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Developmental regulation of GDNF response and receptor expression in the enteric nervous system

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SUMMARY

The development of the enteric nervous system is dependent upon the actions of glial cell line-derived neurotrophic factor (GDNF) on neural crest-derived precursor cells in the embryonic gut. GDNF treatment of cultured enteric precursor cells leads to an increase in the number of neurons that develop and/or survive. Here we demonstrate that, although GDNF promoted an increase in neuron number at all embryonic ages examined, there was a developmental shift from a mitogenic to a trophic response by the developing enteric neurons. The timing of this shift corresponded to developmental changes in gut expression of GFRα-1, a co-receptor in the GDNF-Ret signaling complex. GFRα-1 was broadly expressed in the gut at early developmental stages, at which times soluble GFRα-1 was released into the medium by cultured gut cells. At later times, GFRα-1 became restricted to neural crest-derived cells. GFRα-1 could participate in GDNF signaling when expressed in *cis* on the surface of enteric precursor cells, or as a soluble protein. The GDNF-mediated response was greater when cell surface, compared with soluble, GFRα-1 was present, with the maximal response seen the presence of both *cis* and *trans* forms of GFRα-1. In addition to contributing to GDNF signaling, cell-surface GFRα-1 modulated the specificity of interactions between GDNF and soluble GFRαs. These experiments demonstrate that complex, developmentally regulated, signaling interactions contribute to the GDNF-dependent development of enteric neurons.

Key words: GDNF, Ret, GFRα-1, Enteric nervous system, Receptor signaling, Rat

INTRODUCTION

The enteric nervous system consists of a complex network of neurons and glia that functions autonomously to provide neural control of the gastrointestinal tract (Furness and Costa, 1987). Subpopulations of enteric neurons can be distinguished by their expression of cell-surface markers, transcription factors and neurotransmitters (Blaugrund et al., 1996; Carnahan et al., 1991; Furness et al., 1995; Lo et al., 1998; Pham et al., 1991). The development of this complex array of enteric phenotypes is dependent upon interactions between neural crest-derived progenitor cells and developmental signals provided by the gut environment (Baetge and Gershon, 1989; Coulter et al., 1988; Ernsberger and Rohrer, 1996; Groves and Anderson, 1996; Pisano and Birren, 1999).

One gut-derived cue implicated in enteric development is glial cell line-derived neurotrophic factor (GDNF). Targeted deletion of the *Gdnf* gene results in a dramatic defect in the enteric nervous system characterized by the loss of enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). In vitro, GDNF promotes the development of rodent and avian enteric neurons, working through mechanisms that include increased proliferation and survival (Chalazonitis et al., 1998a; Hearn et al., 1998; Heuckeroth et al., 1998). The absence of neurturin (NTN), another member of the GDNF family, leads to a similar, but less severe, enteric phenotype (Heuckeroth et al., 1999), indicating a role for multiple GDNF family members in enteric development.

GDNF family members signal through the Ret receptor tyrosine kinase (Durbec et al., 1996; Trupp et al., 1996). While all GDNF family members signal through Ret, binding and specificity is mediated through interactions with members of the cell-surface, GPI-linked, GFRα receptor family (Airaksinen et al., 1999; Jing et al., 1996; Treanor et al., 1996). Among the GFRα receptor family members, GFRα-1 mediates GDNF signaling, while GFRα-2 preferentially interacts with neurturin. While affinities are highest between specific GDNF family members and their preferred co-receptors, there is some degree of cross-specificity for the GFRαs (Baloh et al., 1997; Creedon et al., 1997; Horger et al., 1998; Sanicola et al., 1997).

The development of the enteric nervous system depends upon the appropriate expression of GDNF and its receptors. Changes in the expression of Ret and GFRα proteins have been observed in the developing nervous system (Forgie et al., 1999; Pachnis et al., 1993; Schiltz et al., 1999), although the extent to which changes in receptor expression influence the GDNF response is not clear. Here we show that while GFRα-1 is widely expressed throughout the gut of E13.5 rat embryos, expression becomes restricted to regions containing neural crest-derived cells at later developmental times. During this period of changing receptor expression, enteric precursor cells expressing the HNK-1 cell-surface marker consistently respond to GDNF with an increase in neuron number. While this increase in neurons is at least partially a consequence of the mitogenic action of GNDF on E13.5 and E15.5 neuroblasts, GDNF does not promote cell division of E19.5 cells, implying a developmental change in the mechanism of GDNF action. The presence of cell-surface GFRα-1 is required to obtain the maximal effect of GDNF, although soluble GFRα-1 is also capable of mediating GDNF signaling. When GFRα-1 is present on both the cell surface and in a soluble form, interactions between the *cis* and *trans* forms of GFRα-1 act to potentiate the effects of GDNF and to regulate the specificity of ligand/GFRα interactions. These experiments define interactions between GDNF and its receptors that are crucial for the development of the enteric nervous system.

MATERIALS AND METHODS

Immuno-isolation and culture of enteric neural precursor cells

Cells were isolated from embryonic day (E) 13.5, E15.5 and E19.5 embryos from timed-mated pregnant Sprague-Dawley (Charles River Laboratories, Cambridge, MA) or Simonsen Albino rats (Simonsen Labs, Sunnyvale, CA). The intestines, excluding stomach and colon were enzymatically dissociated in 125-250 U/ml type 1 collagenase (Worthington, Lakewood, NJ) at 37°C for 15 minutes (E13.5), 30 minutes (E15.5) or 45 minutes (E19.5). Samples were triturated with fire-polished Pasteur pipettes and filtered though 60 μm sterile mesh. Neural crest-derived cells were isolated from the cell suspension by labeling with the HNK-1 antibody (Abo and Balch, 1981; Erickson et al., 1989; Pomeranz et al., 1993) followed by sequential incubations with para-magnetic bead- (Miltenyi Biotech, Auburn, CA) and FITC-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA). Labeled cell suspensions were passed twice over a column held in a magnetic field, the column was washed, removed from the magnet and the positive cells were eluted. The purification was monitored by counting the percentage of FITC-positive cells in the sample before and after immuno-isolation. Using this technique we obtained cell populations that contained 80-95% HNK-1-positive cells (Pisano and Birren, 1999).

Immuno-isolated cells were plated at 5000 cells per well in 24-well tissue-culture dishes that had been coated with poly-D-ornithine (Gibco BRL) and laminin (Collaborative Biomedical, Bedford, MA) (Pisano and Birren, 1999). Cells were cultured in modified L15N2, serum-free medium, consisting of L15CO_2 (Hawrot and Patterson, 1979) plus 6 μg/ml dextrose (Fisher, Pittsburg, PA), 2 mM glutamine (Gibco BRL), 100 U/ml penicillin (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), 0.5 μg/ml 6,7, dimethyl-5, 6,7,8-tetrahydropterine (DMPH4, Calbiochem), 2.5 μg/ml glutathione (Sigma), and 50 μg/ml L-ascorbic acid (Sigma). N2 additives were included with the exception that insulin was omitted (Bottenstein and Sato, 1979). The following factors were suspended in L15N2 and added 3 hours after plating: 0.05-100 ng/ml human recombinant GDNF (R&D, Minneapolis, MN), 25 ng/ml NTN (PeproTech, Rocky Hill, NJ), 5 μg/ml soluble GFRα-1 (Tang et al., 1998) (Biogen, Cambridge, MA), 20 μg/ml soluble GFRα-2 (Biogen), 10 μg/ml Ret-Fc fusion protein (Ehrenfels et al., 1999) (Biogen), 10 μg/ml anti-GDNF antibody (R&D). Cultures were fed every second day by replacing half the media and factors.

Neuron recovery assays

Immuno-isolated cells were plated in 24-well dishes and incubated for 3 hours. Pre-counts were taken by counting the number of cells in a strip (roughly 200 cells/strip) through the middle of each well using 20× phase-contrast optics. Factors were added to the culture dishes and after five days in culture, the same strip was scanned and cells with a neuronal morphology were scored. Neuron recovery data are shown as the number of neurons divided by the number of cells in the pre-count × 100 (%).

Generation of soluble GFRα-1 and GFRα-2 proteins

Plasmids encoding rat GFRα-1 and human GFRα-2 proteins were generated by cloning DNA fragments encoding amino acids 1-437 of rat GFRα-1 and 1-438 of human GFRα-2 into the Biogen expression vectors pCH269 and pEAG347. The plasmids were transfected into 293-EBNA or CHO cells. Soluble GFRα proteins were purified from conditioned media using SP-Sepharose (Amersham). Ligand-containing fractions were then applied to Lentil Lectin Sepharose and the protein eluted with 2% alpha methyl mannopyranoside. Last, a Q-Sepharose column (Amersham) was used as a polishing step. SDS/PAGE analysis of the GFRα proteins indicated a size consistent with their predicted molecular weight.

Antibody preparation and characterization

To generate an anti-Ret rabbit polyclonal antibody (pAb #R1058), a rabbit was immunized by lymph node injection with rat Ret-Ig fusion protein. To generate rabbit GFRα-1 antiserum (pAb R5), a peptide corresponding to amino acids 416-427 of rat GFRα-1 was used for immunization. The rabbit pAb R5 was purified against the immobilized peptide. The pAb #R1058 anti-Ret antibody was purified by passing the serum over an immobilized control-Ig fusion protein column followed by passage over a Ret-Ig column.

The pAb #R1058 anti-Ret antibody was characterized as follows: 293-EBNA cells were transiently transfected with an expression plasmid containing the full-length sequence for rat Ret in the expression vector CH269. As a negative control, cells were transfected with the expression vector only. To assess the specific binding of antibody to Ret, transfected cells were incubated with anti-Ret, followed by a donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) and analyzed on a Becton-Dickinson FACScan machine for FACS analysis (B-D, San Jose, CA). Ret-expressing cells showed a shift in the fluorescence intensity compared with the vector control cells following incubation with the anti-Ret antibody. This staining of unfixed, non-permeabilized live cells indicated binding of the antibody to an epitope on the extracellular domain of the Ret protein.

The specificity of the pAb R5 anti-GFRα-1 was assessed by western analysis against conditioned medium obtained from 293-EBNA cells transfected with GFRα-1 or GFRα-2. The anti-GFRα-1 antibody gave a positive Western signal for GFRα-1-conditioned medium, but not GFRα-2-conditioned medium (data not shown).

FACS analysis

Tissues were dissected from E13.5, E15.5 and E19.5 rat embryos as described for immuno-isolations. Single cell suspensions were incubated with primary and secondary antibodies in L15N2 medium and analyzed in a Becton-Dickinson FACScan using the Cellquest software package (version 3.1f). The following primary antibodies were used in these studies: rabbit anti-Ret 10 μg/ml (Biogen #R1058),

HNK-1 (Abo and Balch, 1981; Erickson et al., 1989; Pomeranz et al., 1993). Secondary antibodies (Jackson ImmunoResearch) were used at a dilution of 1:100.

Immunocytochemistry

After five days in culture, cells were fixed in 3.7% formaldehyde, 5% sucrose in PBS for 10 minutes at room temperature. Cells were pre-blocked and labeled in 4% goat serum, 0.1% NP40 for 20 minutes at room temperature. Primary antibody incubations were overnight at 4°C, secondary antibody incubations (1:200) (Jackson ImmunoResearch or Molecular Probes) were for 2 hours at room temperature. Immunohistochemistry was performed on 10 μm frozen sections of E13.5, E15.5 and E19.5 rat intestines, excluding stomach and colon. The following antibodies were used in these studies: rabbit anti-peripherin 1:1500 (Chemicon), mouse anti-peripherin 1:100 (Chemicon), rabbit anti-Ret 1:150 (Biogen, #1429) (Ehrenfels et al., 1999), rabbit anti-GFRα-1 2.0 μg/ml (Biogen, #1371) (Ehrenfels et al., 1999), mouse anti-BrdU 1:200 (DAKO). After processing, samples were stored at –20°C covered in n-propyl gallate or Vectashield (Vector Burlingame, CA).

Production of gut-conditioned medium

During the immuno-isolation of enteric neural precursors, the negative flow through (NFT) was collected from the magnet column and plated as an HNK-1-depleted population. NFT cells were plated on poly-D-ornithine/laminin coated dishes and were cultured for 5 days in L15N2. The conditioned media was concentrated fivefold with a Centriprep-10 centrifugal filter device (Amicon, Inc., Beverly, MA) and stored at –80°C. Concentrated NFT was assayed for total protein content by using Pierce's (Rockford, IL) BCA™ Protein Assay Kit following the manufacturer's directions.

GFRα-1 ELISA

Microtiter plates (Nunc MaxiSorp) were coated with 250 ng/ml recombinant rat GDNF (R&D Systems) in 50 mM sodium carbonate, pH 9.6. The plates were blocked for 1 hour at room temperature with 1% BSA in 10 mM Tris·HCl, pH 7.5, 0.05% Tween-20, and 150 mM NaCl, (TBST) and treated with serial dilutions of concentrated NFT-conditioned media in TBST or, as a positive control, soluble rat GFRα-1 in TBST. The plates were washed with 0.05% Tween in PBS and incubated with 1 μg/ml of anti-GFRα-1 #R5 antiserum (Biogen) in TBST for 1 hour at room temperature. The plates were washed and incubated for 20 minutes with a 1:1000 dilution of a HRP-conjugated goat anti-rabbit (H+L) antibody (Biorad, Herules, CA) in TBST. The plate was washed again and developed in 0.1 M glycine, 1 mM magnesium chloride hexahydrate, 1 mM zinc chloride, 0.42 mM TMB (in DMSO) and 0.005% H_2O_2. The reaction was allowed to develop for 5 minutes at room temperature and stopped with an equal volume of 2N H_2S0_4. The plate was read in a SpectraMaxPlus spectrophotometer (Molecular Devices) at 450 nm.

BrdU incorporation

Cultures of immuno-isolated enteric precursors were maintained for 24 or 72 hours, with 1 mM 5-bromo-2′-deoxy-uridine (BrdU, Boehringer-Mannheim) added for the final 24 hours of the culture period. Cells were fixed as described, treated for 10 minutes at room temperature with 2N HCl in PBS, washed and treated for 10 minutes at room temperature with 0.1M $Na_2B_4O_7$-$10H_2O$. Cultures were immunostained as described above.

PIPLC treatment

0.1 Units/ml phosphatidylinositol phospholipase C (PIPLC) (Boehringer Mannheim) was added to each culture well at the time of plating. Factors were added 3 hours after plating, as described. An additional 0.1 Units/ml PIPLC was added to each well at 24, 48, 72 and 96 hours post-plating.

Statistics

Data were analyzed using StatView software (version 4.5 for the Macintosh, Abacus Concepts). ANOVA and subsequent Fisher's Protected Least Significant Difference Test assessed significance.

RESULTS

The HNK-1 antibody recognizes a population of neural crest-derived precursor cells in avian and rodent embryonic gut that have the potential to develop into the neurons and glia of the enteric nervous system. To examine the action of GDNF on the development of enteric neurons we analyzed the number of neurons that developed in cultures of HNK-1-positive, immuno-isolated E15.5 gut cells. Neuron number was counted after 5 days in culture and compared with the number of cells in the dishes 3 hours after plating (% neuron recovery). As previously shown in other studies (Chalazonitis et al., 1998a; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999), GDNF treatment resulted in a robust increase in neuron number (Fig. 1A; Control 72%; GDNF 376% neuron recovery). We tested the specificity of the GDNF effect by

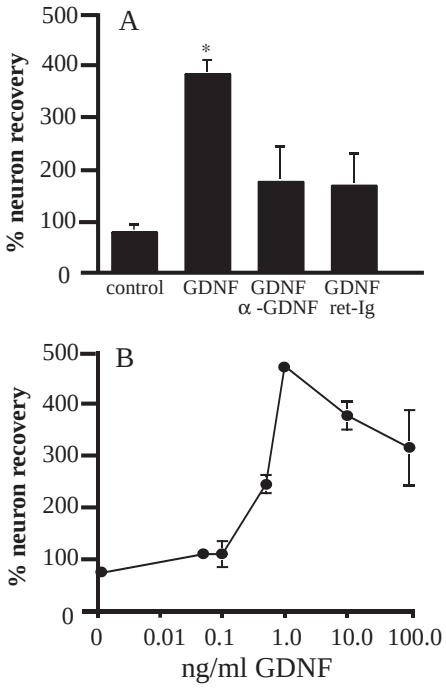

Fig. 1. GDNF treatment results in an increase in E15.5 enteric neuron number. (A) Cells were grown for 5 days in serum-free medium alone or supplemented with 10 ng/ml GDNF, GDNF + 10μg/ml GDNF blocking antibody or GDNF + 10 μg/ml Ret-Ig fusion protein. Cells with a neuronal morphology were counted at the end of the culture period. Neuron recovery is expressed as a percentage of pre-counts taken 3 hours after plating (% neuron recovery). Data are shown as the mean of 2-15 independent experiments, each with duplicate determinations±s.e.m. *Significantly different from medium only control (P<0.001). (B) E15.5 enteric precursors were cultured for 5 days alone or in GDNF (0.05-100 ng/ml) and the percent neuron recovery measured. Data shown represent the mean of 2-18 independent experiments, each with duplicate determinations±s.e.m.

incubating some cultures with a GDNF blocking antibody or with a Ret-Ig fusion protein to scavenge GDNF/GFRα-1 complexes. In both cases, the response to GDNF was substantially decreased, indicating that the increase in neuron number was mediated by the added GDNF (GDNF + blocking antibody 168%; GDNF Ret-Ig 161% neuron recovery). The effects of GDNF on enteric neuron recovery were dose dependent (Fig. 1B). There was no significant effect of 0.1 ng/ml GDNF on enteric neuron recovery and a maximal effect at 1 ng/ml GDNF (6.6-fold increase over the control).

Patterns of GDNF receptor expression

We examined expression of Ret in sections of rat gut isolated from E13.5, E15.5 and E19.5 embryos by immunohistochemistry using the Ret-R1058 antibody (see Materials and Methods). Ret expressing cells were present in the developing gut at all developmental stages examined (Fig. 2). At E13.5, Ret-positive cells were scattered throughout the gut. By E15.5, Ret expression was tightly localized along the outer circumference of the gut in the region of the developing myenteric plexus, a pattern that was maintained at E19.5.

We asked whether HNK-1 and Ret were co-expressed in the same cells at the different developmental stages by using FACS analysis to examine double-labeled cells. As a control, we determined that the enzymatic dissociation of the gut tissue did not result in a loss of cell-surface Ret protein. To do this, cultured Ret-positive rat neuroblastoma cells (NB41A3) were removed from tissue culture flasks with EDTA and treated with collagenase for 30 minutes 37°C. When analyzed by FACS, the enzyme-treated and control cells showed equivalent levels of Ret staining, indicating that enzymatic dissociation did not result in a loss of cell surface Ret (data not shown). Embryonic guts from E13.5, E15.5 and E19.5 rat embryos were then dissociated and double-labeled with HNK-1 and anti-Ret and the percentage of cells that co-expressed HNK-1 and Ret was determined by FACS analysis (Table 1). Expression of Ret was developmentally regulated in HNK-1-positive cells while only a small percentage of HNK-1-negative cells ever showed Ret immunoreactivity. At E13.5, the majority of HNK-1-positive cells also expressed Ret. There was a sharp decline in the percentage of HNK-1-positive cells expressing Ret between E13.5 (66%) and E15.5 (28%), followed by a stabilization of Ret expression in HNK-1-positive cells between E15.5 and E19.5 (25%). This decrease may reflect the development of HNK-1-positive cells into Ret-negative, non-neuronal cells.

While only 28% of the E15.5 HNK-1-positive cells expressed Ret, culture experiments demonstrated that 100% of the neurons that developed from these cells were Ret-positive after 5 days in culture (Fig. 3). Ret was expressed in surviving neurons in cultures grown in the presence or absence of GDNF. These results indicate that the HNK-1-positive cells that were initially negative for Ret die, turn on expression of the receptor during growth in culture or develop into non-neuronal cells.

Developmental changes in GDNF response

Several studies have indicated that enteric neuron survival and/or proliferation is promoted by GDNF (Heuckeroth et al., 1998; Taraviras et al., 1999). Recent work has suggested that the level of this response varies with the developmental stage of the enteric neural precursors (Chalazonitis et al., 1998b; Hearn et al., 1998; Taraviras et al., 1999). We examined the response to GDNF of HNK-1-positive cells isolated at E13.5, E15.5 and E19.5 by growing immuno-isolated neural precursors for 5 days in the presence or absence of GDNF and determining the number of surviving neurons in comparison with the number of cells initially plated. Neural precursors responded to GDNF at all developmental ages tested, with fourfold increase in neuron number in cultures isolated from E13.5 guts (Fig. 4A). Other laboratories have also observed GDNF responsiveness of enteric precursor cells at multiple developmental times and have shown a decrease in the level of the response between rat E12 and E15 (Chalazonitis et al., 1998a; Taraviras et al., 1999). There were no significant

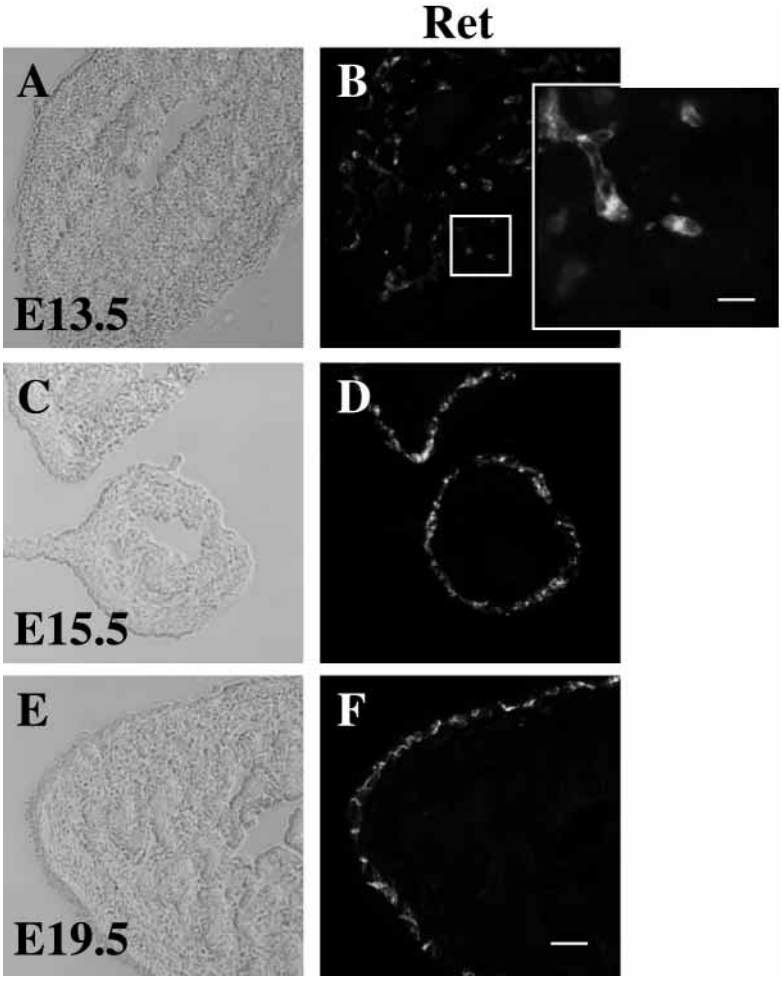

Fig. 2. Localization of Ret expression during enteric development. Cryostat sections from E13.5 (A,B) E15.5 (C,D) and E19.5 (E,F) embryos were stained for Ret. (A,C,E) Bright-field images of the Ret-stained sections. (B,D,F) Ret immunoreactivity. The inset in part B shows a higher magnification of the area in the white box. Scale bar: 50 μm; 10 μm for insert.

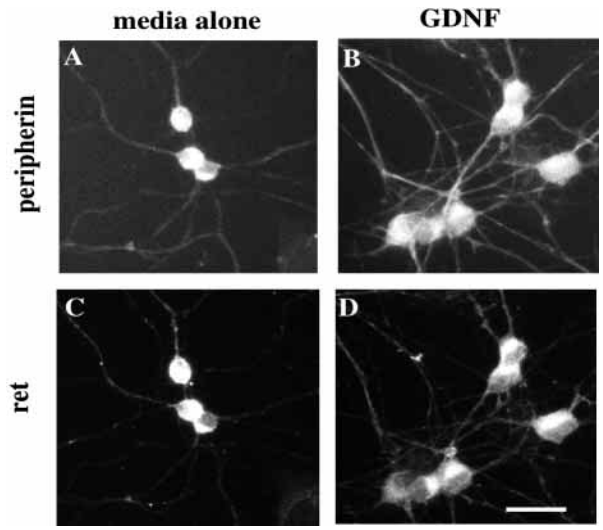

Fig. 3. Neurons that develop from E15.5 HNK-1-positive enteric precursors express Ret. Isolated precursors were grown for 5 days in serum-free medium in the absence (A,C) or presence (B,D) of 10 ng/ml GDNF. The cells were fixed and double-stained for peripherin (A,B) and Ret (C,D). After 5 days in culture, all cells with a neuronal morphology stained for peripherin and all peripherin-positive cells were Ret-positive. Scale bar, 20 μm.

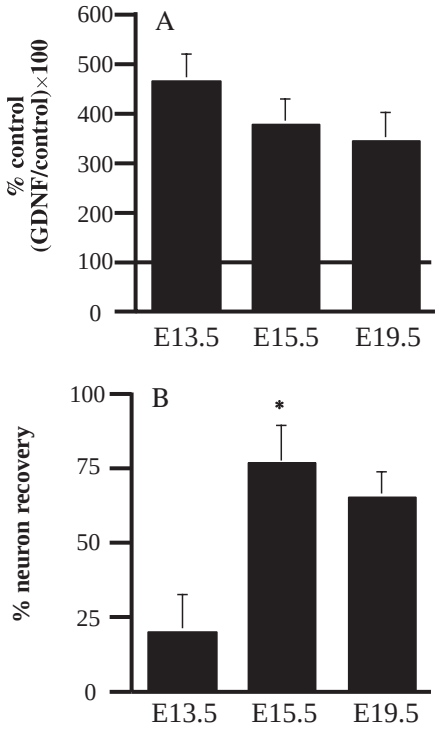

Fig. 4. GDNF increased neuron number at E13.5, E15.5 and E19.5. (A) Enteric precursor cells were cultured for 5 days in the presence or absence of 10 ng/ml GDNF. Cultures grown in GDNF showed a significant increase in neuron number over the control condition at each developmental stage. Data represent the mean±s.e.m. of 5-17 independent experiments, each with a minimum of duplicate determinations. All developmental stages were significantly different than the control (P<0.05). (B) Neuron number in the absence of trophic factors changed with developmental stage. The number of surviving enteric neurons increased between E13.5 and E15.5 in the control condition. HNK-1-positive immuno-isolated cells were cultured for 5 days in serum-free defined medium. The number of neurons at the end of the culture period is expressed as a percentage of the number of cells pre-counted 3 hours after plating. Data are the mean±s.e.m. of 5-17 independent experiments. *E15.5 significantly different from E13.5 (P<0.02).

differences in neuron recovery in our cultures between E13.5, E15.5 and E19.5, although there was a trend towards a smaller response to GDNF at the later stages (neuron number after 5 days in culture in 10 ng/ml GDNF: E13.5, 457% of control; E15.5, 370%; E19.5, 336%).

Although GDNF led to a significant increase in neuron number at different developmental stages, the number of surviving neurons in the absence of GDNF was dramatically different. At E13.5, the number of neurons in control cultures was 20% of the cells initially plated. In contrast, neuron number at E15.5 was 76% and at E19.5 was 65% of cells initially plated (Fig. 4B). These results suggest that in the absence of exogenously added factors, early enteric neural precursor cells exhibit different patterns of proliferation, differentiation and/or survival compared with cells isolated from older embryos.

Developmental changes in intrinsic and GDNF-dependent cell division

GDNF has been shown to increase the proliferation of enteric precursor cells as measured by incorporation of BrdU (Hearn et al., 1998; Heuckeroth et al., 1998; Wu et al., 1999) and by expression of the proliferation marker PCNA (Chalazonitis et al., 1998a). We also observed that GDNF-mediated increases in neuron recovery were a consequence of increased cell division, and investigated whether there were developmental changes in proliferative effects of GDNF. E13.5, E15.5 and E19.5 neural precursors were cultured in the presence or absence of GDNF for 72 hours, with BrdU included for the final 24 hours of the culture period (Fig. 5). At E13.5, GDNF treatment led to an increase in the percentage of peripherin-positive cells that incorporated BrdU (Control, 7%; GDNF, 17% BrdU-positive). At E15.5, GDNF also increased cell division (Control, 1%; GDNF, 7% BrdU-positive), although the intrinsic level of cell division had decreased. In contrast, GDNF had no effect on the division of peripherin-positive cells that developed from E19.5 HNK-1-positive cells (Control, 1.7%; GDNF, 0.9% BrdU-positive). These results suggest that developmental changes occur in the response of developing enteric neurons to GDNF and that the GDNF-dependent increase in neuronal number at E19.5 is mediated through a mechanism other than enhanced proliferation.

Table 1. Expression of Ret in HNK-1-positive and negative populations

	% HNK-1/ Ret-positive	% HNK-1-negative/ Ret-positive
E13.5	66.1±2.6	12.4±2.6
E15.5	28.3±3.9	7.4±1.5
E19.5	25.2±3.1	6.7±2.6

Single cell suspensions from freshly isolated tissues were immunolabeled and analyzed on a FACScan.
Values represent the mean±s.e.m. from two independent experiments.

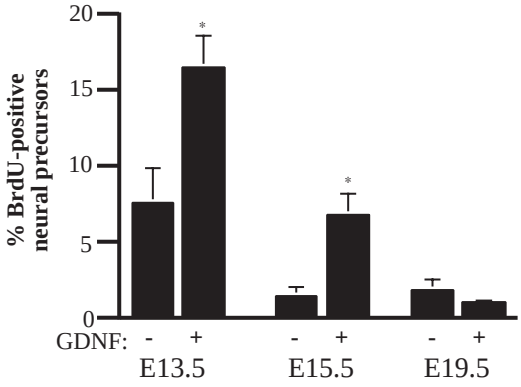

Fig. 5. GDNF promotes enteric cell division at E13.5 and E15.5, but not E19.5. Enteric precursors were grown in serum-free medium for 72 hours, with 1 mM 5-bromo-2′-deoxy-uridine (BrdU) added for the final 24 hours of the culture period. Cells were fixed and the percentage of the peripherin-positive neural cells that were BrdU immunoreactive was scored. GDNF led to a significant increase in the percentage of the peripherin-positive neural cells that were also BrdU-positive at E13.5 and E15.5, but not at E19.5. Data represent the mean±s.e.m. of 2-5 independent experiments, each with a minimum of duplicate determinations. *Significantly different from control (P<0.01).

Soluble GFRα-1 enhances GDNF responses

GFRα-1 functions as a required co-receptor for GDNF signaling through the Ret receptor, although it is not clear how local patterns of expression of GFRα-1 on neuronal and non-neuronal cells contribute to neuronal GDNF responses. We found that neurons that developed from cultured gut-derived E13.5, E15.5 and E19.5 HNK-1 positive precursors cells were all GFRα-1 immunoreactive (data not shown). Immunohistochemistry of fixed cryostat sections through E13.5 embryonic intestines revealed extensive GFRα-1 staining throughout the gut that was not restricted to regions containing Ret-positive cells (Fig. 6, see Fig. 2). At E15.5, GFRα-1 staining showed some localization in a broad band of immunoreactivity along the outer circumference of the intestines. This pattern of expression was even more tightly localized at E19.5 when GFRα-1 immunoreactivity was restricted to the Ret-positive region of the myenteric plexus.

GFRα-1 has been has been shown to promote GDNF action via its *cis* expression on Ret-expressing cells and trans interactions from neighboring cells (Airaksinen et al., 1999). We examined possible trans receptor/ligand interactions by culturing enteric neural precursor cells from different developmental stages with GDNF and 5 μg/ml exogenous soluble GFRα-1. Soluble GFRα-1 enhanced neuron recovery at all stages compared with cultures incubated with GDNF alone (Fig. 7; E13.5, 148% of GNDF condition; E15.5, 198%, E19.5, 178%). GFRα-1, in the absence of GDNF, did not promote neuron recovery (data not shown). These results are consistent with the GDNF-promoting effects of soluble GFRα-1 on midbrain dopaminergic and motoneurons (Cacalano et al., 1998; Treanor et al., 1996), and indicate that GFRα-1 does not need to be associated with the neuronal cell surface in order to participate in GDNF-dependent Ret signaling. Our experiments demonstrate that the effects of soluble GFRα-1 were maintained through prenatal development and suggest

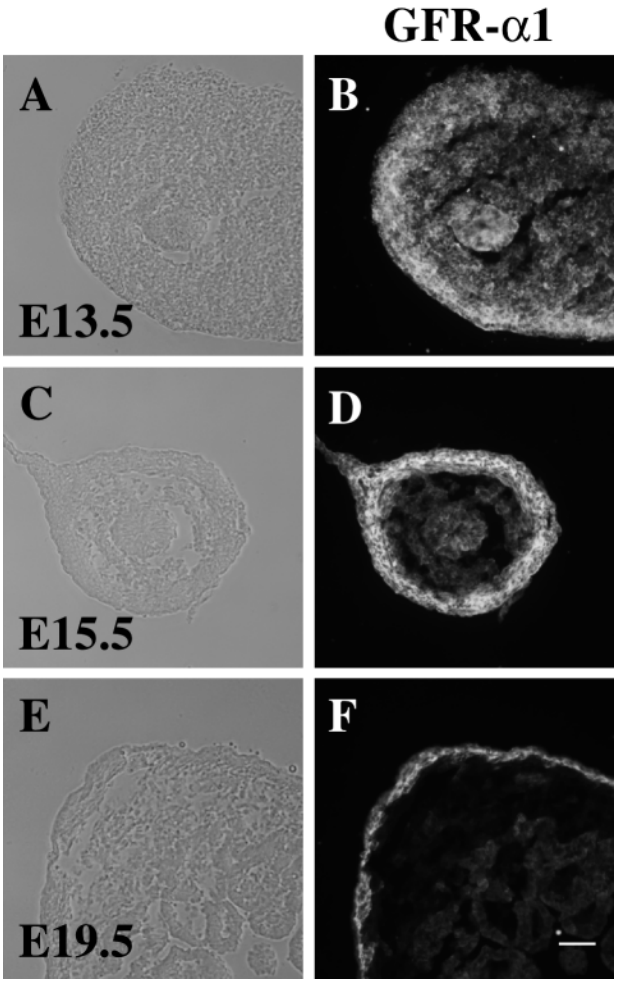

Fig. 6. Localization of GFRα-1 protein expression changes during development of the enteric nervous system. Cryostat sections through E13.5, E15.5 and E19.5 intestine were stained for GFRα-1. (A,C,E) Bright-field images of the GFRα-1-stained sections. (B,D,F) GFRα-1 immunoreactivity. GFRα-1 expression became increasingly more localized over developmental time, becoming restricted to the region of the myenteric plexus by E19.5. Panels (B,D,F) show time-matched exposures. Scale bar, 50 μm.

that GFRα-1 could promote enteric GDNF responses from neighboring cells or as a secreted or shed molecule.

The finding that E13.5 and E15.5 gut displayed a broad pattern of GFRα-1 expression suggested that the embryonic gut could be a source of soluble GFRα-1 in vivo. We investigated whether cells cultured from residual gut cells, obtained as the HNK-1-negative fraction of the immunoisolation, released measurable amounts of GFRα-1 into the medium. At E13.5 and E15.5, the times at which the highest levels of GFRα-1 were observed by immunohistochemistry, GFRα-1 was detectable in the conditioned medium (Table 2). Consistent with the much narrower distribution of GFRα-1-positive cells in E19.5 gut, there was no detectable GFRα-1 present in conditioned medium from E19.5 cultured cells. Together, these results indicate that developing enteric neuroblasts can respond to GDNF through mechanisms that involve both *cis* and *trans*

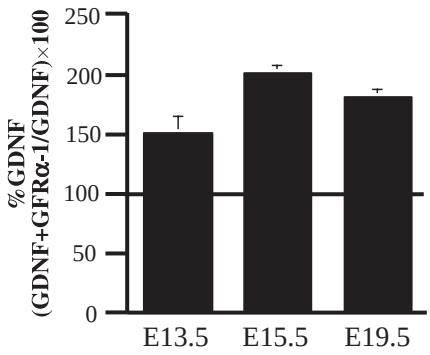

Fig. 7. Soluble GFRα-1 enhances the ability of GDNF to increase neuron number at all developmental stages examined. Enteric precursors were grown for 5 days in 10 ng/ml GDNF in the presence or absence of 5 μg/ml GFRα-1. Cultures grown in the presence of GFRα-1 showed a significant increase in neuron number over that seen in GDNF alone. Data represent the mean±s.e.m. of 3-6 independent experiments, each with a minimum of duplicate determinations. All developmental stages were significantly different from GDNF alone (P<0.001).

expression of GFRα-1 and that soluble GFRα-1 is released by cells in the gut in a developmentally regulated manner.

Specificity of GFRα family members in the GDNF response

The specificity of soluble GFRα-1 in the enteric GDNF response was investigated by examining the survival of enteric neurons in cultures treated with GDNF and GFRα-2, another member of the GFRα family. GFRα-2 forms a complex with Ret and neurturin (NTN) to mediate NTN signaling (Baloh et al., 1997; Buj-Bello et al., 1997). E15.5 enteric neural precursors responded to NTN with increased neuron recovery and the addition of soluble GFRα-2 to the medium resulted in a small but significant further increase in neuron number (Fig. 8A; control, 90% neuron recovery; NTN, 259% neuron recovery; NTN + GFRα-2, 335% neuron recovery). This result demonstrated that the GFRα-2 used was biologically active and that, like GFRα-1, GFRα-2 could enhance Ret signaling in a *trans* configuration. The addition of GFRα-2 to E15.5 neural precursors did not, however, enhance GDNF-mediated neuronal survival, indicating a specific interaction involving soluble GFRα-1 and GDNF (Fig. 8B; GDNF, 311% neuron recovery; GDNF + GFRα-2, 335% neuron recovery).

The observation that soluble GFRα-1 potentiated the effect of GDNF, and the fact that soluble GFRα-1 can be detected in this system, raised the question of how soluble co-receptors interact with cell-surface receptor components. We used the enzyme PIPLC to cleave GFRα-1, as well as other GPI-linked proteins, from the cell surface of immuno-isolated E15.5 enteric neural precursors. Following PIPLC treatment, the effects of NTN and GDNF on neuron recovery were severely attenuated (Fig. 8C). When the cultures were treated with soluble GFRα-1 or GFRα-2 in addition to GDNF or NTN, respectively, the ligand-induced increase in neuronal recovery was restored (Control, 76%; GDNF, 92%; GDNF + GFRα-1, 196%; NTN, 115%; NTN + GFRα-2, 224% neuron recovery). There was a 2.6-fold increase in neuron number over the control in the presence of GDNF + GFRα-1 in PIPLC-treated cultures. While this represents a significant increase in neuron number, the response was not as great as the response of non-PIPLC-treated cultures to GDNF and exogenous GFRα-1. Thus, the enhancement in neuron number may, at least in part, rely on interactions between GFRα-1 and cell-surface GPI-linked proteins.

Unexpectedly, we found that the addition of soluble GFRα-2 to PIPLC-treated cells also rescued the neuron recovery effects of GDNF (Fig. 8C; GDNF + GFRα-2, 203% neuron recovery), even though in cultures not treated with PIPLC GFRα-2 did not lead to an enhancement in GDNF-mediated responses (Fig. 8B). This result indicates that cell-surface GFRα proteins can influence the specificity of interactions between GDNF family members and soluble GFRα components, and raises the possibility of additional levels of regulation in the Ret signaling system.

DISCUSSION

The development of enteric neurons is dependent upon the presence of GDNF in the embryonic gut and the expression of Ret on developing enteric neurons (Baloh et al., 2000). We found that, between E13.5 and E19.5, GDNF promoted a consistent increase in neuron number, despite changes in the pattern of GDNF receptor expression in the embryonic rat gut. While GDNF acted as a mitogen for E13.5 enteric neuroblasts, this proliferative response decreased at later developmental stages and was completely abolished at E19.5. GDNF continued to promote the recovery of enteric neurons at this later time, suggesting that it switches from being a mitogen to a trophic factor for developing enteric neurons. These results confirm and expand the findings of other laboratories showing that GDNF can act as a mitogen and survival factor for enteric precursor cells (Chalazonitis et al., 1998a; Hearn et al., 1998; Heuckeroth et al., 1998; Wu et al., 1999). GFRα-1 is a required co-receptor for GDNF signaling (Cacalano et al., 1998; Enomoto et al., 1998), forming a ligand/receptor complex with GDNF and Ret. We demonstrated the presence of two forms of GFRα-1 in enteric cultures: GPI-linked to cell surfaces, and shed or secreted into the medium. GFRα-1 promoted the effects of GDNF both as a cell-surface molecule and in a soluble form. We have defined a novel role for cell-surface GFRα in maintaining the specificity of GDNF interactions with soluble GFRα-1, demonstrating multiple roles for *cis* and *trans* forms of GFRα molecules. Together, these results begin to define a dynamic pattern of GDNF signaling during the development of the enteric nervous system.

Table 2. GFRα-1 in medium conditioned by HNK-1-negative enteric cells

	pg/ml*	pg/mg total protein‡
E13.5	180±61	137±47
E15.5	383±115	215±63
E19.5	18±10	12±7

*Concentration of GFRα-1 in conditioned medium of gut cells obtained from the negative flow through of the HNK-1 immunoisolation.

‡Amount of GFRα-1 in conditioned medium normalized to the total protein in the medium.

Values represent the mean±s.e.m. from four independent experiments.

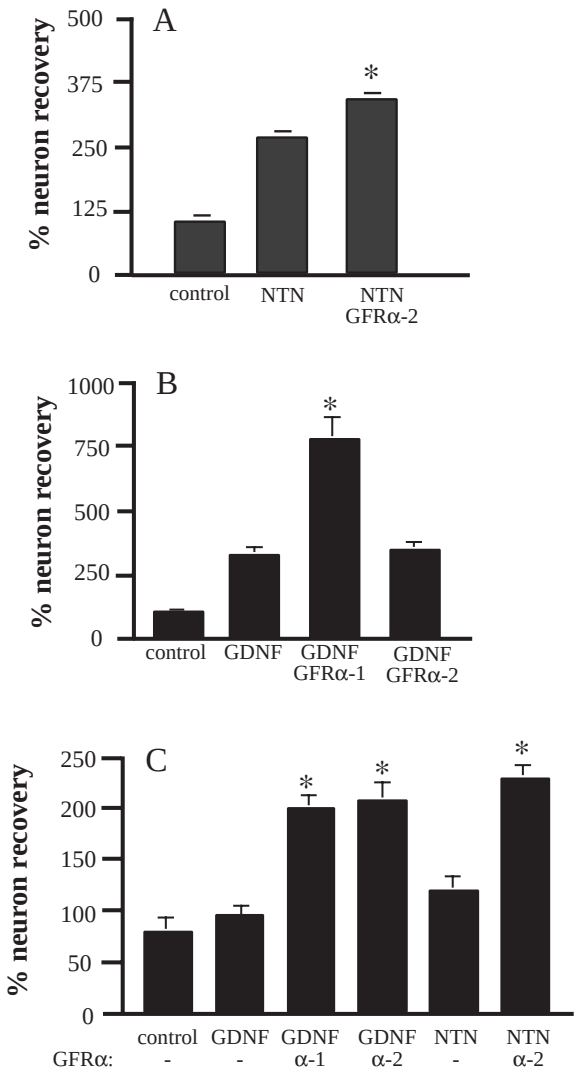

Fig. 8. Soluble GFRαs promote the recovery of enteric neurons in the presence of GDNF and neurturin (NTN) and the specificity of GFRα action is dependent on cell surface GPI-linked proteins. (A) NTN (10 ng/ml) increased the number of neurons that developed from E15.5 HNK-1-positive enteric precursors after 5 days in culture. GFRα-2 (20 μg/ml) enhanced the NTN effect. Data represent the mean±s.e.m. of 2-4 independent experiments each with a minimum of duplicate determinations. *Significantly different from NTN (P<0.006). (B) GFRα-1, but not GFRα-2, enhanced neuronal recovery in the presence of GDNF. Data represent the mean±s.e..m. of 2-4 independent experiments, each with a minimum of duplicate determinations. *Significantly different from GDNF alone (P<0.001). (C) Cleavage of GPI-linked surface molecules abolished GFRα specificity. Cultures of E15.5 enteric precursor cells were treated with phospatidylinositol phospholipase C (PIPLC, 0.1 U/ml) to remove GPI-linked molecules from the cell surface. PIPLC treatment abolished the effects of NTN and GDNF on neuron recovery. Soluble GFRα-1 restored the effect of GDNF and soluble GFRα-2 restored the effect of NTN in PIPLC-treated cultures. Following PIPLC treatment, GFRα-2 also enhanced neuron recovery in the presence of GDNF. Data are the mean±s.e.m. of two independent experiments, each with a minimum of duplicate determinations. *Significantly different from control (P<0.001).

Response of embryonic enteric neural precursor cells to GDNF

The effect of GDNF in promoting the development of neurons from enteric neural precursor cells has been demonstrated under a number of different experimental conditions (Chalazonitis et al., 1998a; Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999). Enteric precursor populations have been immuno-isolated with cell-surface antibodies recognizing the HNK-1 epitope (Hearn et al., 1998), p75 (Chalazonitis et al., 1998a) and Ret (Taraviras et al., 1999), resulting in cultures containing overlapping populations of enteric precursor cells. Despite differences in growth conditions and cell density, these studies all show that GDNF increases the number of enteric neurons that develop and survive in culture. Rat E12-13.5 precursor cells show a greater response to GDNF than cells from later developmental stages, suggesting that the GDNF response is developmentally regulated (Chalazonitis et al., 1998a; Taraviras et al., 1999). Our experiments confirm the neuron-promoting effects of GDNF for E13.5 rat enteric precursors. We see similar increases in neuron number at E15.5 and E19.5, raising the possibility that the developmental decrease in GDNF responsive begins to stabilize by E13.5. Enteric neurons continue to respond to GDNF between E13.5 and E19.5, but the nature of the response changes with developmental time.

Avian HNK-1 positive and mouse p75-positive enteric neuroblasts increase their incorporation of BrdU in response to GDNF, demonstrating that GDNF acts as a mitogen for developing enteric neurons or their precursors (Hearn et al., 1998; Wu et al., 1999). In the rat, Chalazonitis et al. (1998a), used PCNA staining to demonstrate that GDNF promoted cell division of p75-expressing rat enteric precursors at E12. We found that GDNF increased proliferation of rat peripherin-positive neuroblasts at E13.5 and E15.5 (Fig. 5). As enteric neurons continued to mature, GDNF treatment no longer promoted cell division, although it still led to an overall increase in neuron number. These results indicate that embryonic enteric cells undergo a developmental change in their response to GDNF, characterized by the loss of the proliferative response. The maintenance of the neuron recovery response at E19.5, a developmental time at which enteric neurons show no increase in proliferation in response to GDNF, suggests a shift to a survival response to GDNF. This model is consistent with the observation that GDNF treatment leads to an increase in total cell number in cultures of rat E12, but not E14-E16, p75-expressing cells (Chalazonitis et al., 1998a). GDNF has been shown to act as a survival factor for a number of different peripheral neuronal lineages, many of which show different levels of GDNF responsiveness at different developmental times (Buj-Bello et al., 1995; Goldhawk et al., 2000). While GDNF may act to support both enteric survival and proliferation at early developmental times (Heuckeroth et al., 1998), our results provide evidence that the mechanism by which enteric neural precursors respond to GDNF changes with development. This switch from GDNF acting as a mitogen to a trophic factor may take place as maturing enteric neuroblasts become postmitotic, an idea supported by the observation that the loss of a proliferative response to GDNF corresponded to the developmental period in which intrinsic cell division decreased dramatically (Fig. 5).

Developmental changes in expression of GDNF receptor components

Ret-expressing cells were found scattered throughout the developing gut at E13.5, predominantly in cells that co-expressed HNK-1 (Fig. 2, Table 1). Thus, Ret expression is present in neural crest-derived cells, which, by E15.5 are tightly localized to the developing enteric plexuses. A similar expression pattern was observed for GFRα-1 in E19.5 rat gut (Fig. 6). This localization of GFRα-1 is consistent with reports from other laboratories demonstrating expression of GFRα-1 protein and mRNA in the region corresponding to the myenteric plexus at similar developmental stages (Cacalano et al., 1998; Chalazonitis et al., 1998a; Yu et al., 1998). In contrast, we found that at earlier developmental times, GFRα-1 was expressed throughout all of the layers of the gut. This dispersed pattern of brightly GFRα-1-immunoreactive cells in E13.5, and to a lesser extent in E15.5 embryos, suggests that at these developmental stages GFRα-1 is more widely expressed in the gut than had been previously reported in avian intestine (Schiltz et al., 1999). In the developing rat, GFRα-1 mRNA was found in non-neural crest-derived cells in the gut at E14, although protein was not detected by immunocytochemistry (Chalazonitis et al., 1998a). Our results suggest that GFRα-1 protein is initially widely expressed in the rat gut and becomes localized to neural crest-derived cells between E13.5 and E19.5. It is interesting that in situ hybridization in E15.5 rat intestine revealed a fairly broad localization of GFRα-1 mRNA along the circumference of the gut (Treanor et al., 1996), in a pattern similar to the protein expression observed in our experiments. When HNK-1-positive cells were cultured from embryos that showed broad GFRα-1 expression, we observed GFRα-1 immunoreactivity in neuronal cells and not in flat cells at the end of the culture period (data not shown), consistent with the findings of other laboratories (Chalazonitis et al., 1998a).

The high level of GFRα-1 immunoreactivity at early developmental times raises the question of whether GFRα-1 produced by HNK-1-negative cells is available to HNK-1-positive neural precursors. The presence of a soluble form of GFRα-1 in the conditioned media from E13.5 and E15.5, but not E19.5, HNK-1-negative populations parallels the in vivo expression pattern and indicates that a *trans* form of GFRα-1 may be seen by enteric precursor cells. Although *cis* interactions are likely to be the primary mechanism through which GDNF/GFRα-1/Ret signaling occur (Tansey et al., 2000), our data provides evidence that *trans* signaling may play a developmentally regulated role in the GDNF response of enteric neurons.

GFRα-1 acts in *cis* and *trans* to mediate the actions of GDNF

GFRα-1 is an essential part of the GDNF signaling pathway. In the absence of GFRα-1, or following the removal of GPI-linked surface molecules, GDNF did not elicit a response in GFRα-1 transfected cells (Jing et al., 1996) nor did it enhance survival of a number of different types of embryonic neurons (Cacalano et al., 1998; Jing et al., 1996; Krieglstein et al., 1998; Treanor et al., 1996). GDNF forms a signaling complex with Ret and GFRα-1 present on the same cell (Tansey et al., 2000). While such *cis* interactions may form the major signaling complexes for GNDF, several studies have demonstrated that soluble forms of GFRα-1 can act in *trans* to promote a GDNF response (Cacalano et al., 1998; Jing et al., 1996; Treanor et al., 1996). When GFRα-1 was removed from embryonic neurons either by targeted mutation of the GFRα-1 gene, or by PIPLC removal of GPI-linked proteins, GDNF-mediated survival was rescued by the addition of soluble GFRα-1 (Cacalano et al., 1998; Treanor et al., 1996). Here we have shown that, in addition to restoring a response to GDNF in enteric neurons that have been stripped of their cell surface GFRα-1, soluble GFRα-1 can act in *trans* to potentiate the GDNF response of GFRα-1-expressing neurons.

The magnitude of the GDNF response in PIPLC-treated cells cultured with GFRα-1 was smaller than that for untreated cells cultured with GDNF and soluble GFRα-1 (Fig. 8). This result suggests that signaling in *trans* was not sufficient to obtain a maximal response to GDNF, although we cannot rule out the possibility that PIPLC treatment resulted in the loss of additional GPI-linked proteins that are capable of modulating the GDNF response. Our results are consistent with the findings of Tansey et al. (2000), who demonstrated that efficient downstream signaling by GNDF is dependent upon interactions between cell surface GFRα-1 and Ret. Together, our data indicate that GDNF promotes an increase in enteric neuron number by forming signaling complexes with cell-surface GFRα-1 and Ret, and that this response may be potentiated by *trans* interactions with soluble GFRα-1 released by cells in the gut.

In the presence of cell-surface GFRα-1, soluble GFRα-2 did not increase the GDNF response of enteric neural precursors, demonstrating the specificity of GFRα family members for their cognate ligands. Interestingly, this specificity was dependent upon the presence of cell-surface GPI-linked molecules. Following the removal of GPI-linked molecules, including GFRαs, the addition of GDNF and either GFRα-1 or GFRα-2 resulted in the activation of Ret signaling. These experiments suggest that one role of cell-surface GFRα is to limit the specificity of Ret to specific ligand/GFRα combinations. A possible mechanism underlying such regulation is that GDNF can interact with GFRα-1 in a *cis-trans* GFRα-1 configuration. Since the stoichiometry of GDNF, GFRα-1 and Ret is thought to be 1:2:2 (Eigenbrot and Gerber, 1997; Jing et al., 1996), a possible mechanism underlying such regulation is that GDNF interacts with a GFRα-1 dimer composed of a *cis* and a *trans* GFRα-1 component. If this interaction takes place, we would expect to see a stronger GDNF response in the presence of soluble GFRα-1 than GFRα-2 because of the higher affinity of GDNF for GFRα-1 than GFRα-2 (Jing et al., 1997; Klein et al., 1997) and the higher expression of GFRα-1 in the developing gut (Naveilhan et al., 1998; Rossi et al., 1999; Widenfalk et al., 1997). In the absence of cell-surface GPI-linked proteins, preferential *cis-trans* interactions cannot take place and Ret can be activated by GDNF and soluble GFRα-1 or GFRα-2 dimers. These interactions could provide a mechanism to limit the extent of cross-signaling between different receptor components and modulate the influence of multiple GDNF family members during the development of the enteric nervous system.

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