GDNF Is a Chemoattractant for Enteric Neural Cells

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In situ hybridization revealed that GDNF mRNA in the mid- and hindgut mesenchyme of embryonic mice was minimal at E10.5 but was rapidly elevated at all gut regions after E11, but with a slight delay (0.5 days) in the hindgut. GDNF mRNA expression was minimal in the mesentery and in the pharyngeal and pelvic mesenchyme adjacent to the gut. To examine the effect of GDNF on enteric neural crest-derived cells, segments of mouse hindgut containing crest-derived cells only at the rostral ends were attached to filter paper supports and grown in catenary organ culture. With GDNF (100 ng/ml) in the culture medium, threefold fewer neurons developed in the gut explants and fivefold more neurons were present on the filter paper outside the gut explants, compared to controls. Thus, in controls, crest-derived cells colonized the entire explant and differentiated into neurons, whereas in the presence of exogenous GDNF, most crest-derived cells migrated out of the gut explant. This is consistent with GDNF acting as a chemoattractant. To test this idea, explants of esophagus, midgut, superior cervical ganglia, paravertebral sympathetic chain ganglia, or dorsal root ganglia from E11.5–E12.5 mice were grown on collagen gels with a GDNF-impregnated agarose bead on one side and a control bead on the opposite side. Migrating neural cells and neurites from the esophagus and midgut accumulated around the GDNF-impregnated beads, but neural cells in other tissues showed little or no chemotactic response to GDNF, although all showed GDNF-receptor (Ret and GFRα1) immunoreactivity. We conclude that GDNF may promote the migration of crest cells throughout the gastrointestinal tract, prevent them from straying out of the gut (into the mesentery and pharyngeal and pelvic tissues), and promote directed axon outgrowth. © 2001 Academic Press

Key Words: neural crest; migration; sympathetic; dorsal root ganglion; axon growth; enteric nervous system; chemoattraction.

INTRODUCTION

Neurons and glial cells of the vertebrate enteric nervous system arise from neural crest cells. Vagal level (adjacent to somites 1–7) neural crest cells enter the foregut and then migrate rostrocaudally within the gut mesenchyme to colonize the entire gastrointestinal tract (Yntema and Hammond, 1954). Sacral level neural crest cells also contribute some enteric neurons and glial cells to the postumbilical gut (Le Douarin and Teillet, 1973; Burns and Le Douarin, 1998).

Glial-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-β superfamily (for review see Airaksinen et al., 1999). The receptor complex for GDNF consists of the receptor tyrosine kinase, Ret, and a ligand-binding component, GFRα1. Ret and GFRα1 are expressed by enteric neural crest-derived cells (see Table 2 for references), and GDNF is expressed by the gut mesenchyme (Trupp et al., 1995; Hellmich et al., 1996; Moore et al., 1996; Suvanto et al., 1996). The GDNF-Ret/GFRα1 signaling pathway is crucial for the development of the enteric nervous system. Inactivation of the genes encoding GDNF, or either of its receptor components Ret or GFRα1, results in an absence of enteric neurons caudal to the stomach, apart from a small number of cells in the distal rectum (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998; Tomac et al., 2000). GDNF promotes the survival, proliferation, and differentiation of enteric
neuron precursors isolated from the embryonic gut (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Wu et al., 1999). Together these data have been interpreted to indicate that the role of GDNF in development of the enteric nervous system is to ensure that sufficient cells of an appropriate lineage are available to populate the gut.

On the basis of heterotopic transplants of neural crest between vagal and trunk levels, Le Douarin and Teillet (1974) proposed that the developing intestine could specifically attract migrating vagal neural crest-derived cells. Although a number of transcription factors (Southard-Smith et al., 1998; Kapur et al., 1999; Pattyn et al., 1999) and the GDNF signaling pathway have been shown to be essential for the early survival and proliferation of vagal crest-derived cells, molecules within the gut that directly influence the migration process have yet to be identified.

In this study using mouse embryonic cells, we show that GDNF has a chemotactic effect on the migration and neurite outgrowth of enteric neural crest-derived cells, but has little or no chemotactic effect on crest-derived sympathetic or dorsal root ganglia at similar embryonic stages. Hence, in addition to playing a key role in the proliferation, survival, and differentiation of enteric neuron precursors as described previously (see Pachnis et al., 1998; Taraviras and Pachnis, 1999), GDNF expressed by the mesenchymal cells of the gut wall may also be important for driving neural crest cell migration along the gut, in preventing them from straying into the mesentery and other nearby tissues, and in promoting axon outgrowth.

**MATERIALS AND METHODS**

All experiments were performed on embryonic BALB/c mice. The day at which a vaginal plug was found was designated E0.5. Pregnant mothers were killed by cervical dislocation, and the embryos were removed under aseptic conditions. Embryos were then precisely staged using the staging system of Theiler (1989) and described as nominal embryonic days (E).

**In situ hybridization.** The gastrointestinal tract from E10.5–E14.5 mice was fixed for 48 h in 4% paraformaldehyde in 0.1 M phosphate buffer. Whole-mount in situ hybridization of dissected gut tissue was performed as described previously (Thomas et al., 1998). Digoxigenin-labeled antisense mGDNF riboprobes were prepared according to the manufacturer’s instructions (Boehringer Mannheim) using a 320-bp cDNA clone (kind gift from Dr. Heiner Westphal, NIH, Bethesda, MD). The sense GDNF probe was generated by digestion with Fok I and transcription with T7 RNA polymerase. Some whole-mount preparations were subsequently embedded in resin and 3-μm sections cut as described previously (Young et al., 1998). For in situ hybridization on sections, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. After cryoprotection in 20% sucrose/PBS at 4°C, embryos were embedded in OCT compound, and 16-μm sections were prepared using a cryostat. For hybridization, approximately 15 ng of probe/slide was denatured at 70°C for 5 min in a buffer containing 50% formamide, 10% dextran sulfate, 1 mg/ml rRNA, 1 × Denhardt’s 200 mM NaCl, 9 mM Tris–HCl (pH 7.5), 1 mM Tris base, 5 mM NaH2PO4, 5 mM Na2HPO4, 50 mM EDTA and incubated overnight at 60°C under a coverslip. Following hybridization, the coverslips were removed and the sections washed in 1× SSC, 50% formamide at 65°C for 2 × 30 min. After equilibration for 2 × 30 min in MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween, pH 7.5), the sections were blocked for 1 h in 20% blocking agent (Boehringer Mannheim), 20% heat-inactivated sheep serum in MABT and incubated overnight in the presence of anti-DIG Fab fragments (1:4000 dilution; Boehringer Mannheim) in the same buffer. Unbound antibody was removed by washing with 5 × 20 min in MABT and, following equilibration in NTM (100 mM NaCl, 50 mM MgCl2, 100 mM Tris, pH 9.5), the sections were stained for 24 h in BCIP/NBT staining buffer (Moss, Inc.). After staining, sections were washed in PBS and mounted using Aquamount (BDH).

**Catenary (suspended) organ-cultured gut.** Segments of hind-gut from the ileocaecal border to the distal hindgut were removed from E11.5 mice and set up in catenary organ culture as described previously (Hearn et al., 1999). At E11.5, neural crest-derived cells are present in the caecum and slightly caudal to the caecum, but they are not present in the middle or caudal regions of the hindgut (Kapur et al., 1992; Young et al., 1998); thus the explants contained neural crest-derived cells only at the rostral end when first set up in culture (Fig. 2A). The explants were suspended across a “V” cut into a 5 × 5-mm piece of Millipore filter paper, and the orientation of the explant was indicated by removing the corner of the filter paper adjacent to the caudal end. The filter papers with the attached gut segments were placed across Terasaki wells in 20 μl of DMEM (Trace Scientific Ltd., Noble Park, VIC, Australia) with 10% fetal bovine serum (Trace Scientific Ltd). In experimental cultures, the culture medium also contained 100 ng/ml GDNF (PeproTech, Rocky Hill, NJ). The cultures were maintained in a 5% CO2 environment at 37°C for 3 days with a half medium change at day 2. They were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and processed for immunohistochemistry using an antibody to the neuron-specific protein, PGP9.5 (Table 1).

The number of neurons on the filter paper and within the gut explants was quantified in six control and six explants grown in the presence of GDNF. The total number of neurons on the filter paper outside each explant was counted. A digital CCD camera (Image-Point, Photometrics Ltd., Tucson, AZ) attached to a fluorescence microscope (Zeiss Axioskop) was used to take images of the entire piece of filter paper plus gut explant using a ×20 objective lens. A montage was then made of the digital images and the total number of PGP9.5+ neurons present on the filter paper was counted. It was not possible to count the total number of neurons within each explant as the neurons along the bottom and sides of the gut tube could not be clearly visualised because of the tubular shape of the gut explants. Therefore, the density of neurons along the top surface of the gut explants was determined. Digital images were taken of the top surface of the entire gut explant. Montages were made of the images and the number of neurons was counted. For each explant, the area within which the neurons were counted was determined using image analysis software (SigmaScan Pro 4.0), and then the neuronal densities were calculated.

To examine whether GDNF has a direct effect on the mesenchyme, the caudal two-thirds of E11–E11.5 postcaecal hindgut, which lack neural crest-derived cells, were set up in catenary organ culture with or without GDNF (100 ng/ml) added to the culture medium. After 3 days of culture, the explants were fixed and processed for PGP9.5 immunohistochemistry to confirm an absence of neural crest-derived cells in the hindgut segments at the
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TABLE 1
Antibodies Used in This Study

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<th>Primary antibody</th>
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<th>Concentration</th>
<th>Secondary antibody</th>
<th>Source</th>
<th>Concentration</th>
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<td>Goat anti-rabbit Alexa 594</td>
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<td>Goat anti-mouse Alexa 488</td>
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<tr>
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<td>Jackson ImmunoResearch, West Grove, PA</td>
<td>1:200 1:100 1:50</td>
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<tr>
<td>Sheep anti-tyrosine hydroxylase</td>
<td>Chemicon</td>
<td>1:80</td>
<td>Jackson ImmunoResearch</td>
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Primary antibody Source Concentration Secondary antibody Source Concentration
Rabbit anti-PGP9.5 Ultraclone, Isle of Wight 1:1000 Goat anti-rabbit Alexa 594 Molecular Probes, Eugene, OR 1:200
Rabbit anti-Ret plus IBL, Tokyo, Japan 1:50 Goat anti-rabbit Alexa 594 Molecular Probes Both at 1:200
Mouse anti-GFRα1 Transduction Laboratories, Lexington, KY 1:200 Goat anti-mouse Alexa 488
Rabbit anti-PGP9.5 plus Ultraclone, Isle of Wight, UK 1:1000 Biotinylated donkey anti-rabbit followed by Streptavidin-Alexa 594 plus Donkey anti-sheep FITC Jackson ImmunoResearch, West Grove, PA 1:200 1:100 1:50
Sheep anti-tyrosine hydroxylase Chemicon 1:80 Jackson ImmunoResearch

time of removal from the embryo. The hindgut cultures were then incubated in the nuclear dye DAPI (Sigma; 10 µg/ml in PBS), and the total number of nuclei on the filter paper outside the explants was counted.

**Explants grown on collagen gel.** Collagen-coated culture dishes were prepared by restoring acidic collagen solution (4 mg/ml; Boehringer Mannheim, Germany) to normal osmolality with 5× DMEM and normal pH with 200 mM NaOH on ice. This was diluted to 1 mg/ml with culture medium (see above) and 0.6 ml was spread on cold 35-mm-diameter petri dishes (Nunc, Denmark). This was gelled at 37°C for at least 30 min before use. For dorsal root ganglion cultures, NGF (Boehringer Mannheim) was included at 100 ng/ml in the collagen gel.

The esophagus, midgut (both of which contain neural crest-derived cells), lumbar-level dorsal root ganglia, and lumbar-level dorsal aorta with the tissue immediately adjacent to it (containing the paravertebral sympathetic chain ganglia) were dissected from E11.5 mice, and the superior cervical ganglion was dissected from E12.5 mice. Transverse slices, about 500 µm thick, of the gut segments and the dorsal aorta and its adjacent tissue were cut and transferred by pipette with 1 µl of culture medium onto the collagen gel surface. Individual dorsal root ganglia and superior cervical ganglia were also placed on collagen gels. The neural tube plus somites from the level of somite 1 to 6 (vagal level) was dissected from 6- to 11-somite stage (E8.5–9) embryos and placed on collagen gels. A GDNF-impregnated agarose bead (Cibacron, Sigma; 80–110 µm diameter) was placed on one side of each tissue within the explants of vagal neural tube plus somites, frozen sections of some of the explants were cut transverse to the neural tube. The frozen sections were processed for tyrosine hydroxylase and PGP9.5 immunohistochemistry.

**RESULTS**

Expression of GDNF mRNA in the Gastrointestinal Tract of Mouse Embryos

Differences in the timing of developmental events, including the development of the enteric nervous system, are often related to rostrocaudal position along the gastrointestinal tract. The expression pattern of GDNF was examined in whole-mount preparations of gut from E10.5–E14.5 mice. At E10.5, little GDNF mRNA expression was detected in either the midgut or particularly the caecal primordium, and there was only very faint staining in the postcaecal hindgut. From E11.5 onward, staining was observed throughout the intestine (Fig. 1B). The staining in whole-mount preparations appeared most intense in the caecum, followed by the midgut, and the hindgut showed the weakest staining (Fig.
Some of the E11.5 and E13.5 whole-mount preparations were subsequently sectioned to examine the expression of GDNF at the cellular level. Transverse sections revealed that the staining was restricted to the mesenchyme, and the serosal and endodermal epithelial cells were not stained (Figs. 1C and 1D). Although the levels of GDNF mRNA

![Figure 1](https://example.com/figure1.png)

**FIG. 1.** (A, B, E) In situ hybridization localization of GDNF in whole-mount preparations of gastrointestinal tract from E10.5 (A), E11.5, (B), and E13 (E) mice. At E10.5 (A), there is little detectable expression of GDNF, but by E11.5 (B), expression occurs in both the mid- and the hindgut, with the most intense staining in the caecum and midgut. By E13 (E), staining is more intense in all regions. Scale bars, 500 μm. (C, D, F) Resin sections, 3 μm thick, through the whole-mount preparations. Expression of GDNF occurs in the mesenchymal cells, but not the epithelial cells. The levels of expression vary between mesenchymal cells, but the average level of expression per cell does not vary greatly between the different regions. Scale bar, 100 μm (applied to C, D, and F). (G, H) Transverse sections through the pelvic region of an E14.5 embryo showing localization of the antisense probe (G) and the sense probe (H). (G) There is strong GDNF expression by the outer mesenchyme of the gut, but none in adjoining tissues. (H) No staining is observed using the sense probe, apart from weak, nonspecific staining of the gut epithelium. vert. body, body of vertebra. Scale bar, 250 μm.
expression varied between mesenchymal cells, the average level of expression per cell did not appear to vary greatly between the different regions (Fig. 1F). Thus although there was a rapid increase of expression from E10.5 to E12.5, the high intensity of staining in the caecum and low intensity of label in the midgut and particularly in the hindgut in whole-mount preparations were principally due to the differing thickness of the wall of the gut, rather than to a pronounced regionality or rostrocaudal gradient of expression. In transverse sections through the pharyngeal arch regions of E11 embryos, there was no significant nonintestinal GDNF mRNA expression (not illustrated). In sections through the caudal parts of E14.5 embryos, GDNF expression was found only in the kidney primordium (not illustrated) and in outer layers of the gut mesenchyme (Fig. 1G). Expression of GDNF was not detected in any tissues that were adjacent to the gut, including in the pelvic region, where the caudal hindgut is not suspended by mesentery but is enveloped by structures in the pelvic floor (Fig. 1G). Sections stained using the sense probe gave no staining, apart from weak, nonspecific staining of the gut epithelium (Fig. 1H).

Behavior of Neural Crest-Derived Cells in Explants of Gut Grown in Catenary Organ Culture with, or without, GDNF

Explants of hindgut from the ileocaecal border to the caudal end of the hindgut from E11.5 mice were grown in catenary organ culture (Fig. 2A; see Hearn et al., 1999), with or without GDNF (100 ng/ml) added to the culture medium. At the beginning of the culture period of E11.5 hindgut, neural crest-derived cells are present only in the rostral end of the explant (Fig. 2A; see Kapur et al., 1992; Young et al., 1998). After 3 days in culture, many PGP9.5+ neurons are present throughout the control gut explants (Figs. 2C and 2E), indicating that the neural crest cells that are present only at the rostral end at the beginning of the culture period had migrated through the gut explant and differentiated into neurons. A small, but variable, number of neurons was also observed on the filter paper at both the rostral and the caudal end of the control gut explants (Fig. 2C). In the explants grown in the presence of 100 ng/ml GDNF, there was a very low density of PGP9.5+ neurons within the explants (Fig. 2D), and some explants had no detectable neurons. In contrast, there were many more neurons on the filter paper outside of the explant in the presence of GDNF (Fig. 2B), indicating that in the presence of GDNF in the culture medium, enteric neuron precursors had migrated out of the rostral end of the gut explant and differentiated into neurons on the filter paper. Counts were performed of both the total number of neurons on the filter paper outside the explant and the density of neurons within the gut explant in six control explants and six explants grown in the presence of GDNF (Fig. 3). There were significantly (fivefold) more neurons on the filter paper in the GDNF cultures (unpaired t test, t = 3.8, P < 0.05) and a significantly (threefold) lower density of neurons within the gut explants in the presence of GDNF (unpaired t test, t = 4.4, P < 0.05) compared to the control explants. The mean area of the top surface of control explants was 0.21 ± 0.02 µm² (mean ± SEM, n = 6) and that of GDNF-treated cultures was 0.19 ± 0.02 µm² (n = 6), indicating that there was no difference in the overall size of the gut explants between controls and those grown in the presence of GDNF. Thus the neuronal density directly reflects neuron number in the explants.

There were many more nonneuronal cells on the filter paper in the cultures grown in the presence of GDNF than there were in control cultures. The nonneuronal cells presumably include neural crest-derived glial cells and undifferentiated neural crest cells and, possibly, gut mesenchymal cells. Since mesenchymal cells of the gut have been reported to express GFRα1 (Chalazonitis et al., 1998; Worley et al., 2000) and might therefore respond to GDNF directly, we wished to examine whether GDNF is also a chemoattractant for mesenchymal cells of the gut. To distinguish neural crest-derived cells from mesenchymal cells on the filter paper requires simultaneous quadruple labeling (immunohistochemical staining for differentiated neurons, differentiated glial cells, and undifferentiated neural crest cells, plus a nuclear stain to label the entire cell population). As this is not technically possible, we examined whether GDNF has a direct effect on mesenchymal cells of the gut by culturing explants of gut lacking neural crest-derived cells with or without GDNF. Explants of E11–E11.5 postcaecal hindgut, which are not yet colonized by neural crest-derived cells (Young et al., 1998), were grown in catenary organ culture with or without GDNF added to the culture medium. After 3–4 days in culture, no neurons could be detected using an antibody to PGP9.5 in the explants grown either with or without GDNF. Following incubation in the nuclear stain DAPI, the total number of mesenchymal cells on the filter paper outside of the explants was counted in the cultures lacking neural crest-derived cells. There was no significant difference between the numbers of cells on the filter paper outside each explant under control conditions (139.7 ± 20.5 cells; mean ± SEM, n = 4 cultures) and outside explants grown in the presence of GDNF (140.4 ± 27.0 cells, n = 5 cultures; unpaired t test, P = 0.98, not significant). Thus GDNF is not a direct chemoattractant to mesenchymal cells of the gut.

Esophagus, Midgut, Dorsal Root Ganglia, Superior Cervical Ganglia, and Paravertebral Sympathetic Chain Grown on Collagen Gels with GDNF-Impregnated Agarose Beads

Midgut. Explants of E11.5 midgut were grown on collagen-coated culture dishes with a GDNF-impregnated agarose bead on one side and a control agarose bead on the opposite side of the explant. After 4 days in culture, the GDNF-impregnated beads were covered with PGP9.5+ nerve cell bodies (Fig. 4C), whereas most of the control...
beads had no PGP9.5+ cells associated with them. In addition, many groups of PGP9.5+ neurons, forming ganglia, were present on the collagen gel in the vicinity of the GDNF bead (Fig. 4C), but ganglia were sparse in other regions of the cultures. The majority of neurites occurred on the GDNF-bead side of the explant, and most extended directly between the explant and the bead. For each explant, the levels of PGP9.5 immunostaining (pixel intensity) of the neurites between the GDNF bead and the explant, and the control bead and the explant, were quantified (see Fig. 4D). The immunostaining on the GDNF bead side was 2.4 times higher than that on the control bead side, a highly significant difference (Wilcoxon signed rank test, \( P = 0.01, n = 8 \); Fig. 6).

Although many neural derivatives of the neural crest express Ret and GFR\(\alpha_1\), not all of the derivatives are affected in gdnf\(^{--}\), ret\(^{--}\), and gfr\(\alpha_1^{--}\) mice (Table 2). For example, although there are no neurons present in the small and large intestine of the knockout mice, neurons are present in the esophagus. In addition, ret\(^{--}\) mice lack a superior cervical ganglion, but the more caudal sympathetic chain ganglia are unaffected (Durbec et al., 1996). We therefore explanted a range of neural derivatives of the neural crest onto collagen gels to determine for each tissue
whether (i) GDNF is a chemoattractant for neurite outgrowth and cell migration and (ii) Ret and GFRα1 proteins are expressed by the explanted tissues. The effects of GDNF-impregnated agarose beads were examined on E11.5 esophagus, trunk-level paravertebral sympathetic chain ganglia, individual dorsal root ganglia, and individual E12.5 superior cervical ganglia.

**Esophagus.** The GDNF-impregnated beads were covered with nerve cell bodies, and groups of neurons were present on the collagen, distant from the explant, in the vicinity of the beads (Fig. 4B). There was also a profuse outgrowth of PGP9.51 neurites from the explants to the GDNF-impregnated bead, but there were only sparse fibers projecting from the explants in other directions (Fig. 4B). The intensity of immunostaining of the neurites and cells on the GDNF bead side of the explants was significantly (3.7 times) greater than the staining between the control bead and the explants (Wilcoxon signed rank test, \( P < 0.01 \), \( n = 8 \); Fig. 6).

**Lumbar dorsal root ganglia (DRG).** In medium lacking NGF, outgrowth after 3 days was very limited. However, in the presence of NGF, profuse, radial neurite and cell outgrowth occurred from individual DRG, with no detectable preference for outgrowth toward the GDNF-impregnated bead, but there were only sparse fibers projecting from the explants in other directions (Fig. 4B). The intensity of immunostaining of the neurites and cells on the GDNF bead side of the explants was significantly (3.7 times) greater than the staining between the control bead and the explants (Wilcoxon signed rank test, \( P = 0.01 \), \( n = 6 \); Fig. 6).

**Superior cervical ganglion.** The intensity of PGP9.5 immunostaining of neurites on the GDNF bead side of the explants was not significantly different from that on the control bead side of the explants (Wilcoxon signed rank test, \( P = 0.69 \), \( n = 5 \); Figs. 5B and 6). Nerve cell bodies were not associated with either of the beads.

**Lumbar paravertebral sympathetic chain ganglia.** Neurite outgrowth from the explanted ganglia occurred, but the outgrowth was not as radially symmetrical as that of the dorsal root ganglia, possibly because each explant consisted of a chain of ganglion anlagen attached to the aorta (Fig. 5C). The degree to which preferential growth occurred toward the GDNF-impregnated bead varied greatly between explants. When quantified, the intensity of PGP9.5 immunostaining of neurites on the GDNF-impregnated bead side of the explants was slightly (1.2 times), but significantly, higher than that on the control bead side (Wilcoxon signed rank test, \( P = 0.03 \), \( n = 6 \); Fig. 6). Nerve cell bodies were not associated with either the GDNF or the control beads.

**Ret and GFRα1 immunoreactivity of gut, sympathetic ganglia, and DRG explants.** The neurons and neurites present in the gut, sympathetic ganglia, and DRG explants all showed the same pattern of Ret and GFRα1 immunoreactivity (Fig. 7). Regardless of the direction in which they projected in relation to the beads, the neurites extending from the explants were strongly GFRα1+ (Figs. 7A and 7B). Most neurites showed little Ret immunostaining (Figs. 7A and 7A'), but the nerve cell bodies were Ret+ and GFRα1+ (Figs. 7A, 7A', and 7B).

**Neural Tubes Containing Premigratory Vagal Neural Crest Cells Grown on Collagen-Coated Culture Dishes with GDNF-Impregnated Agarose Beads**

To determine whether GDNF is chemoattractive to vagal neural crest cells as they emigrate from the neural tube, the neural tubes and somites, corresponding to somites 1–6, of

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**FIG. 3.** Effects of GDNF on the density of neurons within the explant (left) and total number of neurons on the filter paper outside the explant (right). Data are expressed as means ± SEM from six controls and six cultures grown in the presence of GDNF. In the presence of GDNF, both the density of neurons within the explants and the total number of neurons on the paper are significantly different from those in control cultures (unpaired t tests, \( P < 0.05 \)).
6- to 11-somite mouse embryos were grown on collagen-coated culture dishes for 3–4 days with a GDNF-impregnated agarose bead on one side and a control agarose bead on the opposite side of the explant. The amount of direction of neurite outgrowth varied between explants, but the intensity of PGP9.5 immunostaining on the GDNF bead side of the explant was not significantly different from that on the control bead side of the explants (paired t test, \( P = 0.9, n = 4 \); Figs. 6 and 8). Very few PGP9.5+ neurons were present on the collagen gel outside of the explants. Therefore, to examine whether neural crest cells had emigrated, survived, and differentiated in these cultures, some of the explants were sectioned and processed for tyrosine hydroxylase (TH) and PGP9.5 immunohistochemistry. TH is expressed by sympathetic neural crest derivatives and transiently by 10–15% of vagal crest-derived cells within the E10.5–E11.5 mouse gut (Young et al., 1999). Both TH+/PGP9.5+ and TH−/PGP9.5+ cells were observed in small clusters within the explants, at varying distances from the neural tube (Figs. 8B and 8B'). This indicates that neural crest-derived cells had migrated, survived, and differentiated into neurons in the explants. However, very few had migrated away from the explanted tissue, unlike the case in which GDNF-impregnated beads were placed next to explants of embryonic gut.

**DISCUSSION**

**GDNF Is a Chemoattractant for Enteric Neural Crest-Derived Cells and Neurites**

Previous studies have shown that GDNF promotes the proliferation, survival, and differentiation of enteric neuron precursors isolated from embryonic mice and quail and increases the length of neurites from developing enteric neurons (Chalazonitis et al., 1998; Hearn et al., 1998; Fig. 4.

(A, B) Inverted fluorescence images of slices of midgut (A) and esophagus (B) grown on a collagen-coated petri dish with a GDNF-impregnated agarose bead on one side and a control agarose bead on the opposite side. After 4 days, the explants were processed for PGP9.5 immunohistochemistry. Neurites were associated preferentially with the GDNF-impregnated beads. Note that the control beads were dislodged during processing for immunohistochemistry at the end of the culture period. Scale bars, 500 \( \mu \)m. (C) Higher magnification image of the GDNF-impregnated bead showing that both neurites and migrating neural cells were attracted to the bead. Migrating neural cells covered the bead and were found in groups (arrows) between the explant and the bead. Scale bar, 100 \( \mu \)m. (D) Diagram showing the method used to compare the outgrowth toward the GDNF-impregnated bead compared to the control bead. The average pixel intensities of immunostaining along a line drawn equidistant between the GDNF bead and the explant and on a second line between the control bead and the explant were determined, and the background pixel intensity was subtracted from each.
TABLE 2
Correlation within the Peripheral Nervous System of GDNF Response Elements with the Results of Gene Knockout

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</tr>
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<td>Enteric neurons in the small and large intestine</td>
<td>Yes1,8,9</td>
<td>Yes14</td>
</tr>
<tr>
<td>Sympathetic chain ganglia—(i) superior cervical ganglion (SCG)</td>
<td>Yes4</td>
<td>Yes16</td>
</tr>
<tr>
<td>Sympathetic chain ganglia—(ii) ganglia caudal to SCG</td>
<td>Yes9,14</td>
<td>Yes14,16</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>Yes10,13,14</td>
<td>Yes14,15</td>
</tr>
</tbody>
</table>


Heuckeroth et al., 1998; Schäfer and Mestres, 1999; Taraviras et al., 1999; Wu et al., 1999. The current study demonstrates that GDNF also has a powerful stimulatory effect on the migration of crest-derived enteric neural precursor cells and their neurites and that this stimulation has an important directional component. The clustering of neurons around GDNF beads and the skewing of the paths of growing neurites toward GDNF beads in the cultures on collagen gels, and the depletion of neurons within the gut and the increase of such cells outside the gut in organ culture, strongly suggest that GDNF is a chemoattractant for enteric neural cells and neurites. Additional effects of GDNF which could contribute to the nonrandom cell and neurite distribution pattern, such as chemokinesis (nondirectional stimulation of migration) and improved proliferation or survival of neural cells, are also likely.

The GDNF-Ret/GFRα1 signaling pathway is essential for the development of the kidney as well as the enteric nervous system (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Calamano et al., 1998; Enomoto et al., 1998; Tomac et al., 2000). Ret-expressing MDCK renal epithelial cell lines show GDNF-induced production of locomotory lamellipodia and preferentially migrate toward a localized source of GDNF (Tang et al., 1998). Thus the effect of GDNF on directional migration of enteric neural crest-derived cells is strikingly similar to that on kidney epithelial cell lines.

**GDNF Is Not a Chemoattractant for Vagal Neural Crest-Derived Cells as They First Emigrate from the Neural Tube**

Le Douarin and Teillet (1974) suggested that vagal level neural crest cells are selectively attracted by the intestine because when quail vagal level neural crest cells were transplanted to the midtrunk level of chick embryos, quail cells were found in the gut, even though trunk-level crest cells do not normally reach the gut. The mechanisms by which vagal level neural crest cells navigate from the neural tube to the foregut have yet to be elucidated, but likely to be involved are (a) interactions between specific anchored ligands (for example, extracellular matrix and cell-cell adhesion molecules) expressed on the migratory pathway and their receptors expressed by vagal neural crest cells and (b) expression of diffusable molecules within the gut that are chemoattractive to vagal neural crest cells.

We were unable to demonstrate a chemoattractive effect on mouse vagal neural crest cells as they initially emigrated from the neural tube. This suggests that GDNF does not play a directional role in controlling the migration of vagal neural crest cells as they leave the neural tube. Previous experiments on avian vagal neural tube explants also showed no survival or proliferative effects of GDNF on early emigrating neural crest cells (Hearn et al., 1998). In ret−/− mice, vagal neural crest-derived cells die before, or just as, they reach the foregut (Durbec et al., 1996; Taraviras et al., 1999). We can conclude from these results that the GDNF-Ret/GFRα1 signaling pathway is active in survival before or as the vagal neural crest cells initially reach the foregut. It will be interesting to determine whether the onset of survival responsiveness and chemoattraction to GDNF occur at the same developmental stage.

**Possible Chemoattractive Role of GDNF in Enteric Nervous System Development**

The migratory behavior of enteric neural crest-derived cells, and hence the colonization of the gut by enteric...
neuron precursors, is likely to be influenced by a number of factors including (i) neural crest cell-autonomous factors which give the innate tendency of neural crest cells to migrate (Newgreen, 1992); (ii) interactions between neural crest cells, since when the number of premigratory vagal crest cells is reduced surgically, neural crest cells fail to colonize the most caudal regions of the gastrointestinal tract (Yntema and Hammond, 1954; Peters-van der Sanden et al., 1993); and (iii) the presence of attractive or repulsive molecules in the gut mesenchyme (Le Douarin and Teillet, 1974).

The net movement of most neural precursor cells in the gut mesenchyme is rostral to caudal, as a wave, with a...
defined timetable and with a discrete wavefront (Kapur et al., 1992; Young et al., 1998). The first of these cells to differentiate into neurons transiently express catecholamines (Baetge et al., 1990). The cell bodies of these relatively few, early differentiated neurons lie within a dense population of undifferentiated neural cells (Young et al., 1999). Despite the apparently unoriented surroundings, all these neurons have axons that project caudally for up to 400 μm (Young et al., 1999), which is the same direction as the crest cells are migrating. Thus it is possible that the same mechanism(s) drives both cell migration and axon polarity.

In the current study, enteric neuron precursors showed a strong chemotactic response to GDNF. We therefore propose that mesenchymally derived GDNF, in addition to its role in survival, proliferation, and neuronal differentiation, plays a role in (i) retaining vagal neural crest-derived cells within the gut mesenchyme so that they do not migrate into neighboring tissues via the mesentery and (ii) promoting the migration of neural crest-derived cells along the gut. There were also increased numbers of neurites associated with the GDNF beads compared to the control beads, indicating that GDNF may also be chemotactic to the neurites of differentiating enteric neurons and thus may promote the oriented extension of neurites along the gut. However, we also cannot rule out the possibility that the increased number of neurites associated with the GDNF beads is to some extent an indirect effect, due to increased numbers of neurons associated with the GDNF beads.

The mechanism by which GDNF could promote the rostrocaudal migration and caudally directed neurite outgrowth is not obvious. Since we have shown that GDNF is chemotaxic for enteric neural cells and neurites in vitro, the simplest model is that the rostrocaudal migration of cells and the polarity of the neurites of the differentiating enteric neurons in the gut in vivo could be accomplished by a preceding rostrocaudal wave of GDNF production. Assuming that mRNA level predicts protein production, this simple scenario is unlikely because, although there was an increase in GDNF mRNA with developmental age, the spatiotemporal correlation between GDNF expression and neural crest cell migration was weak. We therefore suggest first that in regions already occupied by neural crest cells, GDNF protein production enables neural cell survival, proliferation, and differentiation to keep pace with the dramatic growth of the gut (see Newgreen et al., 1996). We suggest second that levels of GDNF protein are higher in regions unoccupied by neural cells and neurites, which are

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**FIG. 7.** (A, A', A'') Ret (A, red) and GFRα1 (A', green) immunostaining of an E11.5 dorsal root ganglion after growth on a collagen-coated petri dish for 4 days. The cell bodies show both Ret and GFRα1 immunoreactivity (A, A'), whereas the neurites show intense GFRα1 immunostaining, but little Ret immunostaining (A', A''). (B) The cell bodies and neurites from an explant of midgut that were associated with a GDNF-impregnated bead. Like the dorsal root ganglia, the enteric neural cell bodies (arrows) are Ret+ and weakly GFRα1+, and the neurites (arrowheads) are strongly GFRα1+, but weakly Ret+. Scale bars, 25 μm.
acting as GDNF sinks. This hypothesis requires that the levels of GDNF are limiting. That the levels of GDNF can be limiting is supported by a decreased density of enteric neurons in the gut of gdnf1/2 mice (Shen et al., 1998) and by experiments on renal development in which increased expression of activated Ret ectopically within the kidney (Srinivas et al., 1999) or by neurons projecting to the kidney (Gestblom et al., 1999) resulted in renal malformations which were attributed to a decrease in the available concentration of GDNF around the ureteric bud tips. Thus we propose that the net direction of neural precursor cell migration, and the direction of axon projection pattern of the first enteric neurons, would be determined in part by chemoattraction toward higher levels of untapped GDNF protein.

**FIG. 8.** (A) Explant of E8.5 vagal (somites 1–7) neural tube plus somites grown on collagen with a control bead and a GDNF-impregnated bead placed lateral to the explant. The nerve fiber outgrowth shows no preference for the GDNF bead. Scale bar, 100 μm. (B, B′) Frozen section through an explant of neural tube plus somites processed for tyrosine hydroxylase (TH, B) and PGP9.5 (B′) immunohistochemistry, showing TH+/PGP9.5+ cells (arrow) within the explant, but outside of the neural tube. The location of the border of the neural tube is indicated by the dotted line. Scale bar, 25 μm.

**GDNF Has Little or No Chemoattractant Effect on Early Sensory (DRG) and Sympathetic Ganglion Cells and Neurites**

GDNF has been previously shown to promote the survival of a subpopulation of rat DRG neurons, but their dependency commences only in very late embryonic and early postnatal life (Matheson et al., 1996; Molliver et al., 1997). GDNF has also been shown to both promote the survival of, and promote neurite outgrowth from, E9 chick sympathetic neurons (Ebendal et al., 1995). We did not observe a directional response of neural cells or neurites to GDNF in explants of E11.5 mouse DRG and superior cervical ganglia, but we cannot rule out the possibility that these ganglia from later stage embryos might show a neurite outgrowth response to GDNF.

ret−/− and gdnf−/− mice show very early loss or a reduction in the number of neurons in the superior cervical ganglion (Table 2). Hence, although superior cervical ganglion cells show a survival response to GDNF at early stages, they showed no directional response to GDNF in the present assays. Conversely, survival, position, and differentiation of truncal sympathetic ganglion cells are not dependent on the GDNF system since they are unaffected by ret knockout (Table 2; Durbec et al., 1996). Yet cells in the E11.5 lumbar sympathetic ganglia did show a statistically significant orientation toward a GDNF source, although this was slight in magnitude compared to enteric neural cells. Thus, at least some of the response modalities to GDNF (survival, proliferation, differentiation, and chemoattraction) are separable.

**Lack of Chemoattractive Response to GDNF Is Not Due to Lack of GDNF Receptors**

For a variety of avian embryonic cranial sensory and parasympathetic ganglia and sympathetic ganglia, the survival, differentiation, and neurite outgrowth responses to GDNF and the related neurotrophic factor, neurturin, correlate with expression of the components of their receptors, Ret and GFrα1 (GDNF) or Ret and GFrα2 (neurturin) (Forgie et al., 1999; Hashino et al., 1999). However, in the current study, both the DRG and the superior cervical ganglia showed Ret and GFrα1 immunoreactivity of intensity comparable to that of the enteric neural cells, yet they showed no chemoattractant response to GDNF. Thus, the presence of the GDNF receptor components is not in itself sufficient to guarantee such a response to GDNF. The absence of neurite outgrowth and migration responses to GDNF by murine midembryonic dorsal root and superior cervical ganglion neurons is therefore likely to be due to an absence or inactivity of molecules downstream of the receptors.
CONCLUSION

Our results show that GDNF has a powerful chemoattractive effect on the migration and neurite outgrowth of enteric crest-derived cells. It is likely that GDNF expressed by the gut mesenchyme plays an important role in retaining neural crest-derived cells within the gut, in promoting the rostrocaudal migration of cells through the gut, and in promoting neurite outgrowth.

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