

# Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons *in Vitro*, and Rescue Motoneurons after Spinal Cord Injury

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Interactions between ingrowing nerve fibers and their target tissues form the basis for functional connectivity with the central nervous system. Studies of the developing dental pulp innervation by nerve fibers from the trigeminal ganglion is an excellent example of nerve–target tissue interactions and will allow specific questions regarding development of the dental pulp nerve system to be addressed. Dental pulp cells (DPC) produce an array of neurotrophic factors during development, suggesting that these proteins might be involved in supporting trigeminal nerve fibers that innervate the dental pulp. We have established an *in vitro* culture system to study the interactions between the dental pulp cells and trigeminal neurons. We show that dental pulp cells produce several neurotrophic factors in culture. When DPC are cocultured with trigeminal neurons, they promote survival and a specific and elaborate neurite outgrowth pattern from trigeminal neurons, whereas skin fibroblasts do not provide a similar support. In addition, we show that dental pulp tissue becomes innervated when transplanted ectopically into the anterior chamber of the eye in rats, and upregulates the catecholaminergic nerve fiber density of the irises. Interestingly, grafting the dental pulp tissue into hemisectioned spinal cord increases the number of surviving motoneurons, indicating a functional bioactivity of the dental pulp-derived neurotrophic factors *in vivo* by rescuing motoneurons. Based on these findings, we propose that dental pulp-derived neurotrophic factors play an important role in orchestrating the dental pulp innervation. © 2001 Academic Press

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## INTRODUCTION

Dental pulp innervation occurs at relatively late postnatal ages in rodents compared to that of other trigeminal targets, and pulpal cells produce an array of neurotrophic factors at the time of initiation of the dental pulp innervation. Neurotrophins, glial cell-line derived neurotrophic factor (GDNF), and neurturin are expressed in developing teeth (Nosrat *et al.*, 1996a, 1997a, 1998a; Luukko *et al.*, 1997a,b, 1998). Interestingly, nerve growth factor (NGF),

brain-derived neurotrophic factor (BDNF), and GDNF mRNAs are upregulated in the dental pulp before the onset of dental pulp innervation (Nosrat *et al.*, 1998a). These neurotrophic factors might be crucial in supporting trigeminal nerves as they grow into the developing dental pulp and as the innervation of teeth is established. Indeed, neurotrophic factors are general modulators of neuronal survival, plasticity, and target innervation and play important roles in axon growth and synaptogenesis (see Lewin and Barde, 1996; Poo, 2001; Tucker *et al.*, 2001).

Even though the anatomical relation between developing teeth and trigeminal fibers has been extensively studied in the past (Byers, 1984; Luukko, 1997; Naftel *et al.*, 1999), our knowledge of how the dental pulp becomes innervated is still only fragmentary. Trigeminal axons are present before any structural signs of tooth formation are detectable. By

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the time epithelial changes occur in areas where dental ridges will form (Lefkowitz *et al.*, 1953) axons are present in the maxillary and mandibular processes (Luukko, 1997). Concomitant with dental epithelium thickening and the ecto-mesenchyme condensation underneath the epithelium, axons extend to the mesenchyme and then divide into lingual and buccal branches and grow toward the epithelium (Luukko, 1997). Sensory axons enter the dental pulp in the late bell stage (Pearson, 1977; Kollar and Lumsden, 1979; Mohamed and Atkinson, 1983; Fristad *et al.*, 1994). Nerve ingrowth into the dental pulp corresponds to postnatal day 4–5 in mice and rats. Sensory pulpal axons develop rapidly during the subsequent mineralization, crown and root formation, and tooth eruption. Subodontoblastic, odontoblastic, and predentinal axonal networks are formed and axons are present in cuspal dentinal canals by the time teeth have become functional (Byers, 1980). Interestingly, teeth in the osteopetrotic mutant mice become innervated in spite of the presence of bone abnormalities and failure of root formation (Nagahama *et al.*, 1998), indicating that parts of the dental pulp innervation process might be regulated by local pulpal events and interactions between the developing dental pulp and the ingrowing trigeminal nerve fibers.

To understand the mechanisms behind tooth innervation, we established *in vitro* and *in vivo* models to characterize dental pulp interactions with neuronal tissues. We show that DPC produce neurotrophic factors in culture and provide neurotrophic support for trigeminal neurons *in vitro*. Interestingly, dental pulp-derived neurotrophic factors promote survival of and sprouting from trigeminal and sympathetic ganglia, two cranial ganglia that innervate the teeth *in vivo*. We also show that dental pulp tissue becomes innervated when grafted ectopically into the anterior chamber of the eye in rats and promotes motoneuron survival after spinal cord hemisection.

## MATERIALS AND METHODS

Sprague–Dawley rats (B&K Universal, Sollentuna, Sweden; Charles River, Wilmington, MA) were used. Procedures were approved by the Institutional Animal User Committee (IAUC) at the University of Michigan, and the local Animal Research Committee of Stockholm. Dental pulp tissue from rat pups, 5–6 days old were used in all experiments. The reasons for choosing this particular age were (1) dental pulp does not become innervated prior to P4–5; (2) we have shown that NGF, BDNF, and GDNF mRNAs are upregulated in postnatal teeth (Nosrat *et al.*, 1998); and (3) based on the rather late onset of the dental pulp innervation compared to that of other trigeminal targets, there is a possibility that repellent factors might be present in the dental pulp prior to the initiation of dental pulp innervation. For intraocular experiments, dental pulp tissue was removed under aseptic conditions and placed in Dulbecco's modified Eagle's medium and Ham's F12 nutrients (DMEM/F12; GIBCO BRL) prior to transplantation. For cell culture studies, trigeminal ganglia and dental pulps were dissected and placed in a sterile petri dish containing ice-cold DMEM/F12, 10% fetal bovine serum (FBS; GIBCO BRL),

L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin (2 µg/ml, initial 2 weeks), and gentamicin (50 µg/ml). Tissue pieces were mechanically dissociated and washed twice with Earl's balanced salt solution (EBSS) without calcium or magnesium. Tissue fragments were then trypsinized for 20–30 min with 0.25 µg/ml trypsin, and were further mechanically dissociated with fire-polished Pasteur pipettes and seeded ( $\approx 10^5$ ) on poly D-lysine (100 µg/ml) treated flasks. Dissociated trigeminal tissue fragments were seeded onto chamber slides with dental pulp cells (DPC) that had been subcultured at least once. The chamber slides with DPC had not reached confluency when trigeminal tissue was added. After letting trigeminal cells settle for 30 min, excessive cells were removed by aspirating the medium. This was done to minimize the number of nonneuronal cells in the cocultures, specifically that of Schwann cells (our personal observations).

## Cell Culture Experiments

Cultures were kept in a humidified incubator at 37°C with 95% air/5% CO<sub>2</sub>, and the medium was changed every 2 days. The medium contained either 1% or 10% FBS. When DPC reached confluency, as determined using an inverted microscope, they were subcultured. Cells were subcultured by trypsinization (0.25% trypsin) and were used in the experiments after 1 or 2 passages.

**Skin fibroblast cultures.** The procedures were according to Kawaja *et al.* (1992). Briefly, abdominal skin from an adult rat were shaved, washed with 70% ethanol, excised and cut into fine pieces, washed several times, and added into culture flasks. Fibroblasts generally grow rapidly and migrate from the explants. When confluent, cultures were trypsinized and fibroblasts were transferred onto glass-chamber slides coated with poly D-lysine and laminin.

## Total RNA Extraction and RNase Protection Assay

Total RNA was isolated from dental pulp cells (RNeasy mini kit; Qiagen) and stored at –70°C until use. Both primary cultures and subcultured cells had been grown in culture for at least 4 weeks prior to RNA isolation. NGF, BDNF, and GDNF ribo-probes (for details about the probes see <http://cajal.mbb.ki.se/reagents#probes>) were *in vitro* transcribed and labeled with <sup>32</sup>P-UTP (Amersham) using linearized template DNA fragments. RNase protection assay (RPA) was performed according to manufacturers' protocols (Promega: *in vitro* transcription; Ambion: RPA). A commercially available rat cyclophilin fragment (Ambion) was used as a control and to confirm equal RNA loading in all lanes of a given experiment. A 100 base-pair marker (RNA Century marker templates; Ambion) was also *in vitro* transcribed and labeled with <sup>32</sup>P-UTP. After hybridization and subsequent RNase digestion and gel electrophoresis on a 6% polyacrylamide gel, the protected bands were visualized (Storm 840). NIH-Image was used to quantify the levels of expression of neurotrophic factors relative to each other and to that of cyclophilin.

## Intraocular Transplantations

P6 rat pups ( $n = 20$ ) were ether-anesthetized and decapitated. Tooth pulp tissue was dissected under aseptic conditions, each pulp was dissected into 6–8 pieces and was kept in DMEM/F12 until transplanted. Rats (150-g females,  $n = 20$ ) received 1 drop of 0.1% atropine in each eye for dilating the irises and preventing prolapse. The transplantation procedure was according to Olson

and colleagues (1983). Briefly, intraocular grafting was performed under ether-anesthesia and grafts were introduced into the lateral angle of the anterior chamber. Recipient rats received a small cut on the cornea with an iris scalpel and the pupal tissue was grafted into the eye and positioned in contact with the anterior surface of the host iris. The cornea received an incision in control animals but no pulp tissue was grafted into the anterior chamber of the eye. At the termination of the experiments (5 and 10 weeks), the animals were anesthetized with ether and euthanized by cervical dislocation, and transplants were dissected out and processed. Dental pulp grafts were fixed in 4% PFA in PBS overnight, washed in 10% sucrose in PBS, frozen on dry ice, and cryosectioned. Protein gene product (PGP) 9.5 immunohistochemistry was used to visualize nerve fibers.

### Falck–Hillarp Histochemistry

Iris from the rats in the intraocular transplantation experiments were evaluated using Falck–Hillarp monoamine histochemistry (Falck *et al.*, 1962; Corrodi and Jonsson, 1967) to visualize catecholamine-containing nerve fibers. Dental pulp grafts were removed carefully and the irises were stretch-prepared as whole mounts on glass slides and air-dried. Slides were then reacted with gaseous paraformaldehyde. The catecholaminergic nerve fiber density of irises (controls and dental pulp graft recipients) was estimated semiquantitatively on a blind basis using a fluorescence microscope and appropriate filters. The value 100 represents the normal iris catecholamine nerve fiber density, and is presented in Fig. 6A. Statistical analysis was performed using an unpaired Mann–Whitney *U*-test.

### Immunohistochemistry

**Cell cultures.** Cells were fixed in 4% paraformaldehyde in PBS (PBS, 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3) for 30–60 min, washed with PBS five times for 6 min, preincubated in PBS containing 5% goat serum, 5% bovine serum albumin, and 0.3% Triton X-100 for 1 h, followed by overnight incubation with the different antisera in PBS/0.3% Triton X-100. Antibodies against PGP 9.5 (1:400 dilution; Biogenesis Ltd., UK), growth-associated protein 43 (GAP, 1:500 dilution; Chemicon International, Temecula, CA), and Synaptophysin (1:400; Chemicon International) were used. Chamber slides were subsequently washed four times in PBS, incubated for 1 h with rhodamine or FITC-conjugated secondary antibodies, washed three times in PBS, and mounted. PGP 9.5 antibodies were also detected by biotinylated secondary antibodies, followed by an avidin–biotin complex (Vectastain Elite; Vector Laboratories, Burlingame, CA). The peroxidase activity was visualized with diaminobenzidine (DAB peroxidase substrate kit; Vector Laboratories).

### Cell Nuclei Labeling with Hoechst Nuclear Fluorochrome

To distinguish between the contributions from different tissue components in the coculture experiments, and to distinguish between dental pulp and the surrounding tissues after transplantation into the spinal cord, we incubated dissociated cells from the trigeminal ganglion and dental pulp tissue and cells for 30 min at 37°C in the dark in culture medium containing 10 µg/ml bisbenzimidazole (Sigma). Labeled cells and dental pulp tissue (also incubated for 30 min at 37°C) were rinsed at least three times with HBSS and

twice in culture medium. Cells were resuspended and seeded and tissue fragments were used in transplantation experiments. The labeled cell nuclei appear blue when observed under a fluorescence microscope.

Dental pulp cells and trigeminal cells alone were also labeled and observed for a period of 1 month to study possible cytotoxic effects of bisbenzimidazole on these cells. Cells appeared normal without nuclear condensations, indicating that the labeling technique does not induce apoptosis or other overt cytotoxic effects on cultured cells at the concentration used.

### Spinal Cord Transplantations and Motoneuron Survival Assay

Dental pulp was collected from 5- and 6-day-old Sprague–Dawley rats (B&K Universal). Graft recipients and controls were adult female Sprague–Dawley rats (250–300 g). A subpopulation of motoneurons in the lumbar enlargement was prelabeled with FluoroGold (Fluorochrome, Englewood, CO) under general halothane (Fluothane; AstraZeneca) anesthesia, by cutting the peroneal nerve just above the level of the knee joint and putting the proximal end of the nerve in 2% FluoroGold-Ringer's solution for 30 s. FluoroGold labeling was carried out ipsilateral to the spinal cord hemisection. The general procedure was according to Novikova and colleagues (1996). To determine the relative normal number of motoneurons sending axons into the peroneal nerve, the motoneuron columns of two noninjured animals were also labeled bilaterally with FluoroGold. The peroneal motoneuron pool is located in a column of cells extending through spinal cord segments L4–L6 (Swett *et al.*, 1986). Three days after labeling, rats were placed on a rectal thermometer feedback-adjusted heating pad and operated under general halothane (Fluothane) anesthesia. Incisions were made through skin and muscle, and a laminectomy was performed to expose the L5 spinal cord segment. The exposed spinal cord was hemisected, cutting the right side completely (hemisection,  $n = 8$ ). Dental pulp tissue, collected from first molars, was cut in pieces of about 1 mm<sup>3</sup> and two to three pieces were gently inserted into the spinal cord lesions using fine-tipped forceps ( $n = 4$ ). Hemostasis was achieved and muscle and skin were sutured in layers (3/0 PDSII and 2/0 Ethilon II, Ethicon, respectively). Antibiotics (Borgal; Hoechst) were given immediately before surgery and daily 1 week postoperatively. To avoid urinary tract infections, manual emptying of the urinary bladder was carried out three times daily the first week and twice daily thereafter.

Animals were euthanized and processed 4 weeks after injury. Spinal cords and grafts were dissected, postfixed in fixative for 1 h, and transferred to 10% sucrose solution for cryoprotection. Coronal 20 µm cryostat sections were made from spinal cord segment L2–S1, and a relative number of motoneurons was calculated by counting labeled motoneurons on every other section.

**Statistical analyses.** Data were evaluated using ANOVA and Fischer's post hoc test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

### In Situ Hybridization

GDNF has been shown to be a potent survival factor for motoneurons (Henderson *et al.*, 1994; Oppenheim *et al.*, 1995). Therefore we used *in situ* hybridization to investigate whether the dental pulp explants express GDNF mRNA after transplantation into the spinal cord. Rats were euthanized by decapitation at 1 day ( $n = 2$ ) and 7 days ( $n = 2$ ) after surgery. Spinal cord tissue was

dissected and frozen on dry ice. The procedure for *in situ* hybridization was previously described elsewhere (Dagerlind *et al.*, 1992). An oligonucleotide probe complementary to GDNF (nucleotides 540–589) (Lin *et al.*, 1993) was used. The probe was previously characterized (Nosrat *et al.*, 1996a) and does not match any known sequence in GenBank except that of GDNF. As a control, a 50-mer random probe (Nosrat and Olson, 1995), not complementary to any sequence in GenBank, was used. No specific pattern of hybridization signals was found with this random probe.

## RESULTS

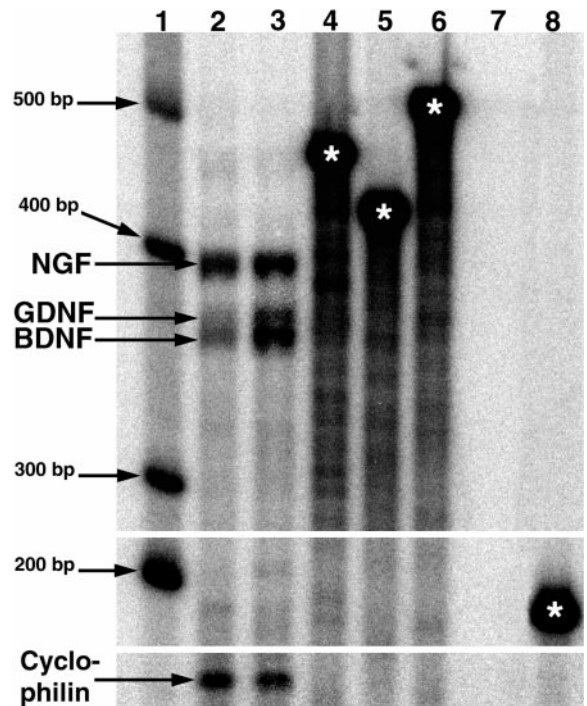
### Dental Pulp Cells Produce Neurotrophic Factors *in Vitro*

NGF, BDNF, and GDNF transcripts are found *in vivo* in the dental pulp at the time of the onset of dental pulp innervation (Nosrat *et al.*, 1998a), that is, at the time when cultures were established. To study whether these factors are expressed by dental pulp cells *in vitro*, we used RNase protection assays (Fig. 1). Cell cultures were established from dissociated dental pulp of 6-day-old rat pups. The cells grew well in culture and were generally fibroblast-like but had a large circumference (Fig. 2A). Small islands of epithelial cells were occasionally observed (10–20 cells) in a few cultures, and were surrounded by confluent dental pulp cells (possibly contamination from the cervical loop area of the developing teeth, which is an epithelial-derived tissue component). Dental pulp cultures (both primary and subcultured) were generally maintained 4–6 weeks prior to RNA isolation. NGF, BDNF, and GDNF mRNA transcripts were expressed by primary cultures of dental pulp cells (Fig. 1). Interestingly, subcultured dental pulp cells also expressed neurotrophic factor mRNA transcripts (Fig. 1). Expression levels of mRNA transcripts were upregulated after subculturing, indicating that this process upregulates neurotrophic factor gene transcription. These results thus suggest long-term neurotrophic competence of the dental pulp cells in culture.

### Trophic and Tropic Effects of Dental Pulp Cells on Cocultured Trigeminal Neurons

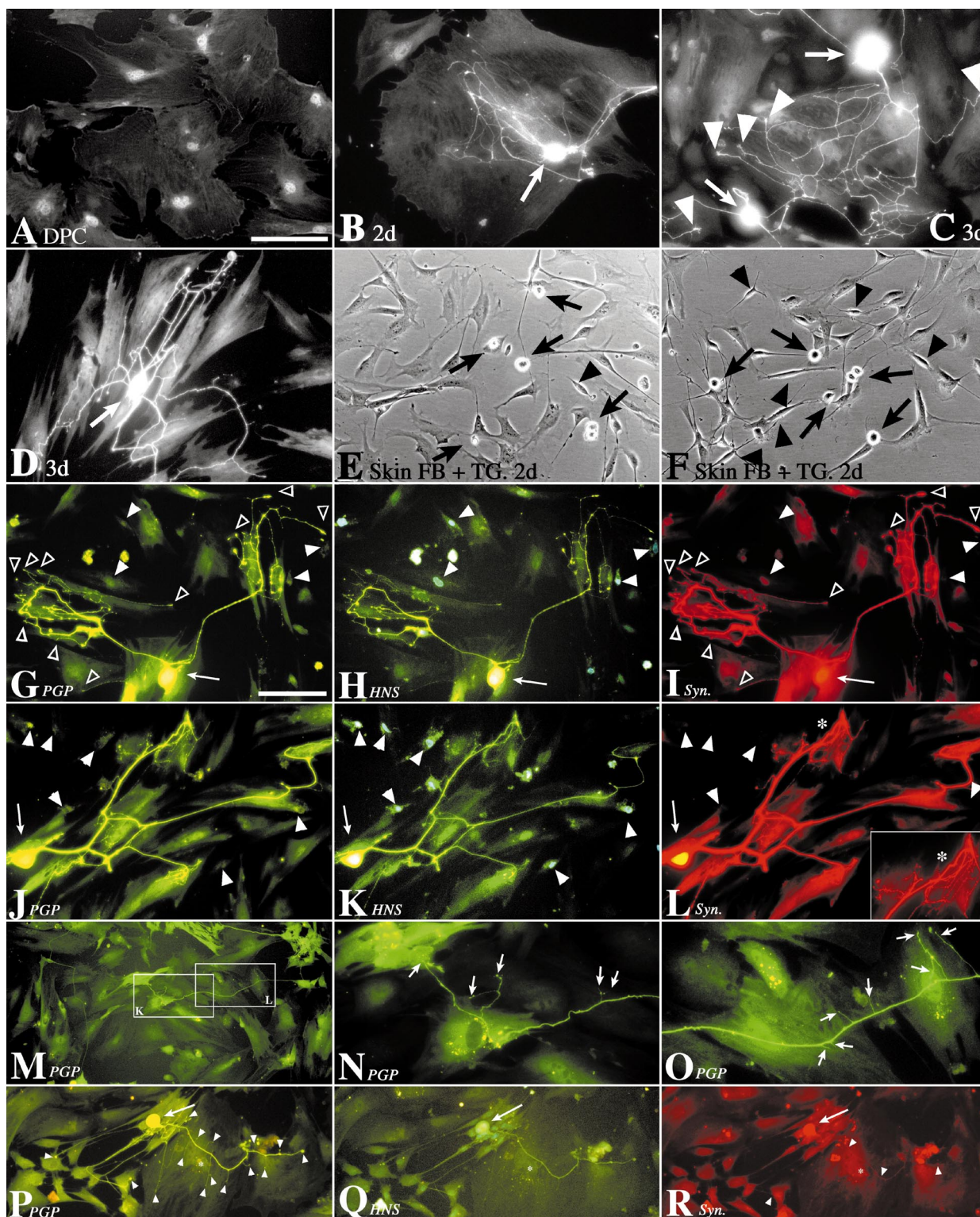
To study possible interactions between dental pulp cells and trigeminal neurons, and based on our finding that neurotrophic factor mRNAs are upregulated in subcultured dental pulp cells, they were cocultured with trigeminal neurons. The mandibular portion of the trigeminal ganglion (or the whole ganglion) was dissected by separating the part of the ganglion attached to the mandibular nerve by blunt dissection, dissociated, and added into the cultures of DPC that had been subcultured at least once and kept in slide chambers a week prior to the coculture procedure.

Trigeminal neurons thrived well in cocultures and sent out neurites, starting after 1 day in culture (see Figs. 2 and 3). Neurites grew toward dental pulp cells, and elaborately

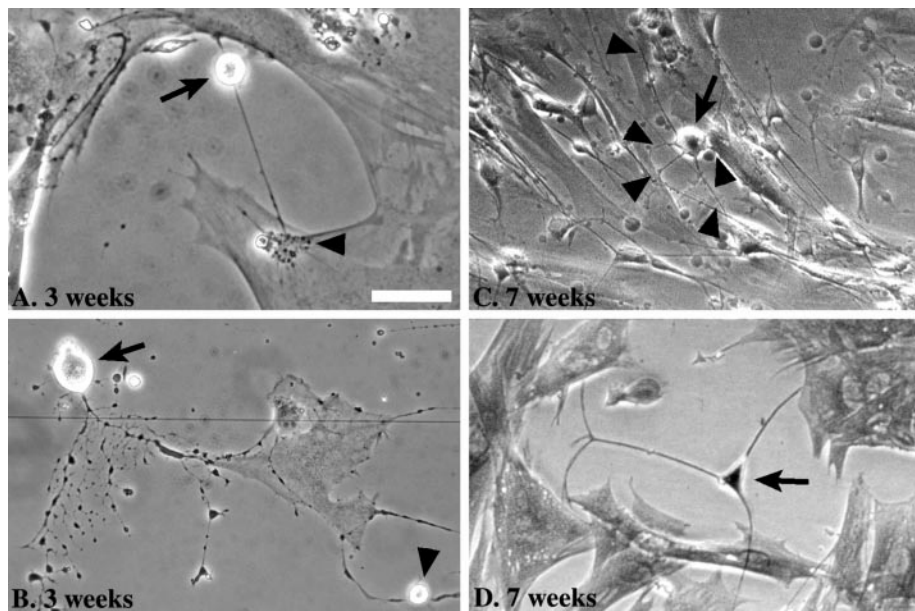


**FIG. 1.** Neurotrophic factor mRNA expression analyzed by RNase protection assay. Primary cultures (lane 2) and subcultured (lane 3) dental pulp cells express NGF, BDNF, and GDNF mRNAs. Arrows point to bands of protected mRNA complementary to the three assayed neurotrophic factors. A riboprobe complementary to cyclophilin was used as an internal standard. Interestingly, when dental pulp cells are subcultured, neurotrophic factor mRNA expression is upregulated. In this example, NGF mRNA is upregulated by 25% content (NIH-image version 1.61), BDNF mRNA by 200%, and GDNF mRNA by 250% relative to mRNA levels expressed by primary cultures of dental pulp cells and standardized to cyclophilin (contrast and brightness were adjusted on lane 3 to visualize neurotrophic factor mRNA upregulation in the subcultured dental pulp cells in this photomicrograph). Lane 1: 100-bp RNA marker; lanes 4–6 and 8: positive controls for the probes; yeast transfer RNA hybridized with riboprobes for NGF, BDNF, GDNF and cyclophilin, respectively; lane 7: yeast transfer RNA was used as negative control.

above some dental pulp cells. It appeared that some pulpal cells were preferred targets compared to other cells, indicating a possible molecular heterogeneity among the pulpal cells in culture irrespective of their similar morphology. The neurite extension patterns were not related to neuron morphologies; neurons of different sizes with different numbers of processes gave rise to neurites that grew toward pulpal cells and ramified above them (arrows in Figs. 2B–2D). Interestingly, this pattern persisted for several weeks (Figs. 3A and 3B: 3 weeks; Figs. 3C and 3D: 7 weeks; Figs. 4J–4O: 2 weeks). No specific and elaborate neurite outgrowth pattern was observed from trigeminal neurons when skin fibroblasts were utilized instead of dental pulp



**FIG. 2.** (A) Photomicrograph of unlabeled dental pulp cells under fluorescence illumination visualizing dental pulp cell morphology in culture. Dental pulp cells grow well in culture and are generally fibroblast-like with large circumference. (B-D) Photomicrographs of trigeminal neurons (arrows) cocultured with dental pulp cells. Culture medium was supplemented with 10% FBS in these experiments. The times after which the cocultures were processed after addition of trigeminal neurons into the cultures are indicated. In B and C, protein gene

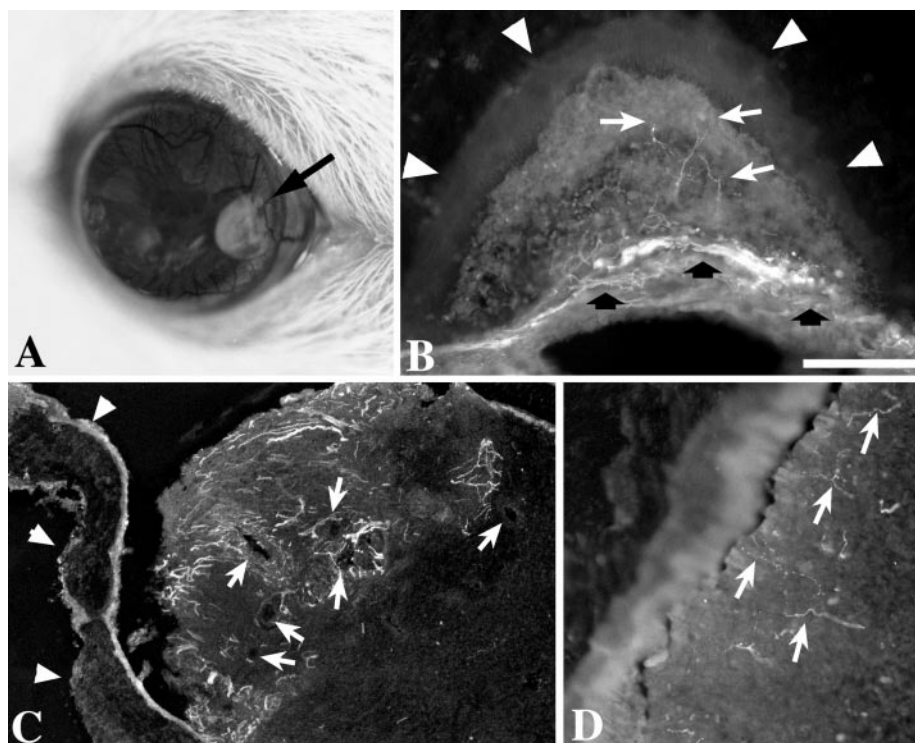


**FIG. 3.** Examples of neuronal morphologies of trigeminal neurons cocultured with dental pulp cells for either 3 weeks (A, B) or 7 weeks (C, D). Photomicrographs were taken with an inverted microscope using phase-contrast illumination. Arrows are pointing at neurons in all figures. (A) A bipolar neuron contacting two flat fibroblast-like cells. One neurite gives rise to terminal ramifications on the edge of the fibroblast-like cell (arrowhead). (B) A large unipolar neuron with extensive processes, some of which are associated with a flat, fibroblast-like cell. A small bipolar neuron is seen in the lower part of the photomicrograph. (C) A multipolar neuron in close association with several fibroblast-like cells. Arrowheads point at neurites extending from this neuron. (D) A multipolar neuron associated with several fibroblast-like cells. The neuron is PGP 9.5 positive (DAB-stained). Scale bar in A = 30  $\mu\text{m}$ .

cells (Figs. 2E and 2F). Indeed, trigeminal neurons were no longer observed after 3 days in culture in the presence of the skin fibroblasts. Fibers were also extended from distant trigeminal neurons to pulpal cells (Figs. 2G–2R), indicating

that soluble long-range axonal guiding molecules might be involved. The preferred pulpal cells that neurites grew toward, and ramified around, demonstrated a fibroblast-like appearance and generally had a large circumference (see

product 9.5 immunoreactivity is visualized, in D synaptophysin immunoreactivity. Figures G–R are color photomicrographs of cocultures of trigeminal neurons with dental pulp cells. Antibodies to protein gene product 9.5 (PGP; G, J, M–O, P), synaptophysin (Syn.; I, L, R), and Hoechst nuclear staining (HNS; H, K, Q) were used. Photomicrographs G–O were taken 3 days after trigeminal neurons were added into the cultures, P–R after 2 weeks. Culture medium was supplemented with 1% FBS in G–O, and with 10% FBS in P–R. (B) A dental pulp cell with a large circumference and a trigeminal neuron (arrow) above it. The trigeminal neuron appears bipolar and has given off elaborate and extensive neurites covering the dental pulp cell. Many neurites have varicosities. (C) Two multipolar trigeminal neurons of different sizes (arrow and arrowhead) are associated with some dental pulp cells. (D) A synaptophysin-positive bipolar neuron (arrow), its branches and end-terminals expanding over several dental pulp cells and giving off several branches. (E, F) Trigeminal neurons (TG, arrows) do not give rise to elaborate patterns of neurite outgrowth, when cocultured with skin fibroblasts (FB). Although some trigeminal neurons survive 2 days in cocultures with skin fibroblasts, they disappear after 3 days in culture. Schwann cells are spindle-shaped and could easily be distinguished in the cultures based on their morphology (arrowheads). (G–L) An extensive pattern of neurite outgrowth develops in the cocultures. Using the stain, it is shown that the cells that neurites have grown toward and ramify on, originate from the dental pulp and are not from the trigeminal ganglion. Cells with blue nuclei (solid arrowheads) are of trigeminal origin and may include Schwann cells, satellite cells, fibroblasts, and neurons. Neurons, however, are intensely bright and therefore their nuclei usually masked. Extensive patterns of neurite outgrowth are not found associated with cells with blue nuclei. Many neuronal branches and their terminal ramifications exhibit varicosities (empty arrowheads). Fine ramifications of the nerve fibers are also synaptophysin positive (I, L). The inset in L is a higher magnification of the area marked with asterisk. (M–O) Neurites extend relatively long distances and branch above some dental pulp cells (arrows in M–O). N and O are higher magnifications of the boxed areas in M. (P, R) Neurites as well as Hoechst staining persist for several weeks in the cultures. Branches have varicosities (arrowheads). Scale bar in A represents 200  $\mu\text{m}$  in A and F–G, and 100  $\mu\text{m}$  in B–E. Scale bar in G represents 100  $\mu\text{m}$  in G–L and N–O, and 50  $\mu\text{m}$  in M and P–R.



**FIG. 4.** (A) A dental pulp graft (arrow) in the anterior chamber of the eye photographed after 9 weeks *in oculo*. Grafts can be visually inspected and are not expected to affect vision (do not cover the pupil). (B) Section of graft and host iris 4 weeks after grafting. PGP-positive nerve fibers (arrows) grow into the dental pulp tissue from the iris (thick black arrows). Upper part of the transplant is covered by a fibrous capsule (arrowheads). (C) Dental pulp tissue becomes extensively innervated after 8 weeks *in oculo*. Many PGP-positive fibers are present in the area of the pulp tissue located toward the fibrous capsule/hard tissue (arrowheads). The part of the pulp tissue toward the iris is less innervated (marked by asterisk). Many blood vessels are also found in the pulp tissue, indicating postgrafting angiogenesis (arrows). (D) The hard tissue that forms has a dentin-like character; it contains patterned tubuli (area of the asterisk) and a predentin zone (marked by arrowheads). (E) Many nerve fibers (arrows) are found adjacent to the hard tissue, resembling the nerve plexus of Raschkow in teeth *in vivo*. (F, G) PGP-positive nerve fibers (arrows) penetrate into the dentinal tubuli in the predentin zone (arrows in F) or into the area of the fibrous tissue covering the dental pulp tissue (asterisk in G). Scale bar in B represents 200  $\mu\text{m}$  in B, 500  $\mu\text{m}$  in C, and 100  $\mu\text{m}$  in D–G.

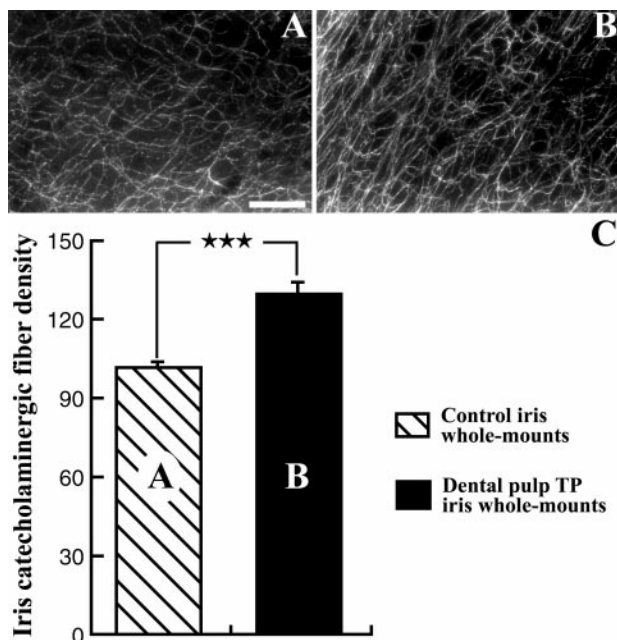
Figs. 2B and 2C for examples). Nerve fibers and some of their contact areas with pulpal cells were synaptophysin positive (Figs. 2D, 2I, 2L, and 2R), indicating a possible formation of synapses/close association between nerve fibers and these cells.

Hoechst nuclear staining enabled us to trace the origin of the fibroblast-like cells that interacted with trigeminal neurons. When trigeminal contributions were labeled, all of the cells from the trigeminal ganglion (i.e., neurons, Schwann cells, satellite cells, and fibroblasts) had blue-labeled nuclei and could be distinguished from dental pulp cells (compare Figs. 4G and 4H, 4J and 4K, and 4P and 4Q). All of the fibroblast-like cells that showed close interaction with trigeminal neurites had unlabeled nuclei (compare Figs. 4G and 4H, 4J and 4K, and 4P and 4Q), indicating a dental pulp origin. Occasionally fusiform spindle-shaped Schwann cells were found in close association with the trigeminal neurites (not shown). The pattern of neurite outgrowth was persistent in the cocultures, whether the

culture medium was supplemented with 1 or 10% FBS (compare Figs. 2B–2D and 4M–4R for culture medium supplemented with 10% FBS and Figs. 4G–4L for culture medium supplemented with 1% FBS).

#### ***Dental Pulp Becomes Vascularized and Innervated Intraocularly and Increases Catecholamine Nerve Fiber Density in Host Irises***

Dental pulp appeared intact in the host eyes (arrow in Fig. 4A) and had become vascularized a few days after transplantation. Already after a week in the anterior chamber of the eye, the dental pulp seemed to resume its programmed development, including hard tissue formation (not shown). However, the size of the transplants did not increase (not shown). We did not see enamel formation, but we observed patterned tubuli in the hard tissue similar to those found in dentin (not shown). Dental pulp transplants were studied



**FIG. 5.** The density of sympathetic, noradrenergic nerve fibers of the irises increases when dental pulp is transplanted (TP) into the anterior chamber of the eye. Scale bar = 100  $\mu\text{m}$ . (A) Normal density of sympathetic ground plexus of control irises as visualized by Falck–Hillarp fluorescence histochemistry. This particular sample received a scoring value of 100. (B) Higher densities of nerve fibers are observed in the irises when dental pulp is transplanted into the anterior chamber of the eye compared to that observed in the irises of sham-operated eyes. The iris in B was scored blindly as 150. (C) The density is increased significantly (Mann–Whitney test, \*\*\*,  $P < 0.0001$ ).

using antibodies to PGP 9.5 to investigate the presence of nerve fibers in the transplants (Figs. 4B–4D). Interestingly, despite the formation of hard tissue and an occasional fibrous capsule surrounding different sides of the grafts (demarcated by arrowheads in Figs. 4B and 4C), nerve fibers entered the dental pulp grafts and were found throughout the transplants (arrows in Figs. 4B–4D). Nerve fibers were present in the pulpal tissue underlying the hard tissue, and occasionally in association with the presumed dentinal tubuli, penetrating them for a short distance (Fig. 4D). At 10 weeks, dental pulps contained more nerve fibers (Fig. 4C) than at 5 weeks (Fig. 4B). The development of the innervation of the dental pulp grafts intraocularly indicates that the major molecular mechanisms that lead to dental pulp innervation already reside in the dental pulp and are orchestrated by the dental pulp. To evaluate the bioactivity of dental pulp-derived neurotrophic factors, we studied the sympathetic nerve fiber density of the host irises. Falck–Hillarp histochemistry was used to evaluate catecholaminergic nerve fiber density of the irises. Irises from dental pulp-grafted eyes after 10 weeks *in oculo* had a significantly

increased number (i.e., density) of catecholaminergic nerve fibers compared to that of controls (Figs. 5A–5C). Increased density of nerve fibers was not restricted just to areas in close proximity to the dental pulp graft; it also had a general character and was found generally in the whole iris (compare Figs. 5A and 5B).

### Motoneuron Survival Is Enhanced by Dental Pulp Grafts

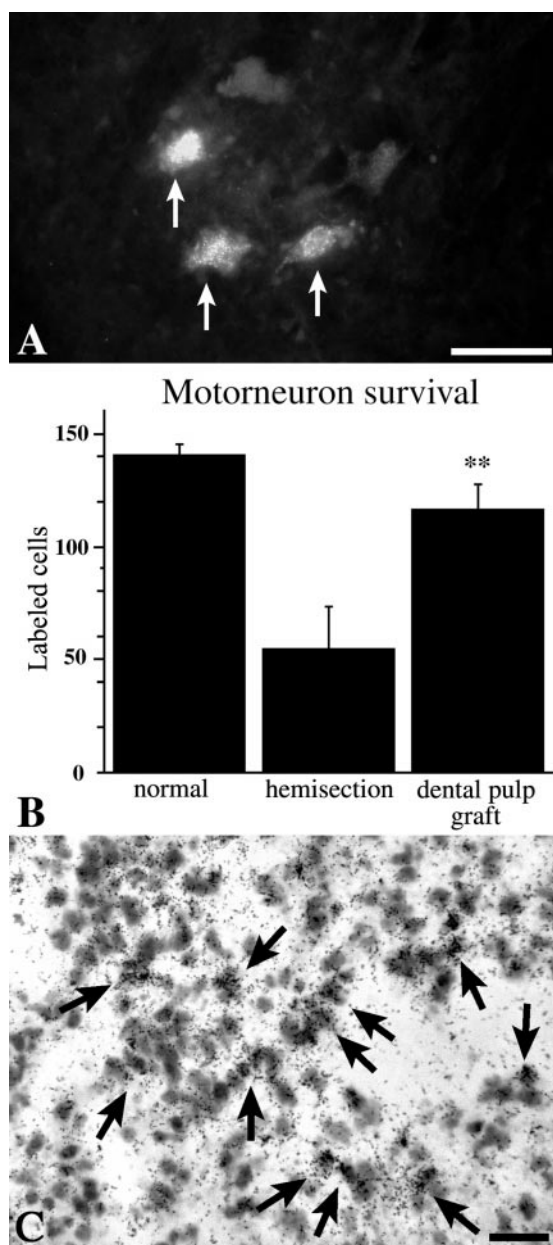
An example of FluoroGold-labeled motoneurons from the lumbar enlargement is given in Fig. 6A. In normal uninjured animals the relative number of FluoroGold-labeled cells was approximately  $140 \pm 5$  (means  $\pm$  SEM,  $n = 4$ , 2 left + 2 right sides, Fig. 6B). A hemisection of the spinal cord resulted in a statistically significant (ANOVA,  $P < 0.01$ ) loss of this motoneuron population, so that  $54 \pm 19$  (39% of normal) cells remained visible at 4 weeks after injury (Fig. 6B). In spinal cords with dental pulp grafts the relative number of labeled neurons was  $116 \pm 12$  (83% of normal). Hence, grafting of dental pulp increased the number of surviving motoneurons by 115% compared to that of the hemisection (ANOVA,  $P < 0.01$ , Fig. 6B).

GDNF has been proposed to be a potent survival factor for motoneurons (Henderson *et al.*, 1994; Oppenheim *et al.*, 1995). We therefore investigated whether GDNF is expressed in the grafts after transplantation into the spinal cord. One day after grafting, strong GDNF mRNA labeling was found in the grafted dental pulp tissue (Fig. 6C). GDNF labeling was strong in most cells of the graft. One week after injury, GDNF mRNA labeling was still observed in dental pulp grafts; however, the labeling had become weaker compared to that at 1 day postinjury.

## DISCUSSION

Here, we show that dental pulp cells produce several potent neurotrophic factors *in vitro*. Messenger RNA transcripts for NGF, BDNF, and GDNF are expressed by dental pulp cells in culture and become upregulated when dental pulp cells are subcultured. When dental pulp cells are cocultured with trigeminal neurons, they promote survival and neurite outgrowth from trigeminal neurons. The neurite outgrowth appears specifically directed toward dental pulp cells. Intraocularly grafted dental pulps become innervated and increase the density of host iris catecholaminergic nerve fibers. Dental pulp grafted to the spinal cord promoted motoneuron survival in hemisected spinal cord.

In the present study, we provide an *in vitro* model in which dental pulp cell–trigeminal neuron interactions could be studied without the addition of exogenous neurotrophic factors into the culture system. This model will enable further studies of the molecular mechanisms under-



**FIG. 6.** (A) An example of FluoroGold-labeled motoneurons from the lumbar enlargement (scale bar = 50  $\mu$ m). (B) The relative number of motoneurons surviving spinal cord hemisection is increased when dental pulp is grafted into the site of the lesion. Four weeks after spinal cord hemisection at the level of the lumbar enlargement, the relative number of FluoroGold-labeled motoneurons is drastically decreased compared to that of normal intact animals ( $P < 0.01$ ). In animals that received dental pulp grafts the relative number of FluoroGold-labeled motoneurons is significantly higher than in animals receiving hemisection only ( $P < 0.01$ ). (C) *In situ* hybridization with an oligo-probe complementary to GDNF mRNA. One-day postgrafting cells in the graft are strongly labeled (e.g., at arrowheads). Scale bar = 50  $\mu$ m.

lying dental pulp innervation *in vitro*. We also utilized the *in vivo* intraocular technique to study nerve fiber ingrowth into dental pulp ectopically grafted into the anterior chamber of the eye. The intraocular technique was previously utilized to characterize tissue interactions involved in tooth development (Kollar and Lumsden, 1979; Lumsden and Buchanan, 1986; Lumsden, 1988).

Neurotrophic factors play an important role in regulating neuronal survival and differentiation (see Barbacid, 1995; Lewin and Barde, 1996) as well as axonal growth and path-finding (Tucker *et al.*, 2001). Exogenous neurotrophic factors are a crucial requirement for *in vitro* survival of central and peripheral nervous tissues (see Davies, 1994a,b; Rochlin *et al.*, 2000). However, no exogenous neurotrophic factors were added into the cocultures of dental pulp cells with trigeminal neurons in the present study. The neurotrophic factor requirement for neuronal survival is eliminated in transgenic mice in which the *Bax* gene is null-mutated. BAX is a regulator of apoptosis and it has been shown that sympathetic neurons from *Bax*<sup>-/-</sup> survive indefinitely *in vitro* without NGF and motoneurons survive neonatal axotomy (Deckwerth *et al.*, 1996). Lentz and colleagues (1999) studied neurite outgrowth patterns from *Bax*<sup>-/-</sup> DRG neurons and showed that neurotrophins influence axonal elongation and distinct axonal morphologies *in vitro*, but they are not required for the survival of *Bax*<sup>-/-</sup> neurons. Only about 5% of wild-type DRG neurons survived without exogenous neurotrophins after 24 h in culture and all had died after 72 h in culture.

When E15 rat trigeminal neurons were studied (Ulupinar *et al.*, 2000) all neurons had died after 48 h in culture without exogenous neurotrophic factors. The culture medium was supplemented with 10% FBS, indicating that neither a high concentration of FBS nor the presence of Schwann cells can promote survival of trigeminal neurons without the addition of exogenous neurotrophic factors. It is well established that Schwann cells (and fibroblasts) grow and proliferate well in culture medium supplemented with 10% FBS. To minimize proliferating nonneuronal cells in the present study (specifically Schwann cells), a low concentration of FBS (1%) was supplemented in the culture medium. Interestingly, we did not observe a difference in neurite outgrowth pattern from trigeminal neurons when either 1 or 10% FBS was supplemented to the culture medium (see Figs. 2 and 4). This suggests that neither low concentrations of serum nor Schwann cells significantly influence survival of trigeminal neurons in our experiments. In addition, Schwann cells present in the cultures did not suffice to promote survival and neurite extension from trigeminal neurons when skin fibroblasts were utilized. Schwann cells have been shown to be an important source of trophic support for developing motor and sensory neurons (Riethmacher *et al.*, 1997). In our culture system, Schwann cells constitute only a small fraction of cells added to the established cultures of dental pulp cells. We also show that dental pulp cells provide neurotrophic support for the trigeminal neurons in the culture system. The

elaborate neurite ramifications we report in the present study resemble Bax<sup>-/-</sup> neurons grown with exogenous neurotrophins. We propose that dental pulp cells provide a potent cocktail of neurotrophic factors that promote survival and influence axonal morphology of dissociated trigeminal neurons in culture.

We also show that dental pulp tissue becomes innervated when ectopically transplanted into the anterior chamber of the eye in rats. The iris has an exceptionally rich innervation, including fibers from the trigeminal, ciliary, and sympathetic ganglia (Olson *et al.*, 1988). In rodents, trigeminal nerve fibers reach the developing teeth during the early stages of tooth development, but do not innervate the teeth until postnatal stages (Mohamed and Atkinson, 1983). These clearly indicate that the major molecular cues for dental pulp innervation are inherent to the dental pulp itself, and these same molecules can regulate ingrowth of several different types of nerve fibers into the dental pulp *in situ* and ectopically, where appropriate nerve fibers are present. Like other peripheral tissues grafted to the eye chamber (Olson and Malmfors, 1970), dental pulp grafts induce collateral sprouting from existing iris nerve fibers and promote innervation of the ectopically transplanted dental pulp tissue. We propose that the dental pulps do so by producing potent molecules such as neurotrophic factors.

Dental pulp grafts increased the density of catecholaminergic nerve fibers of the irises. Increased density of iris nerve fibers could be related to the presence of neurotrophic factors in the anterior chamber of the eye (Giacobini *et al.*, 1993a,b). It has also been shown that dental pulp explants promote neuronal survival and neurite outgrowth from either chick ganglia or rat trigeminal ganglia explants (Nosrat *et al.*, 1996a, 1998a; Lillesaar *et al.*, 1999).

The effects exerted by the dental pulp-derived neurotrophic factors seem to be nonspecific for different types of neuronal populations. Dental pulp-derived neurotrophic factors influence either trigeminal neurons, sympathetic neurons, or spinal cord motoneurons when present in the environment. However, there is also the possibility of combinational coding for neurotrophic factor expression and neuronal interactions, and this might offer specificity for the interactions of the target tissues with different neuronal populations. Interestingly, the general expression of neurotrophic factors in developing teeth diminishes through development and, at the time of the innervation of the dental pulp, only NGF, BDNF, and GDNF are expressed in the dental pulp with specific temporal patterns of expression (Nosrat *et al.*, 1998b), indicating that only these neurotrophic factors might be involved in the innervation of the dental pulp. This is similar to the situation in the lingual gustatory and somatosensory innervations, in which BDNF expression in taste buds brings specificity for the gustatory innervation of taste cells (Nosrat and Olson, 1995; Nosrat *et al.*, 1996b). In parallel, NT-3 expression by surrounding epithelium and somatosensory-related structures in the tongue brings specificity for the somatosensory

innervation of the tongue (Nosrat *et al.*, 1996b; Nosrat and Olson, 1998c). The specificity of gustatory and somatosensory innervation of the tongue has been strengthened by studies of null-mutated and overexpressing animals, in which the innervation is lost in null-mutated mice (Nosrat *et al.*, 1997b; Zhang *et al.*, 1997), and there is a gain of function (in this case promotion of survival of gustatory neurons) in BDNF and NT-4 overexpressing animals (Ringstedt *et al.*, 1999; Krimm *et al.*, 2001).

Although all neurotrophins, GDNF, and NTN are found in the developing teeth (Fried *et al.*, 2000), only NGF, BDNF, and GDNF mRNAs are expressed in the postnatal dental pulp. It was previously suggested that NGF is involved in the guidance of trigeminal axons to developing teeth (Luukko *et al.*, 1997a) and participates in the establishment of a pulpal innervation (Qian and Naftel, 1994, 1996). GDNF has been proposed to be postnatally involved in the regulation of tooth innervation (Luukko *et al.*, 1997b; Nosrat *et al.*, 1998). It is noteworthy that neurotrophic factors seem to be involved in both tooth development per se as well as tooth innervation. For instance, NGF is required for differentiation of cranial neural crest cells into tooth organs, shown by *in vitro* organ culture studies (Amano *et al.*, 1999). At the same time, tooth innervation is reduced in NGF knockout mice (Byers *et al.*, 1997), and anti-NGF treatment of neonatal rat pups reduces the amount of sensory axons in the dental pulp (Qian and Naftel, 1996). Interestingly, NGF mRNA becomes upregulated after experimental tooth injury (Byers *et al.*, 1992; see also Woodnutt *et al.*, 2000). These indeed indicate different roles for neurotrophic factors in the teeth.

Several studies have shown the beneficial effects of neurotrophic factors delivered to the injured spinal cord. BDNF and NT-4 have been shown to promote regeneration of rubrospinal axons (Kobayashi *et al.*, 1997), reduce the necrotic zone, and support motoneuron survival after spinal cord injury (Novikova *et al.*, 1996). NT-3 enhances sprouting of corticospinal tract fibers (Schnell *et al.*, 1994; Grill *et al.*, 1997; Houweling *et al.*, 1998). Both BDNF and NT-3 prevent axotomy-induced death of corticospinal neurons (Giehl and Tetzlaff, 1996). GDNF has been shown to be a potent survival factor for motoneurons (Henderson *et al.*, 1994; Oppenheim *et al.*, 1995). In the present study, we used the disappearance of FluoroGold-labeled cells as a measurement of cell death, although it could also perhaps indicate graft-induced reversal of atrophy. Taken together, our data suggest that dental pulp tissue transplanted into the injured spinal cord provides neurotrophic support to the motoneurons. Indeed, several different properties of the dental pulp make it a potentially viable endogenous source for grafting into injured CNS. Dental pulp produces an array of neurotrophic factors both *in vivo* and *in vitro*. Dental pulp cells are generally fibroblast-like in culture and the major cell type of the dental pulp is neural crest-derived fibroblasts. The risk of graft rejection would also be minimized if autologous grafts could be used. The dental pulp is

easily accessible, and developing human teeth express neurotrophic factor mRNAs (our unpublished observations).

In conclusion, we propose that dental pulp-derived neurotrophic factors play an important role in orchestrating the dental pulp innervation. It might also be possible that nongenetically modified dental pulp cells would constitute a viable source of cells for transplantations where neurotrophic factors have been shown to offer benefits in CNS disorders or injuries, although this aspect requires further studies. The data also indicate how well developmental processes are intermingled with repair mechanisms and emphasize the value of understanding the basic developmental mechanisms for development of regeneration and repair strategies.

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