Age-Dependent Differences in the Effects of GDNF and NT-3 on the Development of Neurons and Glia from Neural Crest-Derived Precursors Immunoselected from the Fetal Rat Gut: Expression of GFRα-1 in Vitro and in Vivo

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No enteric neurons or glia develop in the gut below the rostral foregut in mice lacking glial cell line-derived neurotrophic factor (GDNF) or Ret. We analyzed the nature and age dependence of the effects of GDNF and, for comparison, those of NT-3, on the in vitro development of the precursors of enteric neurons and glia. Positive and negative immunoselection with antibodies to p75NTR were used to isolate crest-derived and crest-depleted populations of cells from the fetal rat bowel at E12, 14, and 16. Cells were typed immunocytochemically. GDNF stimulated the proliferation of nestin-expressing precursor cells isolated at E12, but not at E14–16. GDNF promoted the development of peripherin-expressing neurons (E12 $\rightarrow$ E14–16) and expression of TrkC. GDNF inhibited expression of S-100-expressing glia at E14–16. NT-3 did not affect cells isolated at E12, never stimulated precursors to proliferate, and promoted glial as well as neuronal development at E14–16. GFRα-1 was expressed both by crest- and non-crest-derived cells, although only crest-derived cells anchored GFRα-1 and GFRα-2 (GFRα-1 $\rightarrow$ GFRα-2). GDNF increased the number of neurons anchoring GFRα-1. GFRα-1 is immunocytochemically detectable in neurons of the E13 intestine and persists in adult neurons of both plexuses. We suggest that GDNF stimulates the proliferation of an early (E12) NT-3-insensitive precursor common to enteric neurons and glia; by E14, this common precursor is replaced by specified NT-3-responsive neuronal and glial progenitors. GDNF exerts a neurotrophic, but not a mitogenic, effect on the neuronal progenitor. The glial progenitor is not maintained by GDNF.

Key Words: neural crest; enteric nervous system; gut; intestine; GDNF; Ret; GFRα-1; GFRα-2; NT-3; TrkC; nestin; PCNA; Hirschsprung's disease.

INTRODUCTION

The enteric nervous system (ENS) is formed by precursor cells that migrate to the bowel from three regions of the neural crest (Gershon, 1997). The vagal crest is the only level that provides precursors to the entire bowel (Epstein et al., 1994; Le Douarin et al., 1975; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954, 1955). The sacral crest colonizes only the postumbilical gut (Le Douarin et al., 1975; Le Douarin and Teillet, 1973; Pomeranz and Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991), while the rostral truncal crest provides precursors only to the esophagus and adjoining region of the stomach (Durbec et al., 1996). Many, but probably not all (Henion and Weston, 1997; Lo and Anderson, 1995), crest-derived progenitors of enteric neurons are multipotent when they arrive in the bowel (Lo and Anderson, 1995; Rothman et al., 1990, 1993b; Sextier-Sainte-Claire Deville et al., 1994). Whether or not enteric crest-derived progenitors are multipotent, their fate depends on factors they encounter within the enteric microenvironment. These factors may influence the phe-
notypic choice of multipotent precursors and the development/survival of those progenitors that are fate-restricted.

The developmental potential of enteric crest-derived cells diminishes as a function of age and the cells become sorted into multiple lineages (Blaugrund et al., 1996). Factors that are present in the fetal bowel and have been found to promote the development of enteric neurons include growth factors, such as neurotrophin-3 (NT-3) (Chalazonitis et al., 1994) and the neuropoietic cytokines, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Chalazonitis et al., 1998), and extracellular matrix proteins, such as laminin-1 (Chalazonitis et al., 1997b; Pomeranz et al., 1993). An ENS exists in mice with targeted mutation in genes encoding NT-3 (Fariñas et al., 1994), its high affinity receptor, TrkC (Klein et al., 1994), CNTF (Masu et al., 1993; Takahashi et al., 1994), LIF (Sendtner et al., 1996), or the α (CNTFRα) and β (LIFRβ) components of the tripartite neuropoietic cytokine receptor (DeChiara et al., 1995; Gershon, 1997; Li et al., 1995). Some of these animals (those with mutations in CNTFRα and LIFRβ) lack subsets of enteric neurons that are critical for life; nevertheless, the presence of an ENS in all of these knockout animals establishes that neither NT-3, nor a ligand for the neuropoietic cytokine receptor, are essential for the development of the entire complement of enteric neurons and glia.

In contrast to NT-3 and the neuropoietic cytokines, glial cell line-derived neurotrophic factor (GDNF) appears to be essential for the development of all enteric neurons and glia below the esophagus and proximal stomach. Except for the neurons of the presumptive esophagus and stomach, which are derived from the rostral truncal crest (Durbec et al., 1996), enteric neurons and glia are completely missing in animals that lack either the receptor tyrosine kinase, Ret (Schuchardt et al., 1994), or GDNF (Moore et al., 1996; Jichel et al., 1996; Sánchez et al., 1996). Ret, which is expressed by crest-derived cells in the fetal bowel (Lo and Anderson, 1995; Pachnis et al., 1993), is the signaling receptor that is responsible for mediating the effects of GDNF (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). GDNF does not bind directly to Ret, but to an α component (GFRα), which then forms a transductionally active complex with homodimers of Ret (Jing et al., 1996). There are multiple GFRα proteins, GFRα-1, GFRα-2, GFRα-3, etc. (Jing et al., 1997), and it appears that GDNF preferentially binds to GFRα-1 (Baloh et al., 1997; Davies et al., 1997), while neuturin (NTN) binds preferentially to GFRα-2 ( Creedon et al., 1997; Jing et al., 1997). GFRα-1 has recently been demonstrated to be essential for the formation of the ENS; defects that are similar to those seen in the bowel of GDNF- or Ret-deficient animals are also seen in mice lacking GFRα-1 (Cacalano et al., 1998). Although the effects of the knockouts of Ret, GDNF, and GFRα-1 strongly imply that the development of mammalian enteric neurons and glia is GDNF-dependent, the effects of GDNF on the precursors of these cells have not yet been determined. A recent study has examined the effect of GDNF on mixed cultures of cells from the bowel (Heuckeroth et al., 1998); however, since this investigation was not carried out with isolated crest-derived cells, the direct effects of GDNF on the precursors of enteric neurons could not be distinguished from those that might be modified by the indirect actions of non-crest-derived cells (present in greater numbers) in the same cultures. The location of GFRα-1 in the developing bowel has also not previously been demonstrated.

The fact that there are no enteric neurons or glia below the esophagus and adjacent stomach following the knockout of Ret or GDNF suggests that all of the vagal and sacral crest-derived émigrés in the bowel are GDNF-dependent. Since the initial crest-derived population that colonizes the gut contains multipotent precursor cells, and all enteric precursors are lost in GDNF/Ret-deficient animals, it follows that the survival and/or continued development of the multipotent precursor is GDNF/Ret-dependent. GDNF would thus be expected to promote the development, proliferation, and/or survival of an early precursor that gives rise both to enteric neurons and glia. GDNF might also, in theory, exert effects on committed neural or glial progenitors. Such effects might lead to indirect consequences, since neurons and glia can affect one another's survival. Precursor cells would also be expected to express and/or anchor GFRα-1. To test these hypotheses, the effects of GDNF on the proliferation of enteric neural/glial precursors and on the in vitro development of neurons and glia were characterized as a function of fetal age and compared with those of NT-3. The ability of GDNF to influence responses to NT-3 was also investigated. The effects of GDNF and NT-3 were studied on preparations of crest-derived and crest-depleted cells isolated from the fetal rat gut by immunoselection with antibodies to p75NTR (Chalazonitis et al., 1994, 1997b, 1998; Pomeranz et al., 1993), a marker for crest-derived cells colonizing the fetal rat bowel (Baetge et al., 1990a). The separated crest-derived and crest-depleted populations were also employed to identify the cells of the fetal bowel that express and/or anchor GFRα-1 and GFRα-2. GDNF stimulated the proliferation of enteric neural/glial precursors, but NT-3 did not. GDNF also increased the proportion of crest-derived cells that express TrkC and promoted the development of neurons at E12, an age when NT-3 was ineffective. Although both crest- and non-crest-derived cells were found to express GFRα-1, only crest-derived cells anchored GFRα-1 or GFRα-2. The observations support the view that the ENS below the rostral foregut is derived from a multipotent GDNF-responsive precursor cell. A preliminary report of some of these data has been presented (Chalazonitis et al., 1997a).

MATERIALS AND METHODS

Animals. Fetal rats were obtained from timed pregnant mothers (Sprague Dawley; Charles River, MA). The day at which a
FIG. 1. The efficiency of immunoselection is independent of fetal age. The numbers of total cells (●) and crest-derived cells (▲) obtained by immunoselection from dissociated preparations of fetal bowel increase as a function of age between E12 and E16; nevertheless the proportion of the dissociated cell population that is immunoselected (▲) remains constant during the same period. The immunoselection procedure effectively removes crest-derived cells from the dissociated mixture of cells obtained from the fetal bowel. Even after 1 week in culture, <0.02% of residual cells are p75NTR-immunoreactive and the ratio of p75NTR-immunoreactive cells in the crest-derived cultures to those in the residual cultures is extremely high, 1214:1.

vaginal plug was found was designated as day 0 of gestation. Maternal rats were asphyxiated with CO₂ at gestational day 12, 14, or 16. This procedure has been approved by the Animal Care and Use Committee of Columbia University. The fetal bowel was dissected aseptically from 25 to 30 fetuses per experiment.

Immunoselection. The fetal gut was dissociated as described previously to give rise to a suspension of single cells (Chalazonitis et al., 1994, 1997b, 1998). The dissociated cells were suspended in a medium consisting of αMEM, supplemented with 20% horse serum (HS; JRH Biosciences, Lenexa, KS) and 10% chick embryo extract (CEE; from E11 embryos) and incubated for 1 h at 4°C with a monoclonal antibody to p75NTR (lgG192, diluted to 20 μg/ml; donated by Regeneron Pharmaceuticals Inc., Tarrytown, NY). The incubated cells were centrifuged (at 140 × g for 5 min), washed, and resuspended in CEE and HS-free medium at a density of 10⁶ cells/100 μl. Antibody-coated cells were decorated with magnetic microbeads coupled to goat anti-mouse IgG (Miltenyi Biotec Inc., Auburn, CA) and selected by passage through a magnetic field (~0.6 Tesla; MS, Miltenyi Biotec Inc.). Crest-derived p75NTR-immunoreactive cells are retained by the magnetic field, while the remainder (“residual cells”) pass through. The residual cells are collected first, the magnetic field is then released, and the immunoselected cells are finally flushed from the column with 1.5 ml of medium. Even though more total cells were obtained from the bowel as it enlarged with age, and more crest-derived cells were thus immunoselected, age did not significantly affect the proportion of cells that were immunoselected with antibodies to p75NTR (Fig. 1). At E12, an average of 1.1 ± 0.3 × 10⁶ immunoselected cells were obtained in each experiment (n = 6), which represented 15.7 ± 3.5% of the cells in the starting cell suspension. At E14, an average of 5.0 ± 1.6 × 10⁶ immunoselected cells were obtained in each experiment (n = 3), which represented 17.8 ± 4.7% of the cells in the starting cell suspension at that age. At E16, an average of 13.1 ± 10³ immunoselected cells were obtained in each experiment, which represented 20.1% of the cells in the starting cell suspension.

Tissue culture. Immunoselected and residual cells were always plated for culture at an equal cell density in each experiment. The density at which these cells were plated, however, varied with the type of vessel. Because fewer immunoselected cells could be obtained at E12 than at later ages, cells from the E12 bowel (1.2 × 10⁶ cells/ml) were grown as microcultures on glass coverslips (RESY No. 1001, Germany; 12-mm-diameter). These coverslips were placed in tissue culture wells (Nunc No. 176740). Cells from the E14 or E16 gut (1.8 × 10⁶ cells/ml) were plated in 20-mm-chamber slides (Nunc No. 177429). In all cases, the substrate was rat tail collagen and laminin (isolated from the Engelbreth-Holm-Swarm mouse tumor; Becton Dickinson Labware, Bedford, MA). Cells were grown in Basic Brazeau Medium (BBM) (Ziller et al., 1983), a defined medium used previously to study the development of enteric neurons in vitro (Chalazonitis et al., 1994, 1997b, 1998). At the time of plating, the medium was supplemented with 20% horse serum (HS) to promote the adherence of cells to the substrate. After 18 h, this medium was changed to serum-free BBM. The cultures were then fed on the fourth day in vitro. Cells were cultured for a maximal time of 7 days. When growth factors, GDNF (rat r; 10 ng/ml; R&D Systems, Berkeley, CA) or NT-3 (human r, 40 ng/ml; Regeneron Pharmaceuticals, Tarrytown, NY) were studied, the factors were added to the medium at the time of initial plating and remained present thereafter. Control cultures contained only the vehicle in which the growth factors were suspended (0.5% bovine serum albumin in BBM). Each type of culture was maintained in triplicate. Following the culture period, cells were fixed for 1 h with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate-buffered saline (PBS) to permeabilize the cells. The immunocytochemical demonstration of cell markers. In the case of demonstration of the proliferating cell nuclear antigen (PCNA, see below), fixation lasted 30 min with 1% paraformaldehyde.

Immunocytochemistry. For detection of antigens in cultured cells, fixed cultures were first rinsed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Triton X-100 (Sigma) to permeabilize the cells. The preparations were incubated with PBS-Triton containing 10% HS or 5% goat serum (GS) to reduce nonspecific background staining. Cultures were exposed as whole mounts to primary antibodies suspended in PBS-Triton, containing 4% HS or 2% GS (Table 1). Antibodies to GFRα-1, GFRα-2, and PCNA were incubated from 48 to 72 h at 4°C. All other primary antibodies were incubated overnight at room temperature. PCNA was detected with primary antibodies directly coupled to fluorescein isothiocyanate. All other antigens were visualized indirectly. After treatment with cultures with primary antibodies, the cultures were rinsed and exposed for 3 h at room temperature to an appropriate affinity-purified species-specific secondary antibody. Secondary antibodies included goat anti-rabbit IgG and goat anti-mouse IgG coupled to either alkaline phosphatase (diluted 1:50, Kirkegaard & Perry Labs, Gaithersburg, MD) or biotin (ABC elite kit, Vector Labs, Burlingame, CA). The biotinylated probes were detected with streptavidin–HRP. Sites of bound alkaline phosphatase were visualized with a commercial kit (blue product, Vec-
TABLE 1

Immunocytochemical Markers Used to Identify Cells Differentiating in Vitro

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cell type</th>
<th>Dilution</th>
<th>Antibody</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p75NTR</td>
<td>Cells of neural crest origin</td>
<td>20 ng/ml</td>
<td>Mouse monoclonal</td>
<td>Regeneron, Tarrytown, NY</td>
<td>(Baetge et al., 1990a; Bannerman and Pleasure, 1993)</td>
</tr>
<tr>
<td>Nestin</td>
<td>Neural/gliial precursor</td>
<td>1:500</td>
<td>Mouse monoclonal</td>
<td>PharMingen, San Diego, CA</td>
<td>(Friedman et al., 1990; Hockfield and McKay, 1985; Lendahl et al., 1990)</td>
</tr>
<tr>
<td>Peripherin</td>
<td>Neurons</td>
<td>1:750</td>
<td>Rabbit polyclonal</td>
<td>R. Liem and L. Greene, Columbia Univ., New York, NY</td>
<td>(Baetge et al., 1990a; Portier et al., 1984)</td>
</tr>
<tr>
<td>S-100</td>
<td>Glia</td>
<td>1:400</td>
<td>Rabbit polyclonal</td>
<td>Sigma, St. Louis, MO</td>
<td>(Bishop et al., 1985; Ferri et al., 1982; Kawana et al., 1988; Scheuermann et al., 1989)</td>
</tr>
<tr>
<td>TrkC</td>
<td>Cells responsive to NT-3</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
<td>Barbara Hempstead, Cornell Med. Coll.</td>
<td>(Donovan et al., 1996; Tsouflias et al., 1993)</td>
</tr>
<tr>
<td>GFRα1, α2</td>
<td>Responsive to GDNF family</td>
<td>1:100</td>
<td>Rabbit polyclonal</td>
<td>C.F., Ibañez, Karolinska Inst., Stockholm, Sweden</td>
<td>(Davies et al., 1997)</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cells</td>
<td>1:200</td>
<td>Mouse monoclonal</td>
<td>PharMingen, San Diego, CA</td>
<td>(Garcia et al., 1989; Hyde-Dunn and Jones, 1997)</td>
</tr>
</tbody>
</table>

GDNF, but Not NT-3, Increases both the Number of Neurons and Their Precursors

Cultures of E12 immunoselected and residual cells were exposed for 1 week to GDNF, NT-3, or vehicle. Expression of nestin immunoreactivity was used as a marker for precursor cells, and peripherin immunoreactivity was used to mark cells committed to develop as neurons. The numbers of nestin- and peripherin-immunoreactive cells in cultures of immunoselected cells exposed to GDNF (10 ng/ml) were each about 15-fold greater than those found in controls, in which the primary antibody was omitted or substituted with preimmune serum, were always included with each procedure.

Immunocytochemistry was used to locate GFRα1 in frozen sections of E13 fetal rats and adult rat intestine. Freshly dissected fetuses or segments of bowel were frozen in liquid N2, embedded in OCT medium (Lipshaw), and stored at −80°C until used. Sections (10 μm) were cut at 4°C with a cryostat–microtome and collected on gelatin-coated glass slides. Sections were exposed to primary antibodies to GFRα1 (diluted 1:3000–1:500) for 24 h at 4°C. Sites of immunostaining were visualized with biotinylated secondary antibodies and streptavidin-HRP as described above.

Detection of mRNAs encoding GFRα1. Reverse transcription and the polymerase chain reaction (RT-PCR) were used to identify mRNAs encoding GFRα1 in the p75NTR-immunoselected and residual cell populations. Total RNA was extracted with 6 M guanidinium thiocyanate (Chomczynski and Sacchi, 1987), incubated with Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-Bethesda Research Labs, Bethesda, MD) in the presence of random primers using the protocol provided by the supplier. The resulting cDNA was amplified using primers designed from the sequence of rat GFRα1 (Jing et al., 1996). The forward sequence was 5'-TTGTCAGAAGAATGCTG-3' (corresponding to nucleotides 405–423) and the reverse sequence was 5'-TTGGCCGCTCTTCCTTCCTC-3' (corresponding to nucleotides 889–906). Thirty-five PCR cycles were carried out as follows: 94°C for 0.5 min, 57°C for 45 s, 72°C for 0.5 min. The primers were used at a concentration of 0.34 μM. cDNA encoding the 150 kDa neurofilament protein (NF-M) was amplified (28 cycles carried out as follows: 94°C for 0.5 min, 56°C for 1 min, 72°C for 45 s) using the following primers: 5'-GATACCGAGATCCTCTTAACAG-3' (corresponding to nucleotides 178–198) and the reverse sequence was 5'-GGCCTCTTCCTTCTTCCTC-3' (corresponding to nucleotides 490–507) (Julien et al., 1986). cDNA encoding β-actin was amplified (25 cycles, with an annealing temperature of 56°C) using the following primers: 5'-CTCTTTGATGTCACGCACGATTTC-3' (forward) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (reverse). PCR products were separated by electrophoresis through a 1.5% agarose gel.

RESULTS
cultures exposed either to vehicle or NT-3 (40 ng/ml) (Fig. 2; compare Figs. 3D [vehicle] and 3F [GDNF-treated]). In contrast to GDNF, NT-3 did not significantly change the number of precursors or neurons. These observations suggest that GDNF sensitivity of enteric cells able to develop as neurons arise before these cells become responsive to NT-3 (which had previously been demonstrated in older fetuses; see below).

The immunoreactivity of the precursor marker, nestin, appeared only in scattered islands of cells in control cultures of immunoselected cells (Fig. 3A). In contrast, following exposure to GDNF, nestin-immunoreactive cells seemed to be more evenly dispersed over the culture dish forming a dense cellular mat (Fig. 3B). The nestin immunoreactivity was intracytoplasmic and filamentous (cytoskeletal) in nature, outlining the nonimmunoreactive nuclei, around which it was most dense. Processes were also nestin-immunoreactive; however, the cytoskeletal nature of the nestin immunoreactivity made it difficult to assess cell size or shape in cells stained only to demonstrate this immunogen (Fig. 3C).

In preparations in which double-label immunocytochemistry was used to visualize nestin and peripherin immunoreactivities simultaneously (Figs. 3D–3F), there was very little coincident localization of the two antigens (0.6 ± 0.1% of total cells in control cultures). Again, GDNF increased the number of cells stained individually by the two antibodies; however, even after stimulation with GDNF there was very little coexpression of nestin and peripherin immunoreactivities in the same cells (Fig. 3E; 0.9 ± 0.2% of total cells; P is ns vs control). The total number of cells expressing nestin and peripherin immunoreactivities did not account for all of the cells in either control or GDNF-treated immunoselected cell cultures. The cells that were not immunostained by either antibody constituted 89.0 ± 3% of the total number of cells in control cultures and 58.4 ± 1% of cells in cultures exposed to GDNF. Since the number of cells that were neither nestin- nor peripherin-immunoreactive was increased 2.2 ± 0.1-fold by GDNF, at least some of the nonimmunostained cells must be GDNF-responsive. The nonimmunostained cells may be uncommitted crest-derived precursors that have not acquired nestin. Such cells might do so at a later stage (see also experiments on proliferation below).

A close examination of the cultures of immunoselected cells revealed that the clustering of peripherin-immunoreactive neurons that was evident in control cultures was preserved even after treatment with GDNF (Fig. 3F). What appeared to be a more uniform distribution of cells when cultures were examined at low magnification was caused by the GDNF-induced expansion of the cellularity of the neural islands (Fig. 4 and compare Fig. 3D with 3F). The number of neurons per cluster was greatly increased by treatment with GDNF from 16 ± 2 to 43 ± 3 (n = 50; P < 0.001). The GDNF-promoted clustering of neurons is compatible with the idea that GDNF stimulates the development of colonies of neurons from a common progenitor.

Peripherin-immunoreactive cells were extremely rare (=0.1%) in control cultures of the residual cells, which were not immunoselected at E12 with antibodies to p75NTR (not illustrated). Following exposure of the residual cells to GDNF, however, small clusters of peripherin-immunoreactive cells appeared. Even after treatment with GDNF, however, the proportion of peripherin-immunoreactive cells remained quite low (<2%) in the cultures of residual cells. These observations suggest that small numbers of neuronal precursors are present in the residual cell population, although these cells are not readily detected in the absence of GDNF.

The Developmental Response of Enteric Neural Precursors to GDNF Decreases at the Age When Cells Become Responsive to NT-3

The magnitude of the response of immunoselected crest-derived cells to GDNF decreased significantly (P < 0.001) between E12 and E14 (Fig. 5A). GDNF was still effective at E14 in promoting the development of peripherin-expressing neurons, and it continued to be effective at E16. When cells were isolated by immunoselection at E12 and cultured for a week in the absence of GDNF, the proportion of surviving nestin-immunoreactive cells was quite low (2.3 ± 1.3%; n = 3). In contrast, when cells were isolated by immunoselection at E14 and similarly maintained for 1 week in the absence of GDNF, the proportion of surviving nestin-immunoreactive cells was substantial (26.5 ± 0.5%; n = 3) and significantly greater than at E12 (P < 0.001). The precursor cells that are isolated at E14 are evidently better able to survive in vitro in the absence of GDNF than those isolated at E12, perhaps because the later precursors have been exposed for a longer time to GDNF in situ. Both the effectiveness of GDNF and the dependence of neuronal precursors (nestin-immunoreactive cells) on GDNF, therefore, are maximal at E12.

Responses to NT-3 differed from those to GDNF, in the
sense that NT-3 did not significantly affect the development of neurons at E12 but acquired the ability to do so at E14 (Fig. 5B; \( P < 0.001 \) vs control). NT-3 was thus ineffective at a time when cells were maximally responsive to GDNF and became effective at a time when the effectiveness of GDNF was declining. The ability of NT-3 to promote neuronal development was maintained at E16. It should be noted that even when NT-3 was maximally active at E14 (approximately twofold enhancement), the promotion of neuronal development by NT-3 was less than that of GDNF (approximately fourfold enhancement) at the same age (compare Fig. 5A with 5B).

**GDNF Increases the Proportion of Crest-Derived Cells That Express TrkC Immunoreactivity**

Since crest-derived cells respond to GDNF before NT-3, it is possible that neuronal precursors require GDNF stimu-
lation to become NT-3-responsive. If cells are able to respond to NT-3, then they would be expected to express TrkC (Lamballe et al., 1991; Tessarollo et al., 1993). The effect of GDNF on the expression of TrkC by crest-derived cells was therefore investigated. TrkC-immunoreactive cells were identified immunocytochemically with antibodies that react with the catalytic region of the TrkC tyrosine kinase domain (Buj-Bello et al., 1995; Donovan et al., 1996; Maxwell et al., 1996). These antibodies do not cross-react significantly with TrkA or TrkB. Crest-derived cells were immunoselected from the E12 fetal bowel and cultured for 7 days in the presence or absence of GDNF (10 ng/ml). Very few TrkC-immunoreactive cells were observed in the control cultures (Fig. 6A); however, many cells expressed TrkC immunoreactivity in cultures exposed to GDNF (Figs. 6B and 6C). The GDNF-induced increase in TrkC-immunoreactive cells was about 12-fold.

Promotion of Neuronal Development by GDNF and NT-3 Are Not Additive

To determine whether the effects of GDNF and NT-3 on the in vitro development of enteric neurons are additive, the factors were applied alone and in combination to crest-derived cells immunoselected from the fetal gut at E12, E14, and E16. Supramaximal concentrations of NT-3 (40 ng/ml) (Chalazonitis et al., 1994) and GDNF (10 ng/ml) (Jing et al., 1996) were used. As previously noted, the effect of GDNF in promoting the in vitro development of neurons was maximal at E12, while NT-3 at this age was ineffective (Table 2). The effects of exposing E12 cultures to GDNF plus NT-3 on the development of neurons were not significantly different from those of exposing sister cultures to GDNF alone (Table 2). At E14 and E16, when the relative effectiveness of GDNF decreased, and NT-3 was able, by itself, to promote the development of neurons, the effects of the combination of GDNF and NT-3 were still not significantly different from the effects of GDNF alone (Table 2). GDNF and NT-3, therefore, are not additive in promoting the development of enteric neurons in vitro.

GDNF, but Not NT-3, Increases the Absolute Number of Cells in Cultures of p75 NTR-Immunoselected Cells at E12

When cells that were immunoselected at E12 with antibodies to p75NTR were grown in the presence of GDNF for 7 days the absolute number of cells/culture was over threefold greater than that found in vehicle-treated sister cultures (Fig. 7). In contrast, the addition of GDNF did not affect the total number of cells/culture when it was added to cultures of the residual cells, which remained after the crest-derived cells were removed by the same immunoselection procedure (Fig. 7). In contrast to E12, the total number of cells was not altered by the addition of GDNF to cultures of immunoselected or residual cells at E14 or E16 (Fig. 7). Unlike GDNF, NT-3 was unable to increase absolute cell numbers at any age, no matter whether it was added to cultures of immunoselected or residual cells (Fig. 7). The selective increase in the total number of crest-derived cells induced by GDNF at E12 is consistent with
the possibility that it stimulates the proliferation of at least some of the cells in this population. The proliferative effect of GDNF is not shared by NT-3.

**GDNF Increases the Expression of PCNA in Cultures of p75
NTR-Immunoselected Cells at E12**

The immunoreactivity of PCNA was examined in cultures of cells immunoselected with antibodies to p75
NTR at E12 to test the hypothesis that GDNF stimulates proliferation of cells in this population. PCNA is an accessory protein for DNA polymerases δ and ε and is a good immunocytochemical marker for replicating cells in the S-phase of the cell cycle (Assy et al., 1998; Ellison and Stillman, 1998; Hyde-Dunn and Jones, 1997; Tomlinson and Mackey, 1998). Cultures were maintained for 3.5 or 7 days in the presence or absence of GDNF. GDNF increased the proportion of cells with PCNA-immunoreactive nuclei by more than threefold (Figs. 8 and 9); the effect of GDNF on cells cultured for 3.5 days was virtually identical to that on cells cultured for 7 days. Many, but not all, of the cells with PCNA-immunoreactive nuclei were nestin-immunoreactive (Fig. 9). These data support the view that GDNF stimulates the proliferation of cells in a neuronal or glial lineage. The proliferating cells that do not express nestin could be immature and not yet specified to the point where they display nestin. Alternatively, it is possible that a subset of enteric neurons and/or glia is derived from progenitor cells that do not express nestin.

**GDNF Does Not Promote the Development of Enteric Glia**

Glia were identified by using the immunoreactivity of S-100 as a marker. Crest-derived cells were again immunoselected from the fetal gut at E12, E14, and E16. The cells were cultured for 1 week in the presence of GDNF (10 ng/ml). GDNF did not affect the proportion of S-100-immunoreactive cells in cultures of cells immunoselected at E12 (Table 3); however, there were significantly fewer S-100-immunoreactive cells in the GDNF-exposed E14 and E16 cultures than in the age matched vehicle-treated controls (Table 3). In contrast to GDNF, NT-3 increased the proportion of S-100-immunoreactive cells when added to cultures of cells that were immunoselected at E14 (Table 3). The ability of NT-3 to promote glial development has been reported previously (Chalazonitis et al., 1994). The S-100-immunoreactive cells that developed in culture did not necessarily do so in association with neurons (Fig. 10). The cells tended to form aggregates of small slender stellate

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Image of graph showing the number of neurons per dish with and without GDNF or NT-3 at different stages of development (E12, E14, E16).
cells. The morphology of the S-100-immunoreactive cells developing in the presence of GDNF (Fig. 10A) was similar to that developing in control cultures (Figs. 10B and 10C). These observations indicate that GDNF does not enhance the development of glia as it does of neurons and, when applied relatively late in enteric development, may even skew the developing population toward the neuronal lineage.

**Although Only Neurons Are GFRα-1-Immunoreactive, mRNA Encoding GFRα-1 Is Expressed by both Crest-Derived and Non-Crest-Derived Cells**

Since an "α component" is required for the efficient stimulation of Ret by GDNF or neurturin (Creedon et al., 1997; Davies et al., 1997; Jing et al., 1996; Klein et al., 1997; Treanor et al., 1996; Trupp et al., 1996, 1997), the biosynthesis and localization of GFRα-1 and GFRα-2 were investigated in cells isolated from the fetal bowel. Crest- and non-crest-derived cells were isolated and separated from fetal gut by immunoselection at E14. To determine the effectiveness of immunoselection, RT–PCR was used to analyze mRNA-encoding NF-M, which was employed as a neuron-specific marker protein. mRNA-encoding NF-M was found only in the p75NTR-immunoselected cell population, and not in the residual cells (Fig. 11); therefore, there must be little or no neuronal contamination of the residual cell fraction. In contrast to NF-M, mRNA-encoding GFRα-1 was found to be expressed both by the immunoselected and the residual cell populations (Fig. 11). These observations suggest that both crest-derived and non-crest-derived cells of the E14 fetal gut synthesize GFRα-1.

Immunocytochemistry was employed to locate GFRα-1 protein. Since GFRα-1 is a peripheral protein that becomes linked to the plasma membrane by a glycosylphosphoinositol anchor, it follows that the cells that bind the protein may not be the cells that synthesize it (Buj-Bello et al., 1997; Creedon et al., 1997; Jing et al., 1996; Klein et al., 1997; Yu et al., 1998). In marked contrast to the mRNA, GFRα-1 immunoreactivity was found to be almost completely confined to the immunoselected cultures (Figs. 12 and 13). All of the GFRα-1-immunoreactive cells in these cultures were process-bearing and exhibited the morphology of neurons (Fig. 13A). Both perikarya and neurites were GFRα-1-immunoreactive. Two populations of cells could be defined by the intensity of immunoreactivity. Many more cells were found to be GFRα-1-immunoreactive in cultures treated with GDNF than in controls. After treatment with GDNF, rare GFRα-1-immunoreactive cells were even noted in the cultures of residual cells. Again, however, the GFRα-1-immunoreactive cells were process-bearing and appeared to be neurons. These observations suggest that GFRα-1 selectively binds to the plasma membranes of cells in a neuronal lineage, although both crest- and non-crest-derived cells probably synthesize the protein. The increased

**FIG. 6.** GDNF increases the number of cells expressing TrkC immunoreactivity in cultures of cells immunoselected with antibodies to p75NTR at E12. (A) Control. Media were supplemented only with vehicle. Very few cells express TrkC (arrow) (0.8 × 10^3 cells/12-mm-diameter coverslip). These cells are process-bearing and have a neuronal morphology. (B) GDNF. A massive increase has been induced in the numbers of TrkC-immunoreactive cells (arrow) (9.7 × 10^3 cells/12-mm-diameter coverslip). These cells are process-bearing and have a neuronal morphology. (C) GDNF. At higher magnification, the TrkC-immunoreactive cells (arrow) can be recognized as neurons. Both cell bodies and neurites are TrkC immunoreactive. Calibration bars: A and B, 100 μm; C, 50 μm.

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number of cells found to contain GFRα-1-immunoreactivity in cultures treated with GDNF could reflect either an ability of GDNF to induce GFRα-1 expression or, alternatively, the GDNF-induced increase in neuronal development.

In contrast to GFRα-1-immunoreactive cells, which were abundant in the immunoselected population even in the absence of treatment with GDNF, very few cells in either the immunoselected or the residual populations exhibited GFRα-2 immunoreactivity (~0.4% of total cells). The addition of GDNF to cultures of immunoselected cells did increase the number of cells that were GFRα-2-immunoreactive (~1.6% of total cells); however, even in the presence of GDNF, the proportion of cells that were GFRα-2-immunoreactive was very small (Fig. 14). The GFRα-2-immunoreactive cells were always found in iso-

| Table 2: GDNF and NT-3 Are Not Additive in Promoting Neuronal Development |
|---------------------------------|--------|--------|--------|--------|
| Factors                        | E12    | P      | E14    | P      | E16    | P      |
| GDNF                           | 1342 ± 126 | < 0.001 vs NT-3 | 369 ± 79 | < 0.001 vs vehicle; | 327 ± 9 | —      |
| NT-3                           | 120 ± 20 | ns vs vehicle | 210 ± 15 | < 0.001 vs vehicle | 172 ± 10 | < 0.001 vs vehicle |
| GDNF + NT-3                    | 1075 ± 45 | < 0.001 vs NT-3 | 322 ± 54 | ns vs NT-3 at E14 | 373 ± 46 | < 0.01 vs NT-3 at E16 |

The number of peripherin-immunoreactive neurons as a percentage of the number found in paired vehicle-treated control cultures. The mean neuronal density in vehicle-treated cultures was 39 ± 8 neurons/mm². This represented a mean of 4439 ± 976 neurons per dish in microcultures and 38,713 ± 15,550 neurons/dish in 35-mm Falcon dishes. The number of neurons/mm² found in vehicle-treated microcultures did not differ significantly from the neurons/mm² found in similarly treated cultures grown in Falcon dishes.
lated aggregates. The individual cells were process-bearing and appeared to be neurons.

GRFα-1 immunoreactivity was detected in situ in E13 rats. At this time, small clusters of primitive neurons exhibited GRFα-1 immunoreactivity in the fetal stomach and small, and large intestines (Fig. 15A). Also at this time, they were immunostained by antibodies to GRFα-1, neurons in dorsal root ganglia (Fig. 15C), nerve processes in muscles of the developing body wall (Fig. 15D), spinal motor neurons, periaortic sympathetic neurons, and renal tubules (not illustrated). GRFα-1 immunoreactivity continued to be detectable in neurons of both submucosal (Fig. 15E) and myenteric ganglia (Figs. 15E and 15F) in the adult bowel.

**DISCUSSION**

**GDNF Selectively Affects an Early Precursor Common to the Vagal and Sacral Populations of Crest-Derived Emigrés in the Bowel**

Data obtained in the current study support the hypothesis that GDNF promotes the survival and/or development of an early precursor of enteric neurons. When applied in vitro to crest-derived cells isolated from the fetal rat bowel early in development, GDNF enhanced both the proliferation of neuronal precursors and the in vitro development of neurons. At E12, but not later, GDNF increased the proportion of crest-derived cells that exhibit PCNA, a marker for proliferation (Assy et al., 1998; Ellison and Stillman, 1998; Hyde-Dunn and Jones, 1997; Tomlinson and Mackey,
and nestin, a marker for neuronal (and possibly glial) progenitors (Friedman et al., 1990; Hockfield and McKay, 1985; Lendahl et al., 1990) as well as peripherin, a marker for cells specified to be neurons. GDNF has also been found to stimulate the incorporation of tritiated thymidine by crest-derived cells immunoselected from the murine bowel (Wu et al., 1997) and the uptake of bromodeoxyuridine by cells immunoselected from the quail gut with antibodies to HNK-1 (Hearn et al., 1998). At later ages, E14 and E16, GDNF no longer stimulated the proliferation of precursor cells and was less effective in promoting neuronal development. These data are consistent with the idea that GDNF-responsive cells are present in the early fetal bowel but decline in number as a function of age.

In contrast to GDNF, NT-3, which has previously been found to promote the in vitro development of enteric neurons (Chalazonitis et al., 1994), did not stimulate the proliferation of crest-derived cells at any age and was unable to promote neuronal development prior to age E14. Like NT-3, endothelin-3, another factor that has been demonstrated to exert a mitogenic effect on primary cultures of neural crest cells (Lahav et al., 1996), also fails to stimulate the proliferation of enteric crest-derived cells (Wu et al., 1997). The early mitogenic action of GDNF on enteric crest-derived cells, therefore, is relatively specific. The proliferative response of enteric neural precursors to GDNF is consistent with the idea that stimulation of Ret is required to enable the population of crest-derived émigrés to expand sufficiently to colonize the gut.

A major subset of patients with Hirschsprung's disease, in which the terminal colon is aganglionic, have heterozygous loss-of-function mutations in genes encoding RET (Edery et al., 1994; Pasini et al., 1995; Romeo et al., 1994), and a small subset has mutations in genes encoding GDNF (Angrist et al., 1998; Ivanchuk et al., 1996). In contrast, the homozygous knockout of genes encoding Ret (Schuchardt et al., 1994) or GDNF (Moore et al., 1996; Pichel et al., 1996; Sénchez et al., 1996) causes the entire bowel to become aganglionic below the rostral foregut. We propose that in the setting of decreased Ret activation in patients with Hirschsprung's disease the population of enteric crest-derived cells may be reduced.

FIG. 10. Relatively small numbers of S-100-immunoreactive glia develop in cultures of cells immunoselected with antibodies to p75^{NTR} at E16. (A) GDNF. A small cluster of relatively isolated S-100-immunoreactive cells can be seen (arrow). (B) Control. Media were supplemented only with vehicle. More cells are S-100-immunoreactive (arrow) than in A. (C) Control. At higher magnification, the S-100 immunoreactivity fills the cytoplasm of thin cells that extend short processes. The calibration bar, 50 μm.

FIG. 11. mRNA encoding GFRα-1 is present both in the crest-derived population of cells immunoselected from the fetal rat gut at E14 with antibodies to p75^{NTR} and in the non-crest-derived residual cells. RNA was extracted from the positively and negatively immunoselected cells and used to assay mRNA encoding GFRα-1, NF-M, and β-actin by RT-PCR. Sel, immunoselected cells; Res, residual cells; M, size ladder. The size of the PCR products (GFRα-1, 502 bp; NF-M, 330 bp; β-actin, 540 bp) corresponded to those predicted from the selected primers.
derived cells does not expand to a size sufficient to colonize the entire gut. The terminal colon, which lies at the ends of both vagal and sacral migratory pathways, thus remains uncolonized and becomes aganglionic. This proposal views the pathogenesis of the aganglionosis that occurs in RET- or GDNF-deficient patients as qualitatively different from that which occurs in individuals with mutations in genes encoding endothelin-3 (Edery et al., 1996; Hofstra et al., 1996) or its preferred receptor, endothelin B (Puffenberger et al., 1994). The pathogenesis we postulate for the aganglionosis associated with GDNF/RET mutations would be neural crest autonomous, while that which occurs in mice with natural or targeted mutations in endothelin-3 or the endothelin B receptor is known not to be so (Jacobs-Cohen et al., 1987; Kapur et al., 1993, 1995; Rothman et al., 1993a). Exposure of enteric crest-derived precursors to GDNF in vitro stimulates their proliferation and promotes the development of neurons. In contrast, when added to cultures of crest-derived cells from the fetal bowel, ET-3 fails to stimulate proliferation and inhibits neuronal development (Hearn et al., 1998; Wu et al., 1997). Furthermore, an abnormality of the extracellular matrix of the colon (increased expression of laminin-1) has been demonstrated to occur when ET-3 is deficient that does not occur when Ret is defective (Rothman et al., 1996). These observations suggest that the terminal colon can become aganglionic either because of an

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**FIG. 12.** GFRα-1 immunoreactivity is selectively exhibited by neurons in cultures of crest-derived cells immunoselected from the fetal rat gut at E12 with antibodies to p75NTR. (A) Vehicle-treated immunoselected culture. GFRα-1-immunoreactive cells (arrow) are process-bearing and form an interconnecting neuritic network. Neurites (arrowhead) and cell bodies are GFRα-1-immunoreactive. (B) GDNF-treated immunoselected culture. Many more GFRα-1-immunoreactive cell bodies (arrow) and neurites (arrowhead) are seen. (C) Vehicle-treated residual culture. No cells are GFRα-1-immunoreactive. (D) GDNF-treated residual culture. Rare GFRα-1-immunoreactive neurons appear. Both cells (arrow) and neurites (arrowhead) display GFRα-1 immunoreactivity. (E) In the absence of primary antibodies, neurons (arrow) are not stained. Differential interference contrast microscopy is used to visualize cells. Calibration bars, 50 μm.

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abnormality of the crest-derived cells that colonize the bowel or because of abnormalities of the non-crest-derived elements of the tissues through which these cells migrate.

Enteric Crest-Derived Cells May Have to Be Stimulated by GDNF to Become Responsive to NT-3

Despite the age-related decline in the efficacy of GDNF, its ability to promote neuronal development was always substantially greater than that of NT-3. GDNF, moreover, increased the number of TrkC-immunoreactive cells in the cultures of crest-derived cells; therefore, it is possible that prior exposure of crest-derived cells to GDNF may be a prerequisite for NT-3-responsiveness. Since the maximal responses of crest-derived cells to GDNF and NT-3 were not additive, and were never greater than the effects of GDNF alone, it is likely that the NT-3-responsive cells are a subset of a larger pool of cells stimulated by GDNF. The noncooperative nature of the two factors under the experimental conditions used is likely to reflect the supramaximal nature of the GDNF concentration employed. Essentially, if GDNF by itself induces as many neurons to develop as can possibly do so, then it will be impossible to obtain more neurons, even by stimulating TrkC. Another difference between GDNF and NT-3 was that NT-3 promoted the development of glia as well as neurons. In fact, at E14–16 (but not E12), GDNF actually inhibited gial development; therefore, at later stages GDNF appeared to enhance neuronal development at the expense of that of glia. The early sensitivity of enteric crest-derived cells to GDNF (preceding that to NT-3), the developmental regulation of the response, and the early proliferative effect of GDNF on neuronal precursors all support the idea that a common crest-derived precursor of the ENS is selectively dependent on GDNF at the time when it colonizes the bowel. Since the knockout of Ret affects all of the enteric crest-derived émigrés of the vagal and sacral, but not the truncal lineage of enteric precursors (Durbec et al., 1996), it is apparent that GDNF/Ret stimulation is only required for the development of those parts of the ENS that are formed by vagal and/or sacral crest-derived cells.

The Effects of GDNF Change as a Function of Age

GDNF has been found to exert neurotrophic effects in many different peripheral and central models (Buj-Bello et al., 1995; Henderson, 1996; Trupp et al., 1995). These actions of GDNF frequently change as a function of developmental age (Buj-Bello et al., 1995). The development of specific types of neuron may thus be affected differently by GDNF at different ages. In the current studies, of postmigratory enteric crest-derived cells, two qualitatively different kinds of action of GDNF were found, a mitogenic effect on unspecified precursors at E12 and a neurotrophic effect...
at E14–16. The neurotrophic effect of GDNF, moreover, was different from those of NT-3 or neuropoietic cytokines (Chalazonitis et al., 1998) in that only GDNF altered the ratio of neurons/glia in favor of neurons. These observations are consistent with at least three alternative possibilities. (i) An early common neuron/glia precursor, which is present at E12, could be replaced at E14 by specified neural and glial precursors; GDNF might be able to maintain the neural precursors while the glial precursors might require other factors, such as neuregulins (Shah et al., 1994) which might be supplied by the neurons. Enteric glia, for example, are selectively lost in mice with targeted mutations in the ErbB3 neuregulin receptor (Riethmacher et al., 1997). In the fetal and adult human bowel, moreover, only neurons, and not glia, express RET (Bär et al., 1997). (ii) GDNF could exert an instructive effect at E14–E16 on a retained neuron/glia precursor to follow a neuronal lineage. (iii) Finally, GDNF might promote the generation of a set of specified glial precursors, which die in the absence of a required factor. The observations that cells immunoselected at E14.5 from the fetal mouse gut with antibodies to Ret give rise in clonal cultures to neurons, but not to glia, and that the Ret-immunoreactive cells do not respond to glial growth factor, support the concept that distinct populations of Ret-expressing neuronal and non-Ret-expressing glial progenitors become specified in the fetal bowel (Lo and Anderson, 1995). GDNF has been reported to promote glial expression when applied to mixed cultures of crest- and non-crest-derived cells from the gut (Heuckeroth et al., 1998). GDNF, however, was found in the same study to fail

**FIG. 15.** GFR<sub>α</sub> immunoreactivity is detectable by immunocytochemistry in the fetal rat bowel at E13 and persists into adult life. (A) Cross section through a loop of E13 fetal intestine and immunostained with antibodies to GFR<sub>α</sub>-1. Note the immunostained cluster of neurons (arrow). M, mucosa. (B) Adjacent section through the same loop of E13 fetal intestine illustrated in A, but incubated with preimmune serum. There is no staining. (C) E13 dorsal root ganglion. A subset of neurons exhibits GFR<sub>α</sub>-1 immunoreactivity. (D) Developing muscle of the body wall. Nerve processes exhibit GFR<sub>α</sub>-1 immunoreactivity (arrow). (E and F) Adult rat intestine. Many neurons in both submucosal (arrowhead) and myenteric ganglia (arrow) exhibit GFR<sub>α</sub>-1 immunoreactivity. CM, circular layer of smooth muscle. Calibration bars, 25 μm.
to promote the proliferation of glia. GDNF thus appears not to affect cells in the glial lineage directly. It seems likely that the glial effects of GDNF in the mixed cultures are secondary to its primary action on neurons, which stimulate nonneuronal cells to secrete glial growth factors, such as neuregulins (Riethmacher et al., 1997). Neuregulins promote glial expression (Shah et al., 1993) and are produced by mesenchymal cells (Burden and Yarden, 1997).

Separation of Crest-Derived and Non-Crest-Derived Cells

Crest-derived cells were isolated from the fetal bowel at different ages to be able to examine the interaction of GDNF with these cells, independently of any effects produced by other cells of the gut wall, or effects that nonneuronal cells might exert on the precursors from the neural crest. Since GDNF (Moore et al., 1996; Suvanto et al., 1996; Treanor et al., 1996) and GFRα-1 (current study; see also below) are expressed by non-crest-derived cells of the enteric mesenchyme, it is particularly important to be able to isolate the crest-derived cells in order to investigate direct effects of GDNF. Cellular interactions might confound the interpretation of results in studies of explants of whole gut or dissociated mixed populations of cells. An important feature of the design of the current experiments, therefore, was that immunoselection was employed to obtain relatively pure populations of crest- and non-crest-derived cells.

Immunoselection has been utilized in earlier studies of the development of enteric neurons (Chalazonitis et al., 1994, 1997b, 1998; Lo and Anderson, 1995; Lo et al., 1997; Pomeranz et al., 1993). Four crest-derived cell markers have been employed in these studies, NC-1/HNK-1 immunoreactivity (Chalazonitis et al., 1994; Hearn et al., 1998; Pomeranz et al., 1993), a laminin-binding protein (LBP110) (Chalazonitis et al., 1997b), Ret (Lo and Anderson, 1995; Lo et al., 1997), and p75NTR (Chalazonitis et al., 1997b, 1998). In the earlier experiments, antibodies to p75NTR were shown to select crest-derived cells specifically and efficiently with very little contamination of the immunoselected population by cells that are not of neural crest origin. Neurons and glia developed in the cultures of immunoselected but not of residual cells. A broader population of crest-derived precursors has been found to be immunoselected with antibodies to p75NTR than to Ret (Lo and Anderson, 1995). The Ret-immunoselected population appears to be skewed toward neuronal precursors, and specifically toward the relatively small subset that expresses Mash-1. These neurons, which are transiently catecholaminergic and born early, account for no more than a third of the neurons of the ENS (Blaugrund et al., 1996). Since there are multiple lineages of enteric neuronal precursors, it is advantageous to immunoselect with p75NTR to avoid biasing observations to a restricted set of precursors. In the current experiments, the efficiency of p75NTR immunoselection was demonstrated to be independent of fetal age, although more cells could be obtained from the bowel of older fetuses (see Fig. 1). In contrast to the immunoselected population, the residual population contained almost no p75NTR-immunoreactive cells or mRNA encoding NF-M. In the absence of GDNF, moreover, almost no peripherin- or GFRα-1-immunoreactive cells developed in residual cultures.

GFRα-1 Is Expressed in the Gut by both Crest- and Non-Crest-Derived Cells

For early crest-derived neuronal precursors to be responsive to GDNF, they would have to exhibit both of the components of the GDNF receptor complex, Ret and GFRα-1. Ret is an integral membrane protein and would thus have to be synthesized by the cells themselves; however, since GFRα-1 is a peripheral glycosyl-phosphoinositol-anchored protein (Buj-Bello et al., 1997; Creedon et al., 1997; Jing et al., 1996; Klein et al., 1997; Yu et al., 1998), the enteric crest-derived cells could either synthesize their own GFRα-1 (an interaction in cis) or obtain it from neighboring cells (an interaction in trans) (Yu et al., 1998). Neurons have been demonstrated to obtain GFRα-1 in either of these fashions; in fact, targets can provide GFRα-1 to neurons that innervate them (Yu et al., 1998). Previous studies have established that enteric crest-derived cells express Ret (Pachnis et al., 1993; Schuchardt et al., 1994; Tsuzuki et al., 1995) and that the fetal gut contains mRNA-encoding GFRα-1 (Treanor et al., 1996; Widenfalk et al., 1997; Yu et al., 1998). Prior studies have not established which cells of the fetal bowel express and/or anchor GFRα-1. We now find that mRNA-encoding GFRα-1 is expressed both by the p75NTR-immunoselected and residual populations of cells obtained from the fetal bowel. Both crest- and non-crest-derived cells thus appear to be able to synthesize GFRα-1. On the other hand, in contrast to the mRNA-encoding GFRα-1, GFRα-1 protein could only be immunocytochemically demonstrated on crest-derived cells. We detected GFRα-1 immunoreactivity in the E13 fetal bowel in situ, where it appeared in small clusters of developing neurons. GFRα-1 persisted in enteric neurons of both plexuses through adulthood. These observations suggest that while both enteric crest-derived and non-crest-derived cells synthesize GFRα-1, only crest-derived cells in a neuronal lineage are able to anchor the protein to their membranes.

Since Ret expression in the fetal gut is restricted to crest-derived cells, which also appear to be the only cells of the bowel wall that are capable of anchoring GFRα-1, the effects of GDNF would be expected to be limited to crest-derived cells, as indeed they were. It was not necessary, however, to add exogenous GFRα-1 or medium from non-crest-derived cells to a purified population of crest-derived cells to detect either the mitogenic or the neurotrophic effects of GDNF. The GFRα-1 synthesized by crest-derived cells, therefore, must be sufficient to mediate the action of GFRα-1.
GDNF. This observation provides evidence that GDNF can stimulate enteric crest-derived cells through an interaction in cis. Since non-crest-derived cells synthesize GFRα-1, but do not anchor it in quantities adequate for its immunocytochemical demonstration, these cells probably secrete GFRα-1. It thus seems likely that GFRα-1 interacts with crest-derived cells in trans, as well as in cis; however, direct evidence for an interaction in trans was not obtained. If an interaction with GFRα-1 in cis is sufficient for the activation of Ret by GDNF, the question arises as to why the non-crest-derived cells should synthesize and presumably secrete GFRα-1. One possible explanation is that an interaction of GFRα-1 in trans may amplify the effects of GDNF, serving in situ to enhance its efficacy. The observation that crest-derived precursors can interact with GFRα-1 in cis when exogenous GDNF is presented to them experimentally in supramaximal quantities does not necessarily mean that an interaction in cis is sufficient for the optimal stimulation of neuronal precursors by GDNF in situ. GDNF, as well as GFRα-1, is also secreted in the fetal gut by non-crest-derived cells (Moore et al., 1996; Suvanto et al., 1996; Treanor et al., 1996); therefore, it is possible that the binding of GDNF by GFRα-1 released by nonneuronal cells protects endogenous GDNF and helps it to reach the Ret on the surfaces of neuronal precursors. It is possible that the synthesis and secretion of GFRα-1 by the non-crest-derived cells of the bowel wall prior to the formation of neurons creates an environment that is favorable for the critical proliferative action of GDNF on the early crest-derived émigrés that colonize the gut.

GDNF has previously been shown to promote the development of neurons in primary cultures of mouse trunk neural crest cells (Maxwell et al., 1996). In this case, GDNF was found to enhance the development of neurons with an adrenergic phenotype. The effect of GDNF on the primary truncal crest cells was mimicked by NT-3 and dependent on the presence in the cultures of serum and chick embryo extract. In contrast to these primary crest cells, the effects of GDNF on the postmigratory enteric crest-derived cells of the current study were manifest in serum-free media and did not require the presence of chick embryo extract. It is possible that cultured primary crest cells do not express GFRα-1. If so, then GDNF might be unable to affect these cells unless they are cultured in complex GFRα-1-containing media that interacts in trans with Ret-expressing crest cells. HNK-1-immunoselected cells from the developing avian gut have been found to respond to GDNF in serum-free defined media, but only when they are plated at high density (Hearn et al., 1998). Selected cells that are plated at high density would be expected to contain larger numbers of contaminating non-crest-derived cells, which could be a source of GFRα-1 to interact in trans with the crest-derived cell population. The ability of HNK-1-immunoselected cells from the avian bowel to express GFRα-1 has not been reported.

The number of GFRα-1-immunoreactive cells in cultures was found in the current study to be greatly increased by exposure to GDNF, an effect that could be explained by an ability of GDNF to enhance the expression of its own receptor (C. Ibanéz, personal communication). Alternatively, the GDNF-induced increase in GFRα-1-immunoreactive cells may simply reflect the enhanced development in the presence of GDNF of neurons, which are the cells that anchor GFRα-1. Many fewer GFRα-2- than GFRα-1-immunoreactive cells were found in cultured populations of enteric cells, although GFRα-2 immunoreactivity, like that of GFRα-1, was largely restricted to the crest-derived set of cells and GDNF increased the proportion of cells displaying GFRα-2 immunoreactivity. Since GFRα-1 appears to be the preferred receptor for GDNF, while GFRα-2 is the preferred receptor for neurturin (Baloh et al., 1997; Jing et al., 1997), these observations suggest that GDNF probably plays a larger role than neurturin in the development of the ENS. This conclusion is consistent with the observations made with knockout mice showing that the bowel is totally aganglionic distal to the rostral foregut when GDNF is deficient (Moore et al., 1996), but relatively normal when neurturin is absent (J. Milbrandt and R. O Heuckeroth, personal communication). Since neurturin is expressed in the fetal gut (Widenfalk et al., 1997) and small numbers of cells were found to express GFRα-2, it is possible that neurturin promotes the development and/or survival of a minor subset of enteric neurons. The loss of a small population of neurturin-dependent cells from the ENS would probably be noticed in studies of knockout animals only if the identity of such cells were known so that they could be looked for specifically.

CONCLUSIONS

The observations made in this and prior studies are consistent with the following heuristic model for the formation of the ENS (Fig. 16). We propose that the bowel, distal to the rostral foregut, is colonized by a population of vagal and sacral crest-derived precursors, at least some of which are pluripotent (Rothman et al., 1990, 1993b; Sextier-Sainte-Claire Deville et al., 1994) (Fig. 16, step 1). All of these precursors are GDNF-dependent and thus presumably also express Ret and anchor GFRα-1. Knockout of GDNF (Moore et al., 1996; Pichel et al., 1996; Sénèche et al., 1996) or Ret (Durbec et al., 1996; Schuchardt et al., 1994) thus causes the bowel to be totally aganglionic in the distributions of vagal and sacral crest-derived émigrés (Durbec et al., 1996; Schuchardt et al., 1994). At the early stage, from the time the crest-derived precursor cells enter the rat bowel at E9.5 (Baetge and Gershon, 1989; Baetge et al., 1990a,b) through E12 (Fig. 16, step 2), GDNF stimulates proliferation and expands the number of precursor cells sufficiently to build up a population large enough to completely colonize the bowel. We propose that at least some of the progenitors that GDNF stimulates to divide are
nestin-immunoreactive and are capable of giving rise to neurons and/or glia. Between E12 and E14, the precursor cells that proliferate in response to GDNF are replaced by separate lineages of neuronal and glial progenitors (Fig. 16, step 3). The specified progenitors lose nestin, which is replaced by type-specific cytosolic and intermediate filament proteins. Cells in the neuronal lineage express PGP 9.5 and peripherin or NF proteins. Cells in the glial lineage first express S-100, and later also express glial fibrillary acidic protein (GFAP). GDNF exerts a neurotrophic effect and maintains cells in the neuronal lineage, but does not promote the development of glia. We propose that glial development must be maintained by a factor that was not present in the defined medium used in the present experiments. Since enteric glia are lost in ErbB3 knockout mice (Riethmacher et al., 1997), it is possible that the factor required for glial development is a neuregulin, such as glial growth factor (Shah et al., 1994). In contrast to GDNF, glial development can be promoted by NT-3 and a neuropoietic cytokine (Chalazonitis et al., 1994, 1998). The mature ENS contains many subsets of enteric neurons. This phenotypic diversity arises as the result of complex interactions (which predominantly occur after E14) between multiple lineages of neurally specified progenitors and many different growth factors and extracellular matrix molecules (Fig. 16, steps 4–5). The growth factors may be provided by crest- or non-crest-derived cells. In contrast to GDNF,
which affects the whole ENS, later-acting growth factors, such as NT-3 (Chalazonitis et al., 1994) or a neurotrophic cytokine (Chalazonitis et al., 1998), affect distinct subsets of neurons.

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