orientation of the insert was examined by *Not*I and *Eco*RI digestion. The plasmid pLXSN-GFP was then transfected into the stable viral producer line PA317 utilizing the ecotropic packaging cell line psi-2 as described previously (Blesch et al., 1999; Tuszynski et al., 1996a). Virus-containing medium from the producer cells was collected and used to infect C17.2 cells and rat primary fibroblasts. GFP-expressing C17.2 NSCs were analyzed and sorted following standard procedures by a FACS scan with a standard excitation wavelength of 488 nm. GFP-transduced rat primary fibroblasts were selected using medium containing G418 (400  $\mu$ g/ml, Sigma), as previously described (Tuszynski et al., 1996a).

### Transfection of C17.2 NSCs with the NT-3 gene

A previously generated C17.2 subclone (Liu et al., 1999) was genetically modified to produce and secrete large quantities of NT-3 using the 950-bp human NT-3 gene (gift of Genentech). A new retroviral vector (pLXIE) was generated by digesting the plasmid pLNCX (Milter and Rosman, 1989, Clontech) with *Bcl*I and *Hpa*I to substitute the NeoR gene and CMV promoter with a *Bgl*III-*Hpa*I fragment containing multiple clone sites, an internal ribosome entry site (IRES), and the enhanced green fluorescent protein (EGFP) gene derived from the plasmid pIRES2-EGFP (Clontech). Human NT-3 cDNA was amplified by PCR using the 5' NT-3 primer GTGCTCGAGGTGATGTCCATCTTGTTT containing a *Xho*I site and the 3' NT-3 primer TGTG-GATCCTCATGTTCCTCCGATTTTCT containing a *Bam*HI site from a plasmid containing human NT-3 (pRK5-hu-NT-3). The human NT-3 PCR product was digested with *Xho*I and *Bam*HI and ligated into the plasmid pLXIE. The plasmid was transfected into the Phoenix producer cell line (Kinsella and Nolan, 1996) and NT-3-transduced C17.2 NSCs ("C17.2-NT-3") were fluorescently sorted as described above.

### Differentiation of NSCs in vitro

C17.2 and NT-3-transduced C17.2 NSCs were tested for their differentiation potential in vitro. Cells were seeded onto 12-mm circular coverslips coated with poly-L-lysine or poly-L-ornithine (10  $\mu$ g/ml, Sigma) and cultured for up to 3 weeks. Cells were then fixed on coverslips with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and labeled with cell-specific markers for undifferentiated stem cells (nestin), neural progenitors (MAP-2,  $\beta$ -III tubulin), neurons (Neu N, neurofilament), astrocytes (GFAP), and oligodendrocytes (APC), as listed below. The number of labeled cells was then quantified in three randomly selected fields at 200 $\times$  under a fluorescence microscope and divided

by the total number of cells in that field as determined by phase optics to obtain the mean percentage of labeled cells.

#### *Enzyme-linked immunosorbent assay (ELISA)*

Neurotrophic factor production by C17.2 and C17.2–NT-3 NSCs in vitro was measured by two-site ELISA as described (Conner and Varon, 1996). Conditioned medium from 24-h. cultures was collected after cells were confluent for 2 days, and EIA/RIA plates (Costar 3590) were coated with capture antibodies: anti-NGF (polyclonal, 215/11 [Conner and Varon, 1996], 1:500); anti-BDNF (gift of Amgen, polyclonal, 1:20,000); anti-NT-3 (Chemicon, polyclonal, 1:4000); or anti-GDNF (gift of Amgen, monoclonal, 1:1000). Plates were incubated with secondary antibodies (mouse anti-NGF, Boehringer Mannheim, 1:100; chicken anti-BDNF, Promega, 1:2500; chicken anti-NT-3, Promega, 1:1000; chicken anti-GDNF, Promega, 1:1000) and then with peroxidase-conjugated anti-mouse IgG (Dako, for NGF detection) or anti-chicken IgY (Promega, for BDNF, NT-3, and GDNF detection). Soluble colorimetric product was measured.

#### *Surgery*

A total of 44 adult female Fisher 344 rats (160–200 g) were subjects of this study. NIH guidelines for laboratory animal care and safety were strictly followed. Animals had free access to food and water throughout the study. All surgery was done under anesthesia with a combination (2 ml/kg) of ketamine (25 mg/ml), rompun (1.3 gm/ml), and acepromazine (0.25 mg/ml).

A spinal cord dorsal column lesion was made at the cervical (C3) level using a Kopf microwire knife (Kopf Instruments, Tujunga, CA), as previously described (Weidner et al., 1999). Briefly, after fixation in a spinal stereotaxic unit, a C3 dorsal laminectomy was performed and the microwire knife was stereotactically positioned at the spinal cord midline. A small dural incision was made, and the wire knife was lowered into the spinal cord to a depth of 1.1 mm ventral to the cord dorsal surface and 0.6 mm to the left of the midline. The tip of the wire knife was then extruded, forming a 1.5-mm-wide wire arc that was then raised to the dorsal surface of the cord. To ensure complete axotomy of the dorsal columns, including corticospinal tract and dorsal funicular sensory ascending projections, spinal tissue was compressed against the microwire knife surface using a microaspiration pipette until all visible white matter was transected. Complete axotomy using the wire knife device requires this additional axonal compression and visual verification. The wire arc was then retracted back into the wire knife device, and the instrument was removed from the spinal cord.

After the lesion was completed, 2.5  $\mu$ l of C17.2 NSCs ( $n = 16$  animals), C17.2–NT-3 NSCs ( $n = 16$  animals), or control GFP-expressing rat fibroblasts (FF227, FF501,  $n =$

Table 1  
Experimental groups

Cell types	CST labeling and other axon quantification	CTB labeling and quantification	RT-PCR	Total
C17.2	8	4	4	16
C17.2–NT-3	8	4	4	16
Fibroblasts (FF227, FF501)	4	0	4	8
Lesion only	4	0	0	4

*Note.* CST, corticospinal tract; CTB, cholera toxin B subunit.

8 animals, Table 1), resuspended in PBS at a concentration of  $4 \times 10^4$  cells/ $\mu$ l, was injected into the epicenter of the lesion using a PicoSpritzer II (General Valve, Fairfield, NJ). In addition, 4 animals underwent lesions only (Table 1). Cyclosporin A (10 mg/kg, Sandoz Pharmaceuticals, East Hanover, NJ) was injected intraperitoneally 1 day before surgery and continued daily in all subjects until sacrifice.

The corticospinal tract (CST) was anterogradely labeled with biotinylated dextran amine (BDA, MW 10,000, Molecular Probes, Eugene, OR) immediately after cell grafting in eight animals with C17.2 NSC grafts, eight with NT-3-transduced C17.2 NSC grafts, four with fibroblast grafts, and four with lesions only (Table 1) as previously described (Blesch et al., 1999; Grill et al., 1997). A total of 100 nl of a 10% solution of BDA dissolved in H<sub>2</sub>O was injected into each of 18 sites spanning the rostral-to-caudal extent of the motor cortex with a PicoSpritzer (General Valve, Inc.) using methods and injection coordinates previously reported (Blesch et al., 1999). Animals were perfused 2 weeks after injections. In addition, ascending dorsal column sensory axons were transganglionically labeled with cholera toxin B subunit (CTB, List Biological Lab, CA) injected into both sciatic nerves 2 weeks after spinal cord lesions, as previously described (Bradbury et al., 1999). Briefly, 2  $\mu$ l of a 1% solution of CTB dissolved in H<sub>2</sub>O was injected into each sciatic nerve using a Hamilton syringe (4  $\mu$ l total per animal). CTB was injected in four animals with C17.2 NSC grafts and four with NT-3-transduced C17.2 NSC grafts (Table 1), and subjects were perfused 4 days later.

#### *Histology and immunocytochemistry*

Rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Spinal cords were dissected free, postfixed overnight at 4°C, and then transferred to 30% sucrose for 72 h. Sagittal sections (35  $\mu$ m) of spinal cords from the cervical lesion site were cut on a cryostat, and one of every seven sections was mounted on gelatin-coated slides for Nissl staining. The remaining sections were serially collected into 24-well plates for immunocytochemical labeling.

Immunolabeling was performed on spinal cord sections to identify grafted cells and to determine the number and

phenotype of axons responding to lesions and grafts. All sections were processed free-floating, and endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide as previously described (Grill et al., 1997). Nonspecific antibody reactions were blocked with 5% horse serum (for monoclonal antibodies) or 3% goat serum (for polyclonal antibodies) for 1 h at room temperature. Next, sections were incubated overnight at 4°C with primary antibodies directed against jellyfish green fluorescent protein (polyclonal antibody from Molecular Probes at 1:3000 to label GFP transfected cells), neurofilament (NF; RT97 monoclonal antibody from Boehringer Mannheim at 1:250 to label axons), calcitonin gene-related peptide (CGRP; polyclonal antibody from Chemicon at 1:8000 to label primary sensory axons), tyrosine hydroxylase (TH; monoclonal antibody from Chemicon at 1:1000 to label coeruleospinal axons), serotonin (5-HT; monoclonal antibody from Chemicon at 1:35,000 to label raphespinal axons), choline acetyltransferase (ChAT; polyclonal antibody from Chemicon at 1:500 to label local motor axons), and cholera toxin B subunit (polyclonal antibody from List Biological Lab at 1:10,000 to label ascending dorsal column sensory axons). After washes, sections were incubated with biotinylated secondary antibodies (1:200, Vector Laboratory, Burlingame, CA) for 1 h at room temperature followed by a 1-h incubation in avidin–biotinylated peroxidase complex (1:100, Vector Elite Kit) at room temperature. Diaminobenzidine (0.05%) with nickel chloride (0.04%) was used as chromagen, with reactions sustained for 1–6 min at room temperature. The BDA-labeled corticospinal tract was detected by labeling with avidin–biotinylated peroxidase complex (1:100, Vector Elite Kit) incubated overnight at 4°C and visualized with diaminobenzidine and nickel chloride developed for 5 min at room temperature. The sections were then mounted on gelatin-coated slides, dehydrated, and coverslipped with pro-texx mounting medium (Baxter Diagnostics, Deerfield, IL).

Immunofluorescent labeling was also performed to characterize the state of cellular differentiation and to examine cell–cell interactions at the host–graft interface. After nonspecific antibody reactions were blocked with 5% goat serum for 1 h at room temperature, cells (fixed on slides from *in vitro* studies) or free-floating sections were incubated with primary antibodies directed against Nestin (monoclonal antibody from Chemicon at 1:200 to label undifferentiated neural stem cells), MAP-2 (monoclonal antibody from Chemicon at 1:10,000 to label immature neurons),  $\beta$ III tubulin (monoclonal antibody from Chemicon at 1:750 to label immature neurons), NeuN (monoclonal antibody from Chemicon at 1:250 to label mature neurons), NF (RT97 antibody at 1:175 to label mature neurons and axons), GFAP (polyclonal antibody from Dako at 1:700 to label astrocytes), and adenomatous polyposis coli (APC, monoclonal antibody from Oncogene at 1:200 to label oligodendrocytes) overnight at 4°C. After washes, cells and sections were incubated with Alexa 488 or 594 conjugated goat

anti-mouse or goat anti-rabbit secondary antibodies (1:150, Molecular Probes) for 2.5 h at room temperature. The sections were then washed, mounted on uncoated slides, and coverslipped with Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL).

#### *Quantification of growth of axons within grafts*

The penetration of axons of different phenotypes into grafts was quantified using NIH Image software analysis of immunolabeled sections, as previously described (Tuszynski et al., 1996a). The margins of the lesion were determined with phase contrast optics as a distinct change in the cellular constituency from regions of neurons to a lesion “cavity” devoid of neurons and occupied by grafted cells. Two randomly selected fields within each graft in the lesion site were measured in two separate sections from each subject (four regions total quantified per animal). The number of pixels occupied by immunolabeled axons within a fixed box size of  $640 \times 480$  pixels at  $200\times$  magnification was measured in sections immunolabeled for NF, CGRP, and ChAT in subjects with C17.2 NSC grafts ( $n = 8$ ), C17.2–NT-3 NSC grafts ( $n = 8$ ), and FF227 fibroblast grafts ( $n = 4$ ; see Table 1). Similar measurements were performed in subjects that received CTB injections into the sciatic nerve ( $n = 4$  for C17.2 NSC grafts, and  $n = 4$  for C17.2–NT-3 NSC grafts; Table 1). Thresholding values on video images were chosen such that only immunolabeled axons were measured, and light nonspecific background labeling was not detected. Nonspecific staining of objects other than axons, such as obvious artifactual spots resulting from tissue processing, was edited from images as previously reported (Armstrong et al., 1988). Total labeled pixels were divided by the sample box size ( $640 \times 480$  pixels) to obtain mean axon density per pixel in the graft.

#### *Quantitative real-time PCR for *in vivo* measurement of growth factor mRNA expression in grafted cells*

Two weeks after *in vivo* grafting, C17.2 NSCs ( $n=4$ ), C17.2–NT-3 NSCs ( $n=4$ ), and control GFP-transduced rat fibroblast (FF501,  $n=4$ , Table 1) cell grafts were carefully dissected from their grafting sites and immediately frozen on dry ice for determination of *in vivo* production of growth factor mRNA. Total RNA was extracted using Rneasy Mini Kit (Qiagen, Valencia, CA) and stored at  $-70^\circ\text{C}$ . In addition, total RNA was isolated from cultured C17.2 NSCs, C17.2–NT-3 NSCs, and primary fibroblasts *in vitro* and from one adult rat hippocampus (as a positive control for detection of NGF, BDNF, and NT-3 production). The first-strand cDNA synthesis reaction was carried out from  $2.5 \mu\text{g}$  total RNA using SuperScript II First-Strand Synthesis for RT-PCR Kit with oligo (dT) priming, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). In order to specifically detect *mouse* neural stem-cell-derived growth factor gene expression, mouse-specific primers with

at least one 3' end nucleotide mismatch to rat genes were designed for the neurotrophic factor genes NGF, BDNF, and GDNF, based on BLAST comparison of mouse and rat genes (using Primer Express software, Applied Biosystems, Foster City, CA). To detect expression of the NT-3 gene from C17.2 NSC grafts vs human NT-3-transduced C17.2 NSC grafts, NT-3 primers were designed that were both mouse- and human-specific, but were not rat-specific, by mismatching one 3' end nucleotide of the forward primer to the rat gene. The probes were further designed to detect mouse-specific NGF, BDNF, and GDNF genes and mouse- and human-specific NT-3 genes by including at least one nucleotide mismatch to rat genes in the central portion of the probes, using methods and software described above. The sequence of primers and probes are as follows: NGF primers, 5'CAGACCCGCAACATCACTGTA3' and 5'CC-ATGGGCCTGGAAGTCTAG3', probe, 5'CCAGACTGT-TTAAGAAACGGAGACTCCACTCACC3'; BDNF primers, 5'CGGGACGGTCACAGTCCTA3' and 5'GGGATTAC-ACTTGGTCTCGTAGAAATAC3', probe, 5'AGAAAGT-CCCGGTATCCAAAGGCCAACTG3'; GDNF primers, 5'TTGCAGCGGTTCTGTGAAT3' and 5'TCTTAGAA-TATGGTAAACCAGGTTGTCA3', probe, 5'CGGAGTA-GAAGGCTAACAAGTGACAAAGTAGGCC3'; NT-3 primers, 5'CATTTCGGGGACACCAGGTC3' and 5'TTTG-CACTGAGAGTTCCAGTGT3', probe, 5'AAGCCAG-GCCGGTCAAAAACGGTT3'. Primers and probes were synthesized and purified at Integrated DNA Technologies (Coralville, IA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize expression levels of growth factor genes from different stem cell grafts. The primers and probes of mouse and rat GAPDH were purchased from Applied Biosystems.

To test the specificity of primers for mouse-specific growth factor genes (or mouse- and human-specific for NT-3), nonquantitative PCRs were performed using 2  $\mu$ l of first-strand synthesis cDNA (total 20  $\mu$ l volume) from cultured C17.2 NSCs, C17.2-NT-3 NSCs, primary fibroblasts (FF501), and rat hippocampus. The 100- $\mu$ l PCR reaction additionally contained the following components: 0.2  $\mu$ M of each primer, 0.2 mM dNTP mixture, and 2.5 U Taq DNA polymerase (Qiagen, Valencia, CA). The PCRs were conducted in a programmable thermocycler (Ericomp) using an initial denature temperature of 94°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. A total of 20  $\mu$ l of final PCR product was separated in a 1.5% agarose gel, stained with ethidium bromide, and photographed.

Quantitative real-time PCR was performed at the Genomics Core Facility of the University of California at San Diego using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s plus 60°C for 1 min. The PCR reagents, except primers and probes, were from the TaqMan PCR Core Reagent Kit. The targets and endogenous control

GAPDH were amplified in separate tubes according to manufacturer's instructions, and standard curves were prepared for both the target and the endogenous reference. For samples of C17.2 and C17.2-NT-3 grafts, mouse GAPDH was amplified as an endogenous control using mouse-specific primers and probes; for samples of rat primary fibroblast (FF501) grafts (to ensure specificity of the growth factor probes for mouse genes), rat GAPDH was amplified as an endogenous control using rat-specific primers and probe. For each sample, the amount of target and endogenous reference was determined from the appropriate standard curve. Then, a standardization factor of endogenous control GAPDH was determined by finding the maximum value of GAPDH among the same group of samples and then dividing each value by that maximum value. The target amount (in nanograms) was calculated as a normalized value by multiplying the standardization factor by the amount of target found from the curve.

#### *Statistical analysis*

In all quantification procedures, observers were blinded to the nature of the experimental manipulation. Statistical differences between two groups were determined by two-tailed Student's *t* test. Multiple group comparisons were made by ANOVA and post hoc Fisher's tests, using a significance level of 95%. Data are presented as means  $\pm$  standard error of the mean.

## **Results**

### *C17.2 NSCs survive grafting to spinal cord lesion sites and migrate for short distances*

Histological examination revealed that wire knife lesions completely transected the dorsal columns and the dorsal corticospinal tract and that C17.2 and C17.2-NT-3 NSCs readily survived grafting to the lesion cavity and were well vascularized (Fig. 1A–E; Fig. 2F). Fibroblasts also survived grafting (not shown), whereas animals with lesions alone exhibited cystic cavities (Fig. 1F). Grafts of mouse C17.2 NSCs into these cyclosporin-treated rats did not elicit notable cellular immune responses compared to grafts of syngenic rat primary fibroblasts (Fig. 1). Lesion size and graft size were similar among all grafted groups. Most grafted stem cells remained localized to the injection site, migrating for maximal distances of only 1–2 mm from the lesion site (Fig. 1E; Fig. 2F). Transduction with NT-3 did not affect the distance of stem cell migration. In no case did tumors form at the lesion/grafting site.

### *In vitro and in vivo differentiation of NSCs*

In vitro, C17.2 NSCs were small and round, with little morphological evidence of differentiation (Fig. 2A). C17.2

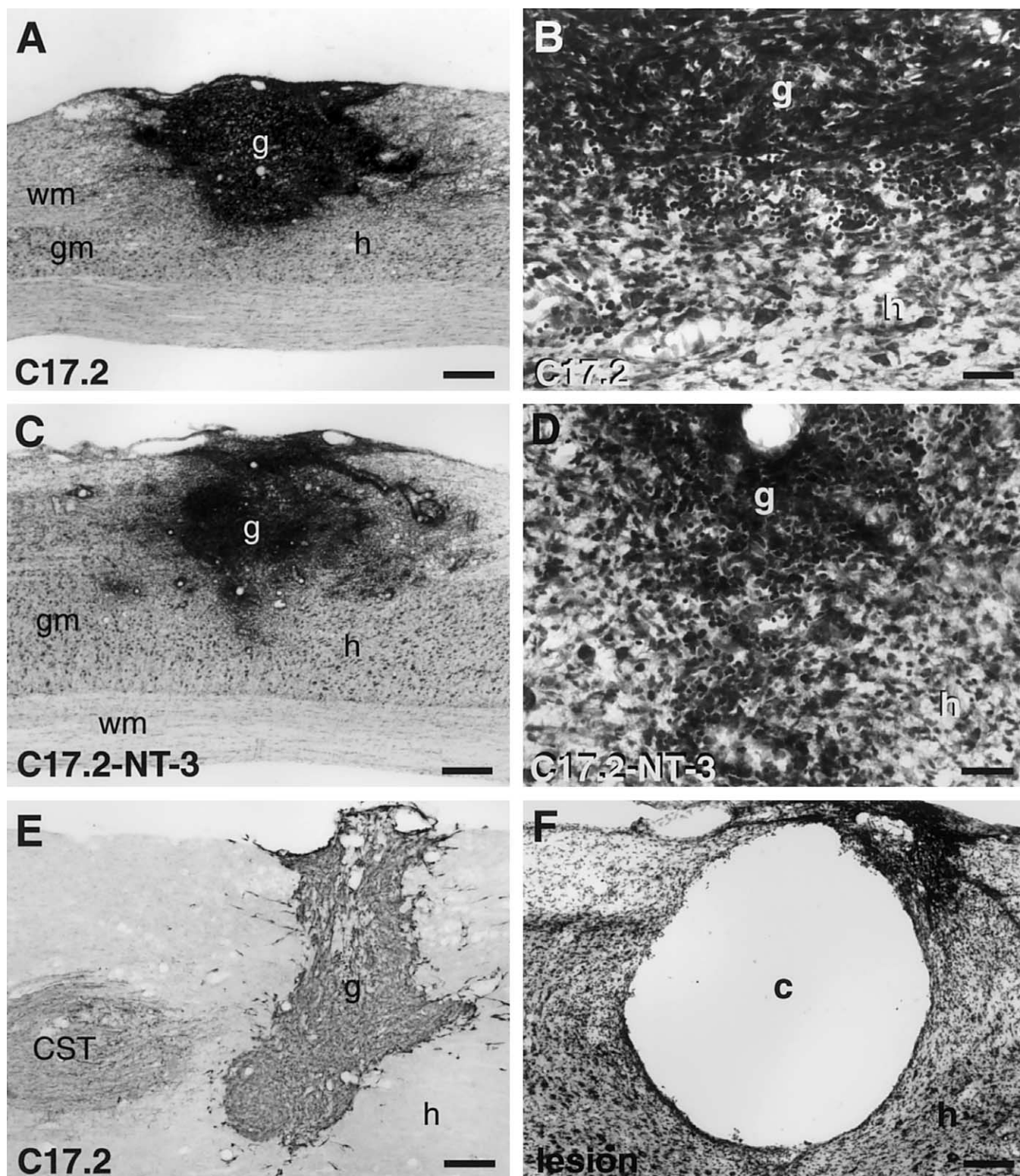
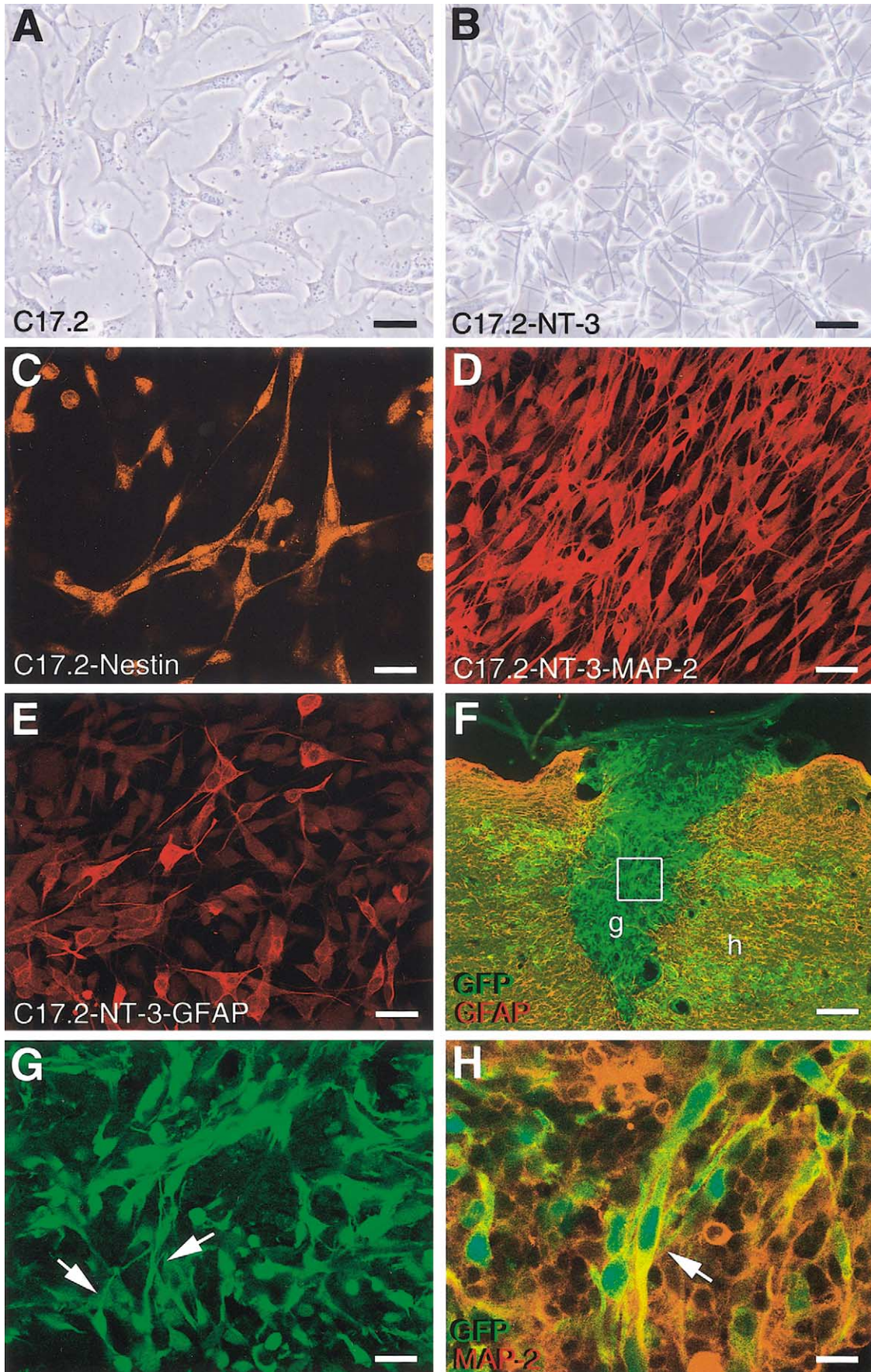


Fig. 1. In vivo morphology of lesion and graft. Nissl-stained sagittal spinal cord sections showing a C17.2 NSC graft (A, B) and C17.2-NT-3 NSC graft (C, D) in low- and high-magnification view 2 weeks after grafting. (E) GFP immunostaining showing GFP-expressing C17.2 NSCs grafted into a C3 wire knife lesion and lesioned corticospinal tract (CST). (F) A Nissl-stained sagittal spinal cord sections showing extent of C3 dorsal wire knife lesion cavity in a lesion-only animal. c, lesion cavity; g, graft; h, host; gm, gray matter; wm, white matter. Scale bar = 390  $\mu$ m (A, C), 44  $\mu$ m (B, D), 177  $\mu$ m (E, F).





cells expressed the neuroepithelial stem cell marker nestin in vitro (Fig. 2C), but did not label for the early neuronal markers MAP-2 and  $\beta$ -III tubulin, the mature neuronal markers NeuN and neurofilament, the astrocytic marker GFAP, or the oligodendrocytic marker APC after 2–3 weeks in culture (data not shown). In contrast, C17.2 NSCs transduced to express human NT-3 exhibited extensive neuronal differentiation in vitro (Fig. 2B, D, and E), consistent with a previous report (Liu et al., 1999). A total of 60% of C17.2 NSCs transfected with NT-3 exhibited MAP-2 labeling after 2–3 weeks in vitro (Fig. 2D), with occasional cells also expressing  $\beta$ -III tubulin but not neurofilament or NeuN. A total of 3–4% of NT-3-transfected cells were GFAP-positive (Fig. 2E). The remaining cells expressed the primitive neuroepithelial stem cell marker nestin. Notably, after in vivo grafting, C17.2 NSCs possessed a small round-shaped morphology and exhibited no evidence of differentiation (Fig. 1E). Indeed, C17.2 NSCs expressed neither stem cell (nestin) markers nor specific neuronal or glial markers (MAP-2,  $\beta$ -III tubulin, NeuN, NF, or GFAP and APC) at the postgrafting time point of 2 weeks. On the other hand, many NT-3-transfected C17.2 NSCs displayed more mature neuronal-type morphology after 2 weeks in vivo, containing bipolar or multipolar extending processes (Fig. 2F, G). Double fluorescent immunolabeling showed that occasional NT-3-transduced C17.2 cells expressed the neuronal marker MAP-2 in vivo (Fig. 2H). However, the majority of NT-3-transduced C17.2 NSCs in vivo did not express either nestin or neuronal/glial cell markers (MAP-2,  $\beta$ -III tubulin, NeuN, NF, or GFAP and APC).

*C17.2 NSCs support robust growth of spinal motor and sensory axons after injury; NT-3 transduction amplifies and extends this growth-promoting effect*

NF immunolabeling was entirely absent in lesion sites in animals that lacked cell grafts. Control, GFP-expressing fibroblast grafts were modestly penetrated by NF-labeled axons (Fig. 3A, B). Notably, the extent of axonal penetration increased 3-fold in recipients of C17.2 NSC grafts compared to control fibroblast grafts ( $P < 0.05$ ; Fig. 3C, D, and G). Further, grafts of NT-3-secreting C17.2 NSCs were penetrated by 4-fold greater numbers of neurofilament-labeled axons relative to unmanipulated C17.2 NSCs ( $P <$

0.0001; Fig. 3E, F, and G), a 12-fold increase relative to fibroblast grafts.

The origin of axons that penetrated grafts was determined by BDA-, CTB-, and immunolabeling. C17.2 NSC grafts were penetrated by CGRP-labeled sensory axons (Fig. 4B) and ChAT-labeled spinal motor axons (Fig. 5B). Notably, control GFP-expressing fibroblast grafts were completely devoid of ChAT-labeled axons (Fig. 5A, B). The density of sensory and motor axons in NSC grafts significantly exceeded that of GFP-expressing control fibroblast grafts when measured by NIH Image analysis ( $P < 0.001$ , Fig. 4A, B, and D;  $P < 0.01$ , Fig. 5A, B, and D). Thus, NSCs enhanced both the density and the diversity of axonal growth after spinal cord injury. These axons were relevant to local spinal circuits, however, rather than to descending or ascending spinal projections. Compared to control fibroblast grafts, C17.2 NSC grafts were not penetrated by significantly greater numbers of coerulospinal axons (TH-immunolabeled axons), raphespinal axons (5-HT immunolabeled axons), corticospinal axons (BDA-labeled axons), or CTB-labeled dorsal column sensory axons (see also below).

Transduction of the NSCs to express NT-3 augmented the density and diversity of axons penetrating spinal cord lesion sites (Figs. 3–6). A significantly greater overall number of axons penetrated C17.2–NT-3 NSC grafts compared to C17.2 NSC grafts or fibroblast grafts ( $P < 0.0001$ ; Fig. 3). Ascending dorsal column sensory axons penetrated C17.2–NT-3 NSC grafts more extensively than C17.2 NSC grafts ( $P < 0.05$ ; Fig. 6). On the other hand, penetration of CGRP-labeled nociceptive axons into C17.2–NT-3 NSC grafts was unchanged compared to C17.2 NSC grafts (yet significantly exceeded penetration of control fibroblast grafts; Fig. 4), whereas fewer ChAT-labeled motor axons penetrated C17.2–NT-3 NSC grafts compared to C17.2 NSC grafts alone ( $P < 0.05$ ; Fig. 5B, C, and D). Corticospinal axons did not penetrate grafts.

*C17.2 NSCs naturally secrete neurotrophic factors that are known to promote sensory and motor axon growth*

Previously it has been reported that CGRP-expressing sensory axons are NGF-responsive (Lindsay and Harman, 1989; Otten et al., 1983; Tuszynski et al., 1994) and that

Fig. 2. Differentiation and migration of C17.2 and C17.2–NT-3 NSCs in vitro and in vivo. (A) Undifferentiated morphology of C17.2 NSCs after 1 week in culture (reseeded for 24 h, phase contrast optics). (B) In contrast, after 1 week in culture many C17.2–NT-3 NSC-derived cells exhibit pyramidal morphology and extend processes. (C) Cultured C17.2 NSCs expressed nestin after 1 week in culture (reseeded for 24 h). (D) Sixty percent of C17.2–NT-3 NSCs in vitro express the neuronal marker MAP-2 after 2 weeks in culture, whereas C17.2 NSCs in vitro lacking the NT-3 gene do not express MAP-2 (data not shown). (E) A small proportion (3–4%) of C17.2–NT-3 NSCs in vitro also express the astrocytic marker GFAP, whereas C17.2 NSCs lacking the NT-3 gene do not express GFAP (data not shown). (F) Fluorescent double immunolabeling after in vivo grafting reveals that C17.2–NT-3 NSCs (GFP label, green channel) completely fill C3 wire knife lesion cavity (outlined by GFAP immunolabeling, red channel) and migrate for short distances from the graft site in rostral and caudal directions. (G) Higher magnification of F (rectangle window) reveals that many grafted C17.2–NT-3 NSCs have pyramidal morphology with long cellular processes (arrows), similar to in vitro observations. (H) Occasional C17.2–NT-3 cells (green channel) grafted in vivo were found to express MAP-2 (red channel), illustrated by fluorescent double immunolabeling at high magnification (arrow). However, most grafted C17.2–NT-3 cells did not label for MAP-2 or  $\beta$ -III tubulin. g, graft; h, host. Scale bars = 44  $\mu$ m (A, B), 35  $\mu$ m (C, D), 230  $\mu$ m (E), 23  $\mu$ m (F), and 14  $\mu$ m (H).



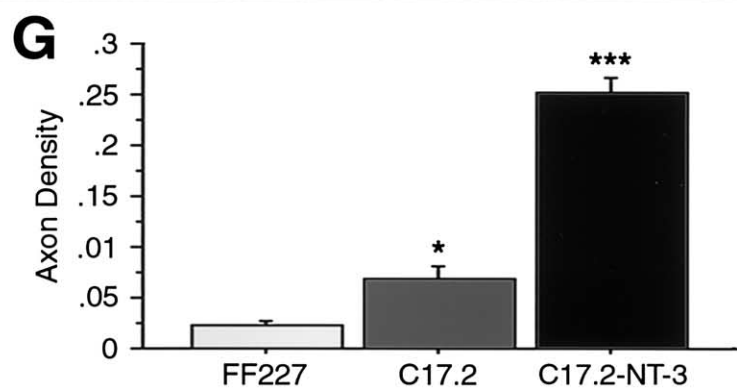
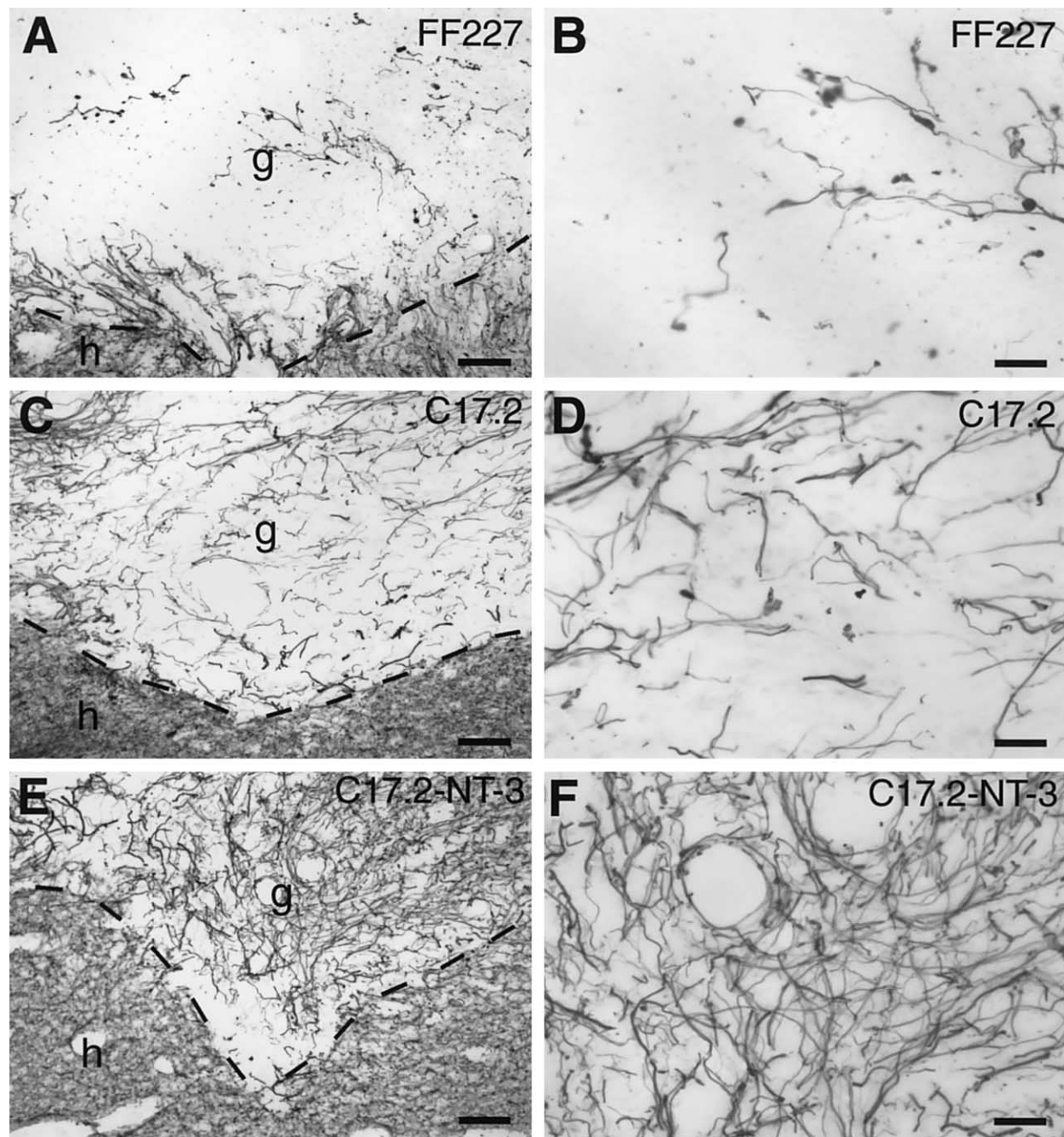


Fig. 3. Axonal penetration into fibroblast, C17.2, and C17.2-NT-3 grafts to C3 dorsal column lesion sites. (A, B) NF immunolabeling in sagittal spinal cord sections of control GFP-expressing fibroblast graft (FF227) recipients show modest penetration of axons at low (A) and higher (B) magnification. (C, D) Significantly increased numbers of axons penetrate C17.2 NSC grafts, compared to fibroblast graft recipients. (E, F) NT-3-transduced C17.2 NSC grafts consistently exhibit the greatest degree of axonal penetration. Scale bars = 88  $\mu$ m in A, C, E and 27.5  $\mu$ m in B, D, F. g, graft; h, host; dashed lines indicate host/graft interface. (G) Quantification of NF-labeled axon penetration of grafts indicates significant differences between each group (overall ANOVA  $P < 0.0001$ ;  $*P < 0.05$ ,  $***P < 0.0001$ ).

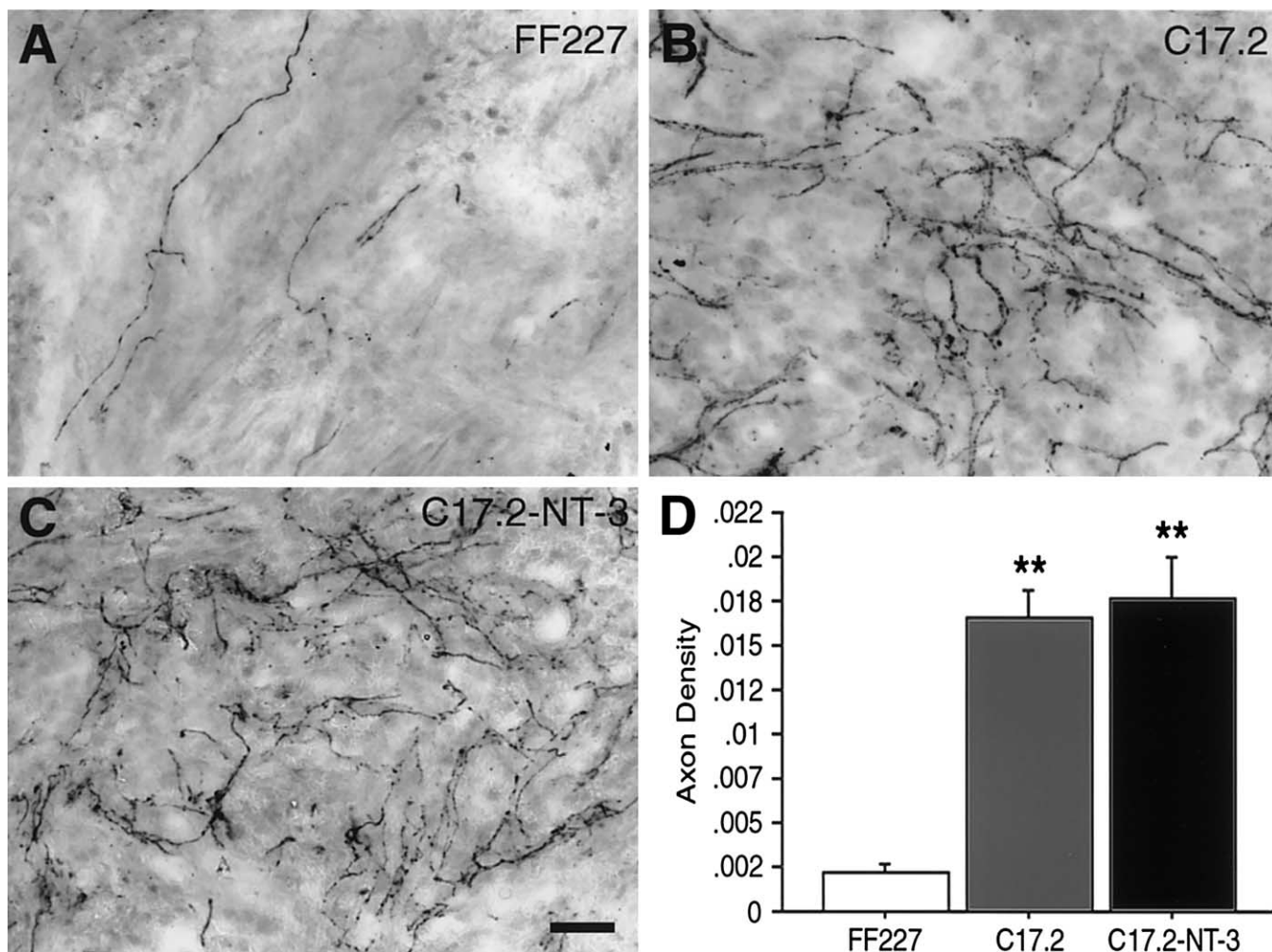


Fig. 4. CGRP immunolabeling and quantification. (A) CGRP-labeled sensory axons only modestly penetrate fibroblast grafts (FF227), but robustly penetrate (B) C17.2 grafts and (C) C17.2-NT-3 grafts. Scale bar = 27.5  $\mu$ m. (D) Quantification shows that both C17.2 and C17.2-NT-3 grafts contain significantly more CGRP-labeled axons than control fibroblast grafts (\*\* $P < 0.001$ ).

motor neurons are both BDNF- and GDNF-responsive (Tuszynski et al., 1996b; Vejsada et al., 1998; Yan et al., 1992). To determine whether a mechanism of NSC-enhanced growth of spinal sensory and motor axons was growth factor secretion by these cells, specific ELISAs for NGF, BDNF, NT-3, and GDNF protein levels were performed on C17.2 NSCs, C17.2-NT-3 NSCs, and GFP-expressing fibroblasts in vitro. Whereas primary fibroblasts expressed no detectable levels of these growth factors, conditioned medium from C17.2 NSCs contained significant quantities of NGF ( $7.5 \pm 2.5$  pg/ $10^6$  cells/day), BDNF ( $7.1 \pm 0.1$  pg/ $10^6$  cells/day), and GDNF ( $70 \pm 1$  pg/ $10^6$  cells/day) (Fig. 7A). NT-3 expression was not detectable in nontransfected C17.2 NSCs. Interestingly, transduction of C17.2 NSCs with NT-3 not only resulted in significant NT-3 production from transduced cells ( $12,592 \pm 8$  pg/ $10^6$  cells/day), but also resulted in a significant increase in NGF ( $51.5 \pm 7.5$  pg/ $10^6$  cells/day) and BDNF ( $84.1 \pm 0.15$  pg/ $10^6$  cells/day) production and a decline in GDNF pro-

duction to undetectable levels (Fig. 7A). These findings were replicated in two separate sets of assays.

To confirm that C17.2 NSCs and NT-3-transduced C17.2 NSCs express NGF, BDNF, NT-3, and GDNF in vivo, cDNA from these NSC grafts, as well as from control rat primary fibroblast grafts and rat hippocampus, were amplified using quantitative real-time PCR. By choosing mouse- and human-specific primers and probes (as described under Materials and Methods), only mouse and human growth factor genes and not rat genes were detected. Analysis of standard, nonquantitative RT-PCR products confirmed the specificity of mouse and human primers: cDNAs of growth factor genes were amplified only from cultured C17.2 and human NT-3-transduced C17.2 NSCs, but not from rat hippocampus (where growth factor genes are normally expressed (Ceccatelli et al., 1991; Ernfors et al., 1990; Lenhard and Suter-Crazzolar, 1998; Pochon et al., 1997) or control rat fibroblasts (Fig. 7B). The pattern of growth factor gene ex-

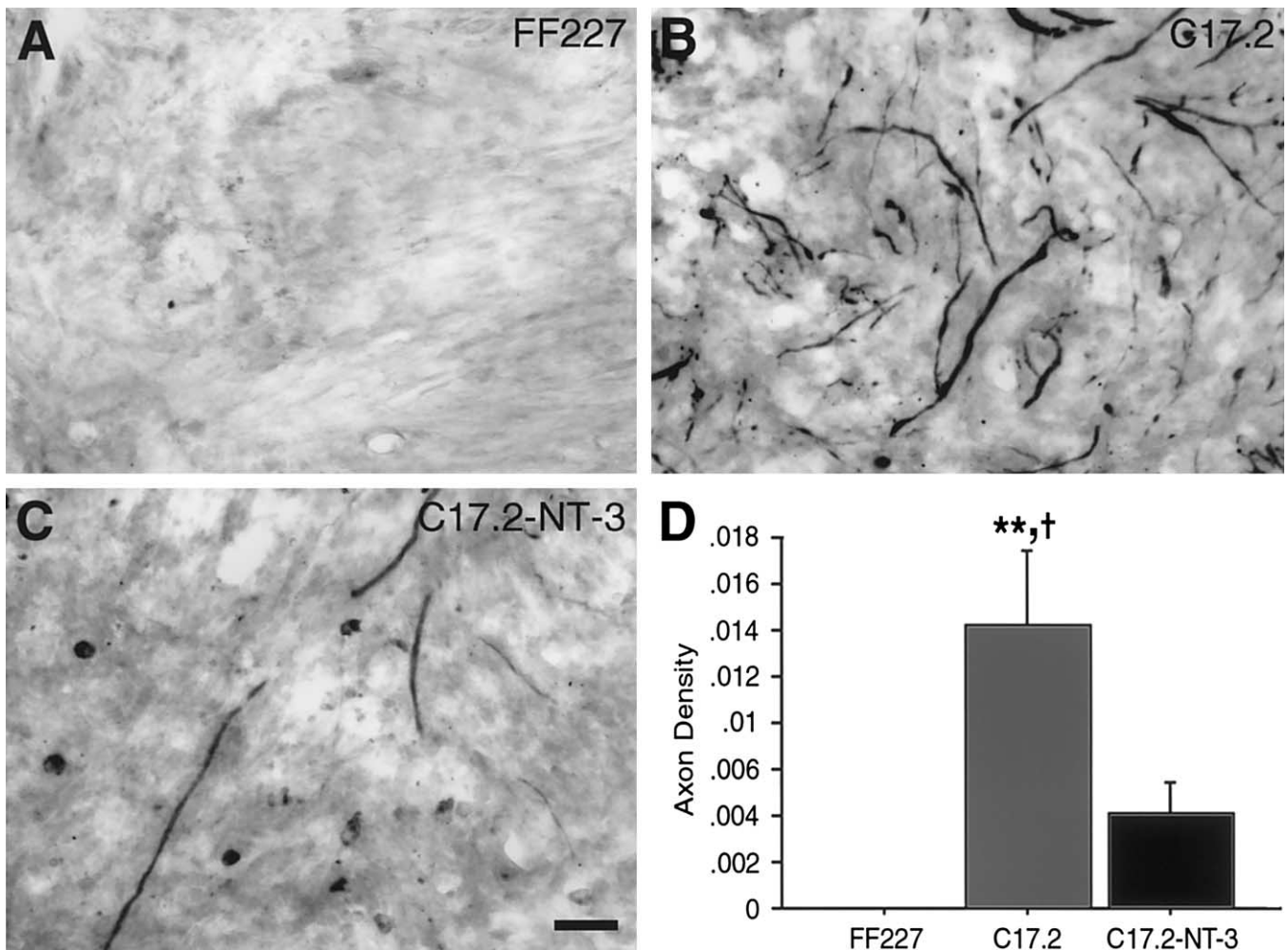


Fig. 5. ChAT immunolabeling and quantification. (A) ChAT-labeling motor axons fail to penetrate fibroblast grafts (FF227), but (B) extensively penetrate C17.2 grafts and (C) moderately penetrate C17.2-NT-3 grafts. Scale bar = 27.5  $\mu$ m. (D) Quantification reveals that the density of ChAT-labeled axons was significantly greater in C17.2 grafts than in fibroblast grafts (\*\* $P < 0.01$ ) or C17.2-NT-3 grafts ( $\dagger$ ,  $P < 0.05$ ). ChAT-labeled axons do penetrate C17.2-NT-3 grafts, whereas they are entirely absent in fibroblast grafts.

pression in cultured NSCs was similar to the ELISA data (Fig. 7A, B). Quantitative neurotrophic factor in vivo gene expression was compared using real-time PCR among C17.2 graft recipients ( $n = 4$ ), C17.2-NT-3 graft recipients ( $n = 4$ ), and control rat primary fibroblast (FF501) graft recipients ( $n = 4$ , Table 1) 2 weeks after lesions and cell transplantation. Both mouse and rat GAPDH cDNAs were amplified as endogenous controls to normalize growth factor gene expression levels among samples. With mouse-specific primers and probes, NGF was expressed in C17.2 ( $130.6 \pm 21.7$ ) and NT-3-transduced C17.2 NSC grafts ( $217.9 \pm 9.7$ ,  $P < 0.01$ , Fig. 7C). BDNF expression was significantly higher in C17.2 NSC grafts ( $150.8 \pm 24.4$ ) than in C17.2-NT-3 NSC grafts ( $52.4 \pm 4.0$ ,  $P < 0.001$ ). Similarly, GDNF expression was higher in C17.2 NSC grafts ( $348.9 \pm 46.0$ ) than in C17.2-NT-3 NSC grafts ( $94.5 \pm 10.7$ ,  $P < 0.0001$ ). As expected, human NT-3-transduced C17.2 NSC grafts

contained far greater levels of NT-3 mRNA ( $114 \times 10^6 \pm 33.6 \times 10^5$ ) than C17.2 NSC grafts ( $226.7 \pm 7.1$ ,  $P < 0.0001$ ). Rat primary fibroblast grafts contained no detectable levels of growth factor mRNAs using the same mouse- and human-specific primers and probes (Fig. 7C). Patterns of growth factor gene expression in vivo were similar to those of neurotrophic factor protein measurements obtained by ELISA in vitro and confirmed that C17.2 neural stem cells secrete significant levels of growth factors that correlate with observed patterns of growth from motor and sensory axons. Only one difference in a single group was found between in vitro growth factor protein and in vivo mRNA measurements: levels of BDNF gene expression in NT-3-transduced C17.2 cells were lower in vivo than predicted by in vitro ELISA (Fig. 7A, B). Nonetheless, overall findings were consistent in vitro and in vivo. Notably, the reduction in GDNF expression by NT-3-transduced NSCs correlated

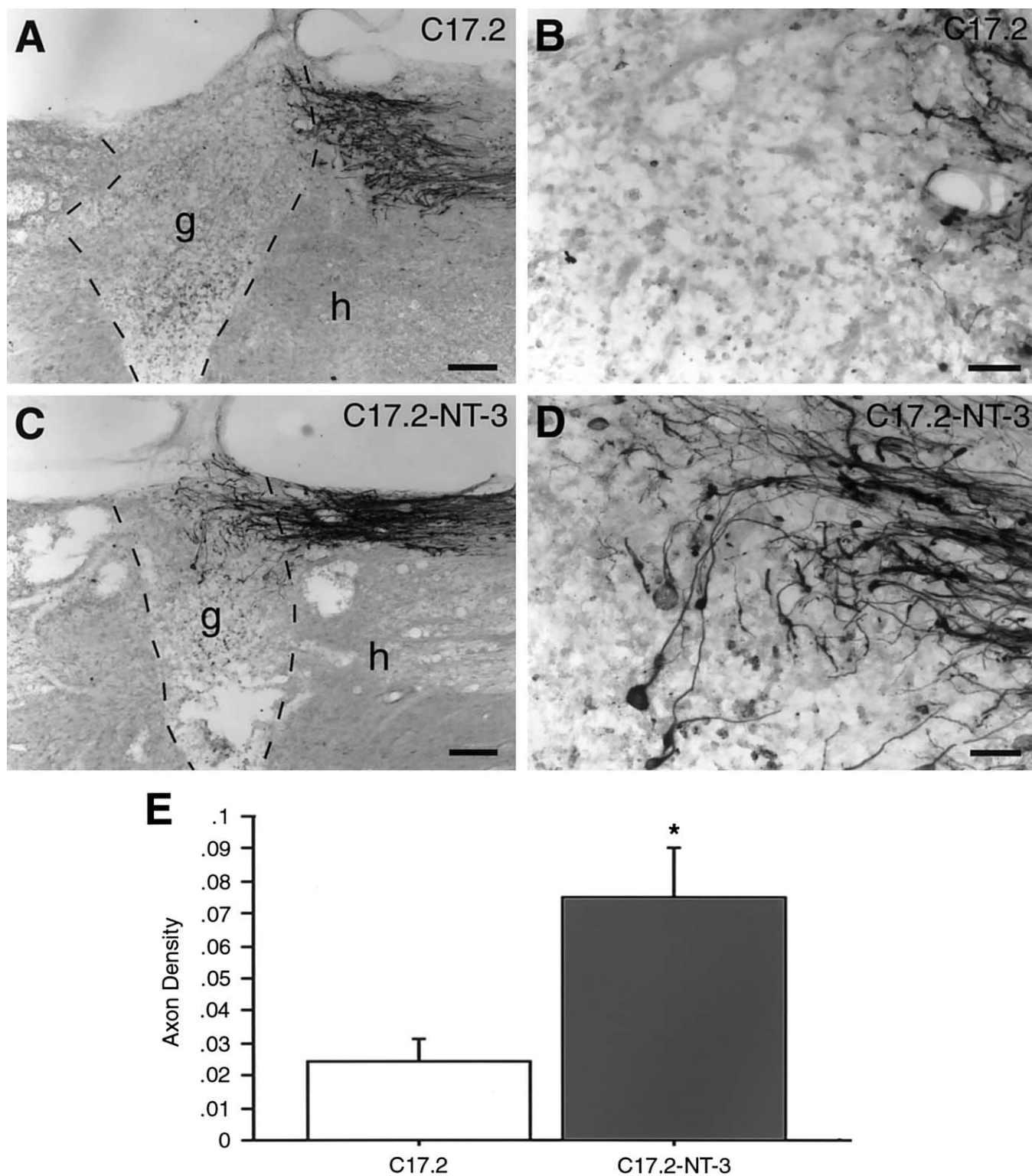


Fig. 6. Dorsal column sensory axons penetrate C17.2-NT-3 grafts. (A) CTB-labeling of dorsal column ascending sensory axons in C17.2 NSC grafts reveals axons at the host/graft interface, but axons rarely penetrate grafts. (B) The failure of extensive axonal penetration of C17.2 NSCs is also evident at higher magnification. (C) In contrast, C17.2-NT-3-secreting grafts are penetrated by CTB-labeled axons, which readily cross the host/graft interface. (D) At higher magnification, axons are clearly visible within the C17.2-NT-3 graft. g, graft; h, host; dashed lines indicate host/graft interface. Scale bars = 177  $\mu\text{m}$  in A, C; 44  $\mu\text{m}$  in B, D. (E) Quantification of CTB labeling demonstrates significantly greater number of axons in C17.2-NT-3 grafts than C17.2 grafts (\* $P < 0.05$ ).



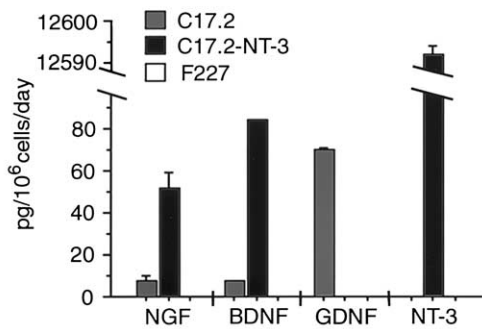
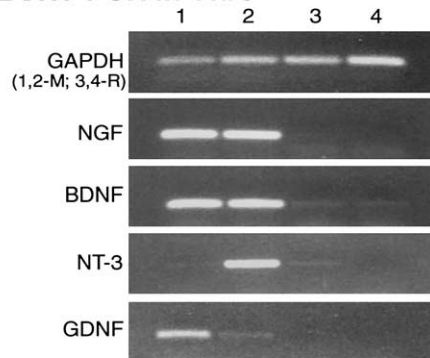
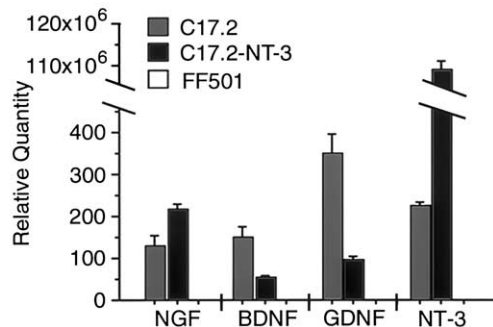
**A. ELISA In Vitro****B. RT-PCR In Vitro****C. Real-time RT-PCR In Vivo**

Fig. 7. C17.2 NSCs naturally produce neurotrophic factors. (A) In vitro specific ELISA of conditioned medium collected from C17.2 NSCs, NT-3-transduced C17.2 NSCs (C17.2-NT-3), and control GFP-transduced fibroblasts (FF227). Results represent mean values of two independent assays, with each assay containing samples at three different dilutions (1:1; 1:5; 1:50). (B) Nonquantitative RT-PCR in vitro demonstrates amplification of neurotrophic factor genes using mouse-specific primers (for NT-3, both mouse- and human-specific) from cDNA of C17.2 cells (lane 1) and NT-3-transduced C17.2 NSCs (lane 2), but not from cDNA of control rat hippocampus (lane 3) or rat fibroblasts (lane 4). The endogenous control GAPDH is amplified with mouse-specific primers in lanes 1 and 2 and with rat-specific primers in lanes 3 and 4. (C) Quantitative real-time RT-PCR with mouse-specific primers and probes (for NT-3, both mouse- and human-specific) shows expression of the growth factor genes NGF, BDNF, GDNF, and NT-3 at different levels in mouse-derived C17.2 NSC grafts (C17.2,  $n = 4$ ) vs NT-3-transduced C17.2 NSC grafts (C17.2-NT-3,  $n = 4$ ). Gene expression is not detectable in control GFP-transduced primary rat fibroblast grafts ( $n = 4$ ). The expression of growth factor genes is normalized (nanograms) relative to endogenous control GAPDH gene expression (see Materials and Methods for details).

with the reduction of motor axon penetration into the same grafts.

**Discussion**

The present study demonstrates that C17.2 neural stem cells can elicit significant host axonal growth after SCI, presenting a cellular substrate to the lesion site that supports axonal extension even in the absence of stem cell differentiation. The grafted C17.2 NSCs support extensive growth of axonal systems of several classes, including motor and sensory axons that have previously been reported to exhibit sensitivity to NGF, BDNF, or GDNF (Lindsay and Harnar, 1989; Otten et al., 1983; Tuszynski et al., 1994, 1996b; Vejsada et al., 1998; Yan et al., 1992). Indeed a likely mechanism contributing to stem cell-induced growth of axons in this study is the constitutive production of the growth factors NGF, BDNF, and GDNF both in vitro and in vivo. Genetically modifying stem cells to express another growth factor, NT-3, induces stem cell differentiation and expands the density and diversity of host axonal growth, yet reduces expression of GDNF and BDNF in vivo which in turn leads to a reduction (but not elimination) of local motor axon growth. Thus, growth factor expression in stem cells is dynamically regulated, likely as a function of differentiation state, and is further influenced by the production of other growth factors in the cell.

Recently it has been reported that embryonic stem cell grafts to the spinal cord promote functional recovery after injury (McDonald et al., 1999) and that one potential mechanism of this beneficial effect may be reduction of demyelination (Liu et al., 2000). Although demyelination contributes to functional loss after SCI, the majority of functional deficit after injury is attributable to loss of axonal connectivity. Thus, the present demonstration that stem cells augment host axonal growth presents an important mechanism whereby neural stem cells can contribute to reconstitution of neural structure independent of their differentiation fate.

The grafting of NSCs to specific regions of the nervous system has previously been reported to induce differentiation of cells to lineages appropriate to the environment in which they are placed (Brüstle et al., 1997; Gage et al., 1998; Lundberg et al., 1996; Snyder et al., 1997a; Suhonen et al., 1996). For example, hippocampal-derived NSCs placed in the ventral migratory stream adopt the phenotype of olfactory system neurons (Suhonen et al., 1996). Clone C17.2 NSCs grafted to other models of injury and degeneration, including phototoxic-induced cortical neuron degeneration (Snyder et al., 1997a) or demyelination (Yandava et al., 1999), induce differentiation and adoption of mature CNS cellular markers intrinsic to each model. In this study, C17.2 stem cell grafts to the injured spinal cord did not express mature markers of either neuronal or glial lineages; thus these cells did not reconstitute the cellular char-

acter of the preinjured spinal cord. This lack of differentiation may be a function of complexities intrinsic to SCI, including glial activation, inflammation (including altered cytokine levels), and extensive regional cellular degeneration. Yet the failure to differentiate did not compromise the ability of NSCs to promote neural repair via another mechanism: promoting host axonal growth. Indeed, it is possible that more extensive differentiation of stem cells could downregulate the expression of some genes that contribute to host axonal regeneration, including growth factors. When C17.2 NSCs were transduced to express NT-3, they differentiated more extensively and down-regulated expression of GDNF both in vivo and in vitro. This loss of GDNF expression correlated with a significant reduction of GDNF-sensitive motor axon penetration of the graft/lesion site. (It is not the case that transfection with NT-3 simply overwhelmed the protein synthesis capacity of stem cells resulting in a loss of GDNF production, because expression of NGF increased.) This reciprocal relationship between the enhanced expression of one growth factor at the expense of the expression of the other is intriguing. One explanation for this observation may be that GDNF is expressed by NSCs in their more immature state; NT-3 induces the cells toward a neuronal phenotype in which GDNF secretion is not characteristic. Alternatively, an as yet undefined complex reciprocal interplay may exist between growth factor signal transduction pathways.

Having established the feasibility of engineering NSCs to overexpress various growth factors to pattern host axonal regrowth, one can tailor the specific choice of growth factor to the class of axon that is targeted. For example, enhancement of NT-3 secretion is well suited to dorsal column sensory axons and is less appropriate for motor neuron projections. For the latter, augmentation of GDNF would be appropriate, a growth factor that is constitutively produced by the NSCs. Interestingly, one will need to coordinate the administration of these growth factors such that their application is additive and not antagonistic. The simultaneous cointegration of subclones of NSCs, each engineered to overexpress a different growth factor, may yield the most optimal and compatible therapies. Future investigations will address this issue.

A number of studies have reported a propensity of neural stem cells to migrate in the adult central nervous system. Cells of the subventricular zone of adult mammals repopulate the olfactory bulb throughout life by migrating through the rostral migratory stream (Luskin et al., 1997). Hippocampal precursor cells, if implanted into the rostral migratory stream, also migrate to the olfactory bulb (Gage et al., 1998; Suhonen et al., 1996). C17.2 NSCs implanted into the developing nervous system reportedly distribute to diverse locations of the brain and spinal cord (Snyder et al., 1992, 1997b). In the present study, grafted C17.2 NSCs remained within close proximity to the lesion/implantation site, filling the lesion cavity and migrating only short distances in the host cord. Although the specific “tropic” sig-

nals that influence stem cell migration in the nervous system remain to be defined, the restriction of NSCs to the lesion site in this SCI model may be optimal for initiating and supporting axonal growth through the lesion cavity. Indeed, the growth of NSCs in this lesion model was well-controlled, with no tumor formation and no cord distortion. Future studies will determine whether cell migration occurs at longer time points after grafting to sites of SCI.

The ability of stem cells to support axonal regeneration, as demonstrated in the present study, taken together with the previously demonstrated ability of NSCs to integrate in a stable fashion in other regions of the nervous system, suggests their potential utility as vehicles for gene therapy to promote CNS repair. The present findings indicate that stem cells can be transduced to express very high levels of neurotrophic factors, resulting in modification of their axonal growth-promoting properties in the injured spinal cord. Compared to previous studies utilizing grafts of genetically modified fibroblasts (Grill et al., 1997), these NSCs elicited responses from a broader range of host axons, most likely attributable to their constitutive production of growth factors. Future studies will examine the effects of overexpressing other growth factors in NSCs through extended time points in vivo to further characterize the potential of these multipotent cells to constitute useful vehicles for CNS gene therapy in a variety of CNS disorders.

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