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Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system

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SUMMARY

RET is a member of the receptor tyrosine kinase (RTK) superfamily, which can transduce signalling by glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) in cultured cells. In order to determine whether in addition to being sufficient, RET is also necessary for signalling by these growth factors, we studied the response to GDNF and NTN of primary neuronal cultures (peripheral sensory and central dopaminergic neurons) derived from wild-type and RET-deficient mice. Our experiments show that absence of a functional RET receptor abrogates the biological responses of neuronal cells to both GDNF and NTN.

Despite the established role of the RET signal transduction pathway in the development of the mammalian enteric nervous system (ENS), very little is known regarding its cellular mechanism(s) of action. Here, we have studied the effects of GDNF and NTN on cultures of neural crest (NC)-derived cells isolated from the gut of rat embryos. Our findings suggest that GDNF and NTN promote the survival of enteric neurons as well as the survival, proliferation and differentiation of multipotential ENS progenitors present in the gut of E12.5-13.5 rat embryos. However, the effects of these growth factors are stage-specific, since similar ENS cultures established from later stage embryos (E14.5-15.5), show markedly diminished response to GDNF and NTN. To examine whether the in vitro effects of RET activation reflect the in vivo function(s) of this receptor, the extent of programmed cell death was examined in the gut of wild-type and RET-deficient mouse embryos by TUNEL histochemistry. Our experiments show that a subpopulation of enteric NC undergoes apoptotic cell death specifically in the foregut of embryos lacking the RET receptor. We suggest that normal function of the RET RTK is required in vivo during early stages of ENS histogenesis for the survival of undifferentiated enteric NC and their derivatives.

Key words: RET, Receptor tyrosine kinase, Glial cell-line derived neurotrophic factor, Neurturin, Mouse, Rat, Enteric nervous system, Apoptosis

INTRODUCTION

The *c-Ret* proto-oncogene encodes a member of the RTK superfamily (Takahashi et al., 1988; Takahashi and Cooper, 1987), which is expressed during vertebrate embryogenesis in the developing excretory system, in all lineages of the peripheral nervous system (PNS) and in motor and catecholaminergic neurons of the central nervous system (CNS), including ventral midbrain dopaminergic neurons (VMDNs) (Avantaggiato et al., 1994; Durbec et al., 1996b; Marcos and Pachnis, 1996; Pachnis et al., 1993; Trupp et al., 1997; Tsuzuki et al., 1995; Young et al., 1998). Despite the widespread expression of *c-Ret* in the nervous system of vertebrates, mutations of this locus affect, albeit drastically, only a subset of PNS ganglia. Thus, loss of function mutations of *c-RET* in humans lead to congenital megacolon (Hirschsprung's disease), a condition characterised by absence of enteric ganglia from the terminal colon (Edery et al., 1994; Romeo et al., 1994). Also, mice homozygous for a targeted mutation of *c-Ret* (*Ret.k*$^{-}$) die within 12-24 hours of birth and lack the superior cervical ganglia (SCG) and enteric ganglia posterior to the proximal stomach (intestinal aganglionosis) (Durbec et al., 1996b; Schuchardt et al., 1994). Outside the nervous system, RET-deficient mice have severe hypodysplasia or aplasia of the kidneys, consistent with high levels of expression of *c-Ret* in the developing metanephros (Robertson and Mason, 1995; Schuchardt et al., 1994, 1996).

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN) and persephin (PSP) belong to a small family of closely related neurotrophic factors that is distantly related

to the transforming growth factor-β (TGF-β) superfamily (Kotzbauer et al., 1996; Lin et al., 1993; Milbrandt et al., 1998). The first member of this family (GDNF) was identified as a potent survival factor for VMDNs in culture (Lin et al., 1993), but subsequently it was shown that it can also support the survival of a wide variety of neuronal cell types from the CNS and PNS, such as motor neurons and subsets of peripheral sensory, sympathetic and parasympathetic neurons (Buj-Bello et al., 1995; Henderson et al., 1994; Oppenheim et al., 1995; Trupp et al., 1995). Furthermore, exogenous administration of GDNF has potent protective effects in vivo on motor neurons and VMDNs compromised by axotomy or toxicity (Beck et al., 1995; Choi-Lundberg et al., 1997; Gash et al., 1996; Henderson et al., 1994; Tomac et al., 1995; Yan et al., 1995). The second member of the family, NTN, is 40% identical to GDNF and was originally isolated as an activity capable of promoting the in vitro survival of sympathetic neurons (Kotzbauer et al., 1996). However, the neurotrophic effects of this molecule also extend to peripheral autonomic and sensory neurons as well as VMDNs and motor neurons of the CNS (Horger et al., 1998; Klein et al., 1997; Kotzbauer et al., 1996). Finally, PSP, which is 40% identical to GDNF and NTN, has strong neurotrophic effects on motor neurons and VMDNs and has the potential of promoting the survival of SCG neurons isolated from postembryonic day 4 (P4) chicken (Enokido et al., 1998; Milbrandt et al., 1998).

All members of the GDNF-related family of neurotrophic factors can activate the RET RTK in in vitro cell culture assays (Durbec et al., 1996a; Trupp et al., 1996). This activation is not direct, but is mediated by members of a family of glycosylphosphatidylinositol (GPI)-linked cell surface molecules, called GFRα (for GDNF Family Receptor). Four members of this family have been described so far; GFRα-1 and GFRα-2 can bind to and mediate activation of RET by both GDNF and NTN (although some preference of GDNF and NTN for GFRα-1 and GFRα-2, respectively, has been reported), while GFRα-4 mediates signalling by PSP (Baloh et al., 1998, 1997; Creedon et al., 1997; Enokido et al., 1998; Jing et al., 1996, 1997; Klein et al., 1997; Sanicola et al., 1997; Treanor et al., 1996). In addition to these cell culture studies, a series of genetic experiments have further supported the hypothesis that GDNF, GFRα-1 and RET are components of a single functional signalling pathway. Thus, targeted mutations of *Gdnf* or *Gfrα-1* in mice result in phenotypes which, characterised by kidney agenesis and intestinal aganglionosis (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), are remarkably similar to the phenotype of RET-deficient animals. At present, no information is available regarding the effects of mutations of the loci encoding the other GFRα receptors or NTN and PSP.

Despite strong evidence that RET mediates signalling by GDNF, several observations have raised the possibility that some of the biological effects of GDNF itself or the other members of its family (NTN and PSP), could be mediated by other receptor systems and thus be RET-independent. First, several neuronal cell types that respond to GDNF and NTN in culture (such as VMDNs and peripheral sensory neurons) appear to be unaffected by mutations in the *c-Ret* locus ((Durbec et al., 1996b; Marcos and Pachnis, 1996; Schuchardt et al., 1994) and M. S. and V. P., unpublished). Second, the phenotype of GDNF-null mouse embryos (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) is very similar (and possibly more severe) compared to that of RET-deficient embryos (Durbec et al., 1996b; Marcos and Pachnis, 1996; Schuchardt et al., 1994). This observation, in addition to suggesting that GDNF may be capable of activating non-RET-related receptors, indicates that it is also the main ligand activating RET during mammalian embryogenesis and that if NTN and PSP have any function(s) during this period, it is likely to be mediated by other cell surface receptors. Third, some of the biological effects of GDNF may be mediated directly by the GFRα family of coreceptors. This hypothesis has been strengthened recently by the realisation that GPI-linked cell surface proteins can be clustered in living cells in microdomains that have been implicated in signal transduction (Simons and Ikonen, 1997). Finally, the GDNF family of neurotrophic factors belongs to the superfamily of TGF-β signalling molecules (Lin et al., 1993), which generally activate serine/threonine kinase receptors (Josso and di Clemente, 1997). It is therefore possible that, in addition to signalling through RET, GDNF and its relatives may be capable of activating receptors with other biochemical (i.e. non-tyrosine kinase) activities.

The ENS in vertebrates is the most complex part of the PNS. It contains a large number of neurons organised into an intricate array of interconnected ganglia which are present throughout the gut wall and control contractility of its musculature and secretions of its glands (Gershon et al., 1994). The majority of the precursors of the ENS are derived from the vagal (corresponding to somites 1-5) and anterior truncal (corresponding to somites 6-7) neural crest (NC) (Durbec et al., 1996b; Epstein et al., 1994; Le Douarin and Teillet, 1973; Peters-van der Sanden et al., 1993; Yntema and Hammond, 1954). NC cells destined to colonise the bowel emerge from the dorsal aspect of the neural tube at E8.0-8.5, invade the foregut mesenchyme 24 hours later (enteric NC) and, migrating in a rostro-caudal direction, colonise the entire bowel (Durbec et al., 1996b; Jacobs-Cohen et al., 1987; Kapur et al., 1992; Rothman et al., 1984; Young et al., 1998). Prior to entry into the foregut, prospective enteric NC cells induce expression of c-Ret mRNA (RET+) (Durbec et al., 1996b; Lo et al., 1997). Formation of the ENS in mammals depends on a series of overlapping developmental processes which are controlled by a complex set of interactions between the enteric NC and the surrounding mesenchyme (Gershon, 1998; Le Douarin and Dulac, 1992). Thus, upon entering the gut wall, NC cells respond to signals which promote their survival, proliferation, migration along the entire length of the gut and differentiation into mature neurons and glia (Gershon et al., 1993). Despite the indubitable role of RET, GFRα-1 and GDNF in the development of the mammalian ENS, their mechanism of action and the cellular processes they control in vivo remain largely unknown.

Here we report on studies which address the in vitro and in vivo roles of the RET signal transduction pathway. Using primary cultures of neuronal cells derived from wild-type and mutant (*Ret.k⁻/Ret.k⁻*) mouse embryos, we demonstrate that functional deletion of *c-Ret* abrogates the biological responses of the analysed cell types to both GDNF and NTN. We suggest that RET, in addition to being sufficient (in conjunction with the GPI-linked coreceptors), is also necessary for signalling by GDNF and

NTN even in cells that remain unaffected by *c-Ret* mutations in vivo. In addition, in vitro studies have been performed to address the role of GDNF and NTN on the development of the mammalian ENS. Our findings suggest that GDNF and NTN promote the survival, proliferation and differentiation of RET-expressing enteric NC immunoselected from the gut of rat embryos. However, the response of enteric NC to GDNF and NTN was age-dependent in that RET+ cells isolated from relatively late stages of embryogenesis (E14.5-15.5) showed a diminished response to both neurotrophic factors when compared to similar cell populations derived from younger (E12.5-13.5) embryos. Finally, we present evidence that the in vitro function(s) of the RET signalling pathway is likely to represent its in vivo role(s), as embryos homozygous for the *Ret.k*$^{-}$ mutation show a dramatic increase in programmed cell death of enteric NC.

MATERIALS AND METHODS

Animals

Mice carrying the *Ret.k*$^{-}$ mutation (Durbec et al., 1996b; Schuchardt et al., 1994) were maintained on a mixed genetic background by random genetic crossing. The genotype of embryos and animals was determined by PCR, as has been described previously (Schuchardt et al., 1994). The day of the vaginal plug was considered as E0.5. Transgenic mice carrying the MS6 transgene will be described elsewhere (M. Sukumaran, unpublished). The MS6 transgene was introduced into the *Ret.k*$^{-}$ genetic background by standard genetic crosses. For cultures of enteric NC, RET+ cells were isolated from Wistar rat embryos.

Cell culture

P0.5 DRG neurons were isolated from individual wild-type (+/+), heterozygous (+/*Ret.k*$^{-}$) or mutant *Ret.k*$^{-}$/*Ret.k*$^{-}$ mice. Preliminary experiments showed that the response of wild-type or heterozygous DRG neurons to both GDNF and NTN was identical and therefore cells from the two genotypes were combined in all subsequent experiments. We performed three experiments and in each experiment, control and mutant cells were plated in triplicate. In total, we analysed DRG neurons from 31 wild-type or heterozygous and 23 mutant animals. DRG neurons were isolated and cultured as described previously (Davies, 1995). Neurons were plated in a defined medium that was based on Ham's F14 (Gibco) and supplemented with 2 mM glutamine, 60 μg/ml penicillin, 100 μg/ml streptomycin, 0.35% bovine serum albumin (Pathocyte-4, ICN), 16 μg/ml putrescin (Sigma), 60 ng/ml progesterone (Sigma), 30 nM selenious acid (Sigma), 340 ng/ml triidothyronine (Sigma) and 400 ng/ml L-thyroxine (Sigma). The survival response of DRG neurons to GDNF or NTN was expressed as the percentage of neurons surviving after 48 hours relative to the number of neurons present 6 hours after plating. Cultures of DRG neurons were fixed and stained for β-galactosidase activity as previously described (Natarajan et al., 1999). NGF, NTN and GDNF were added to the cells 1-2 hours after plating at a concentration of 50 ng/ml.

VMDNs were isolated from individual +/+ (n=10), +/*Ret.k*$^{-}$ (n=21) and *Ret.k*$^{-}$/*Ret.k*$^{-}$ (n=13) E13.5 mouse embryos and cultured for 7 days in the absence or presence of GDNF (5 ng/ml) or NTN (50 ng/ml) (Barker and Johnson, 1995). 20 control (+/+ or +/*Ret.k*$^{-}$) and 9 mutant (*Ret.k*$^{-}$/*Ret.k*$^{-}$) embryos were analysed for their response to GDNF, while 11 control and 4 mutant embryos were analysed for their response to NTN. At the end of the culture period the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained using an antibody against tyrosine hydroxylase (TH, Affiniti) and anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody.

RET+ cells were isolated from the gut of mouse and rat embryos as previously described (Lo and Anderson, 1995; Natarajan et al., 1999). The purified RET+ cells were cultured in a chemically defined medium based on L15-CO_2 (Gibco) that was supplemented with 100 μg/ml transferrin (Sigma), 16 μg/ml putrescin (Sigma), 60 ng/ml progesterone (Sigma), 1 mM sodium byturate (Sigma), 30 nM selenious acid (Sigma), 0.174% bovine serum albumin (Pathocyte-4 ICN) and 67.5 ng/ml triidothyronine (Sigma). RET+ cells were plated on poly-D-lysine (Sigma; 0.5 mg/ml) and laminin (Gibco; 20 μg/ml) 3-cm gridded dishes (Nunc) as low density (5-8×10^2 cells/dish) or high density (8-10×10^3 cells/dish) cultures. Growth factors were added to the culture medium 1-2 hours after the plating at a final concentration of 50 ng/ml. High-density cultures survived for at least 10 days while the overall viability of low-density cultures was 3-4 days. For cell counting and immunostaining, RET+ cells were fixed at various time points (6 hours, 1 day, 2 days or 3 days) after plating with 4% paraformaldehyde (in PBS) and permeabilised with 0.2% Triton in PBS containing 1% sheep serum. Neuronal cells were identified morphologically and by using a monoclonal antibody against neuron-specific class III b-tubulin (Tuj1; Sigma). Staining was revealed using an HRP-conjugated anti-mouse secondary antibody (Boehringer). The total number of cells and the number of neurons present at each time point were expressed relative to the numbers present 6 hours post-plating. For each time point we analysed four duplicate dishes and each experiment was repeated three times. For the bromo-deoxyuridine (BrdU) incorporation experiments, 10 μM of BrdU (Boehringer-Mannheim) was added to the culture medium for 24 hours at 37°C, followed by a period of 48 hours in BrdU-free medium before fixation (Chalazonitis et al., 1994). BrdU incorporation was detected as described previously (Natarajan et al., 1999).

In situ hybridisation

Non-radioactive in situ hybridisation on fresh-frozen sections of mouse embryos was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). For antisense riboprobes we used the following templates: a full-length GFRα-1 cDNA, a 1120 bp-long PCR-amplified fragment of GFRα-2 containing the open reading frame, and the insert of pmcRet7 that corresponds to the proximal extracellular and the entire intracellular domain of RET (Pachnis et al., 1993).

Cell apoptosis assay

Apoptotic cells were identified on sections of E10.5 mouse embryos by TUNEL histochemistry using the ApopTag kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. For double-label experiments, sections were also incubated with a polyclonal antibody against TH (Affiniti) and expression of this protein was revealed by using an alkaline-phosphatase-conjugated anti-rabbit secondary antibody (Boehringer-Mannheim).

RESULTS

The RET receptor is necessary for the response of peripheral sensory neurons to GDNF and NTN

To examine whether GDNF or NTN can elicit biological responses in cells lacking a functional RET receptor, we compared the effects of these neurotrophic factors on various groups of postmitotic neurons isolated from wild-type or RET-deficient mouse embryos or neonates. First we assessed the response of peripheral sensory neurons isolated from dorsal root ganglia (DRG). To facilitate our analysis, we used as a reporter of RET-expressing DRG neurons a transgene (MS6) which, composed of the nLacZ gene (encoding

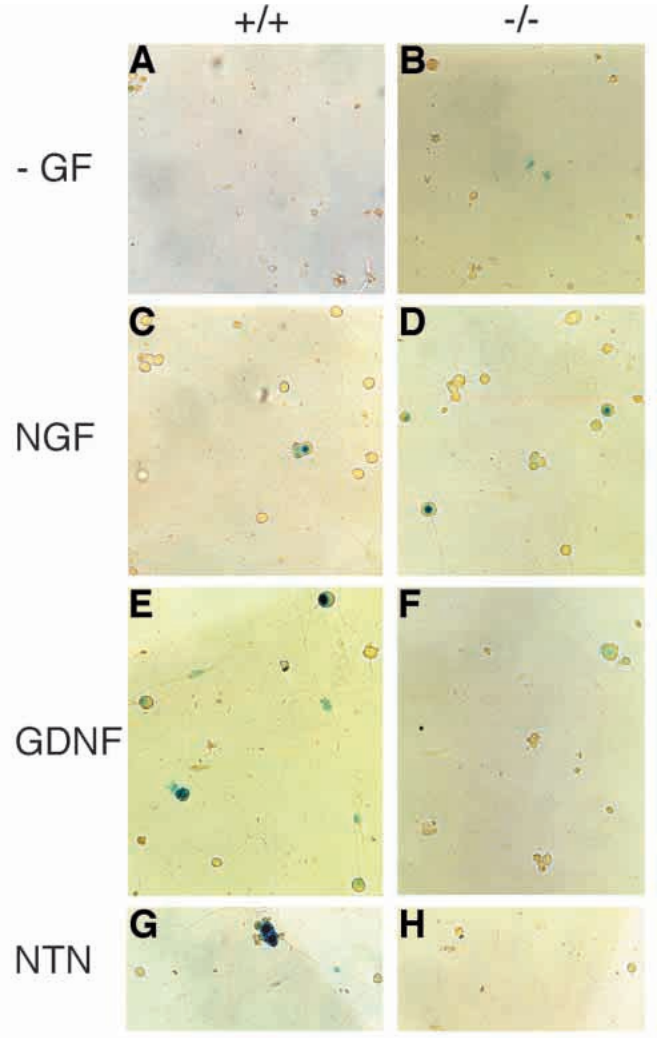

Fig. 1. RET-deficient DRG neurons do not respond to GDNF and NTN in vitro. Wild-type (+/+; A,C,E,G) and RET-deficient (–/–; B,D,F,H) DRG neurons were isolated from P0.5 mice derived from intercrosses between *+/Ret.k⁻;MS6* animals and cultured for 2 days in a defined medium containing no neurotrophic factors (–GF; A,B) or 50 ng/ml of NGF (C,D), GDNF (E,F) or NTN (G,H). After treatment, cultures were fixed and stained for β-gal histochemistry. Cells with positive nuclei represent RET-expressing neurons.

nuclear-localised β-galactosidase; β-gal) under the control of regulatory sequences from the murine *c-Ret* locus, has been shown to be expressed by the majority of the RET-expressing DRG neurons. Using this transgene as a reporter, we have also shown that the *Ret.k⁻* mutation has no detectable effect on the development and differentiation of the RET+ neurons of DRG during mammalian embryogenesis (M. S. and V. P., unpublished). These findings are consistent with analysis of GDNF- and GFRa-1-null mice, which also show no significant abnormalities of DRG neurons (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). To compare the responses of wild-type and RET-deficient sensory neurons to GDNF and NTN, cultures of dissociated DRGs were established from newborn animals derived from *+/Ret.k⁻;MS6* intercrosses. In the absence of growth factors, DRG neurons of all genotypes failed to survive and underwent apoptotic cell death (Figs 1A,B and 2). In contrast, a large proportion of sensory neurons (approx. 70%) from wild-type and mutant animals survived in the presence of nerve growth factor (NGF; Figs 1C,D and 2), indicating that the intracellular mediators of RTK signalling remain functional in *Ret.k⁻* neurons. Also, the percentage of β-gal+ (i.e. RET+) neurons present in these cultures was similar and independent of their genotype, consistent with the lack of effect of the *Ret.k⁻* mutation on sensory DRG neurons. However, the effects of GDNF and NTN were genotype-dependent. In the presence of 50 ng/ml of either neurotrophic factor, 25-30% of neurons were rescued from apoptosis in cultures established from wild-type or *+/Ret.k⁻* animals (Figs 1E,G and 2) (Kotzbauer et al., 1996; Matheson et al., 1997). The fraction of rescued neurons, which is similar to the fraction expressing *c-Ret* in newborn DRGs, and the enrichment of β-gal+ neurons observed, suggest that GDNF and NTN act on the RET-expressing subpopulation of cells. Contrary to these findings, very few neurons were rescued by GDNF and NTN in cultures derived from *Ret.k⁻/Ret.k⁻* animals and among those none expressed β-gal (Figs 1F,H and 2). Finally, combination of GDNF and NTN failed to rescue a higher percentage of wild-type or mutant neurons (Fig. 2).

GFRα-1 and GFRα-2 have been described as obligatory coreceptors required for activation of RET by GDNF and NTN (Baloh et al., 1997; Creedon et al., 1997; Jing et al.,

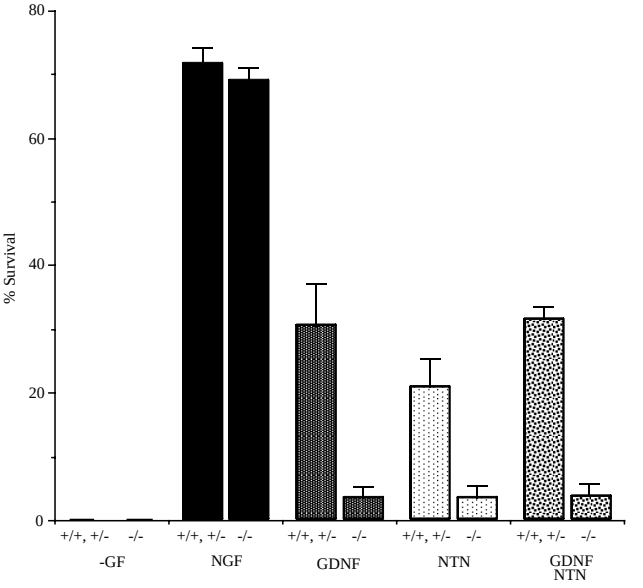

Fig. 2. Quantification of the survival of DRG neurons isolated from wild-type or heterozygous (+/+ or +/–) and mutant (–/–) P0.5 animals at the end of a 2-day culture period. Neurons were incubated in the absence of neurotrophic factors (–GF) or in the presence of NGF, GDNF, NTN or GDNF and NTN. % survival indicates the percentage of neurons at the end of the culture period relative to the number present 6 hours after plating. No difference was detected in the response of wild-type or heterozygous animals and the data for these two genotypes have been combined. The apparent difference in the percentage of neurons surviving in the absence or presence of GDNF and NTN is not significant ($P>0.05$). +/–, *+/Ret.k⁻* and –/–, *Ret.k⁻/Ret.k⁻*.

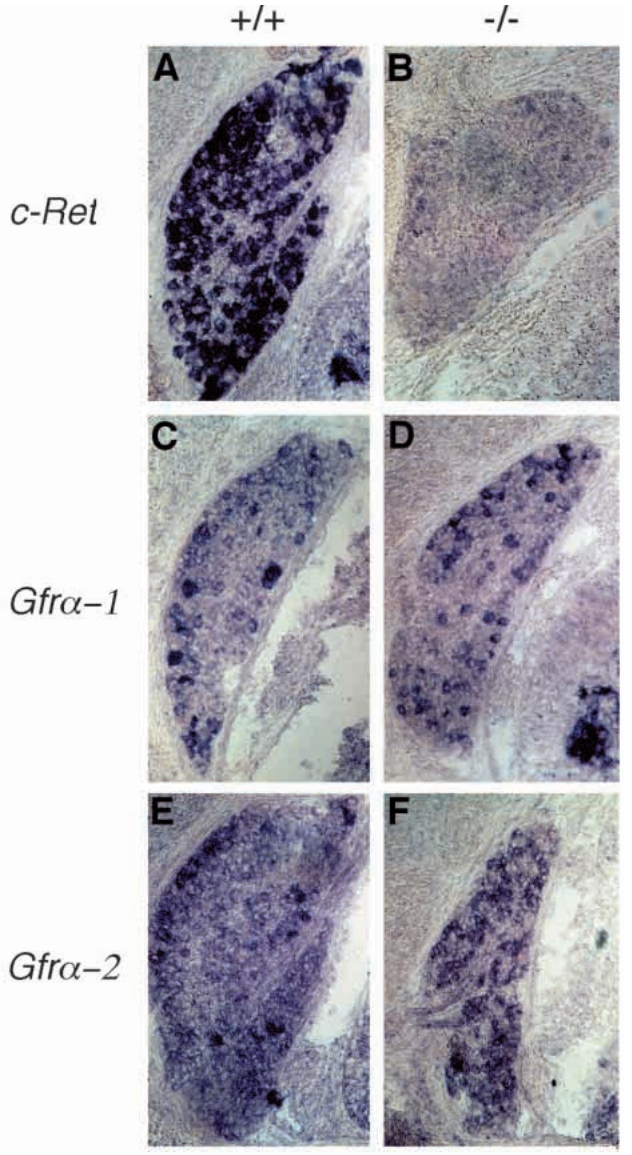

Fig. 3. The *Ret.k*⁻ mutation does not affect the expression of *Gfrα-1* and *Gfrα-2* in DRG. In situ hybridisation analysis of *c-Ret* (A,B), *Gfrα-1* (C,D) and *Gfrα-2* (E,F) expression in wild-type (A,C,E) and mutant (B,D,F) animals.

1996; Klein et al., 1997; Sanicola et al., 1997; Treanor et al., 1996). To determine whether the failure of *Ret.k*⁻ DRG neurons to respond to GDNF and NTN is due to an indirect effect of the mutation on expression of *Gfrα-1* or *Gfrα-2*, the expression of these genes was compared between wild-type and mutant embryos. As shown in Fig. 3, both *Gfrα-1* and *Gfrα-2* were expressed in DRG of newborn mice. More importantly, no difference was detected in the levels or distribution of Gfrα-1 or Gfrα-2 mRNAs in DRG (or in any other tissue we examined) of wild-type and *Ret.k*⁻ mutant animals. Our results indicate that the lack of response of DRG sensory neurons isolated from RET-deficient newborn animals is unlikely to be caused by downregulation of the GPI-linked coreceptors and suggest that the RET RTK is necessary for signalling by GDNF and NTN in peripheral sensory neurons.

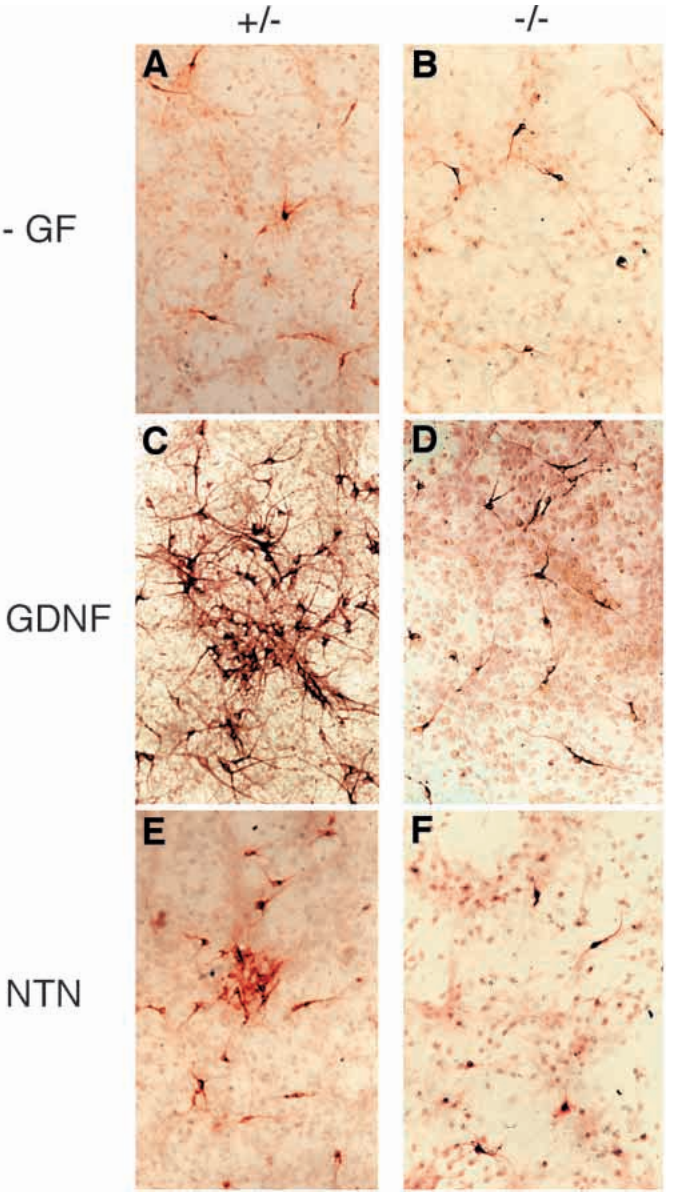

Fig. 4. RET-deficient VMDNs fail to respond to GDNF and NTN in vitro. VMDNs were isolated from E13.5 mouse embryos derived from intercrosses between *+/Ret.k*⁻ parents and cultured for 7 days in the absence of any neurotrophic factors (–GF; A,B) or in the presence of GDNF (C,D) or NTN (E,F). At the end of the culture period, cells were fixed and processed for TH immunocytochemistry. Note the effect of GDNF and NTN on the number and the morphology of the TH+ neurons.

The RET receptor is required for the response of midbrain dopaminergic neurons to GDNF and NTN

To further analyse the requirement of *c-Ret* for GDNF and NTN signalling in the nervous system, we compared the response to these neurotrophic factors of cultures of CNS neurons derived from control and RET-deficient mouse embryos. For this, we chose to study VMDNs which, in addition to responding to GDNF and NTN in vitro and in vivo (see Introduction), remain unaffected by mutations of the *c-Ret* locus ((Marcos and Pachnis, 1996; Schuchardt et al., 1994) and

our unpublished data). Individual cultures of VMDNs were established from +/+, *+/Ret.k⁻* or *Ret.k⁻/Ret.k⁻* E13.5 embryos and their survival response to GDNF and NTN was assayed by determining the number of tyrosine hydroxylase-expressing (TH+) cells at the end of the culture period (Lin et al., 1993; Poulsen et al., 1994). Since no difference was observed in the response of cultures from wild-type and heterozygous embryos (not shown), the data obtained from these two genotypes were combined. Cultures of VMDNs isolated from +/+ or *+/Ret.k⁻* embryos responded to both GDNF and NTN by increased survival and enhanced axonal outgrowth. However, no such response was observed in parallel cultures established from *Ret.k⁻/Ret.k⁻* embryos (Figs 4, 5). This, together with analysis of expression of *Gfrα-1* and *Gfrα-2* in the brain of wild-type and mutant embryos which showed that the *Ret.k⁻* mutation does not alter the levels or pattern of expression of these genes (data not shown), suggested that the failure of *Ret.k⁻* VMDNs to respond to GDNF and NTN was due exclusively to the absence of the RET RTK. Our results thus far are consistent with the hypothesis that loss-of-function mutations of *c-Ret* abrogate the survival and differentiation responses of central and peripheral neurons to GDNF and NTN, indicating that a functional RET receptor is necessary for signalling by these neurotrophic factors.

GDNF and NTN promote the survival, proliferation and differentiation of enteric neural crest immunoselected from the gut of mammalian embryos

In both neuronal lineages examined thus far (i.e. VMDNs and DRG neurons), expression of *c-Ret* is restricted to differentiated postmitotic neurons, thus precluding the analysis of the effects of RET activation on undifferentiated neuronal progenitors. However, in the ENS of mammalian embryos (the development of which depends on normal RET function), *c-Ret* is expressed in both undifferentiated multipotential progenitors as well as in postmitotic neurons (Avantaggiato et al., 1994; Lo and Anderson, 1995; Natarajan et al., 1999; Pachnis et al., 1993; Tsuzuki et al., 1995; Young et al., 1998). To further examine the role(s) of the RET signal transduction pathway at various stages of cell commitment and differentiation of the vertebrate ENS, NC-derived cells were isolated from the gut of mammalian embryos and their response to GDNF and NTN was analysed in vitro. For the purification of the highly dispersed population of enteric NC, we used the strategy that was originally developed by Anderson and his colleagues. Using monoclonal antibodies specific for the extracellular domain of RET, these investigators imunoselected a population of cells from the gut of E14.5 rat embryos (RET+ cells) that was highly enriched in NC-derived progenitors of the ENS (Lo and Anderson, 1995; Lo et al., 1997). More recently, we have used a similar strategy and isolated RET+ cells from the gut of E11.5 mouse embryos (Natarajan et al., 1999). In a series of preliminary experiments we established that, although cultures of RET+ cells isolated from the gut of rat and mouse embryos had qualitatively identical responses to all experimental manipulations, the overall quality and viability of rat cultures were superior compared to similar cultures established from equivalent-stage mouse embryos (not shown). This difference is likely to be due to species-specific responses of ENS cells to the in vitro cell culture conditions and therefore all experiments reported here were performed with cultures established from rat embryos. RET+ cells were isolated from the gut of E12.5-15.5 rat embryos and seeded at high or low densities in defined medium in the absence or presence of neurotrophic factors (for experimental details see Materials and methods). In the absence of neurotrophic factors, the number of cells present in high density cultures decreased progressively and by the end of the third day no live cells could be identified (Fig. 6A). However, in the presence of 50 ng/ml of GDNF or NTN, the plated cells survived for at least 10 days and increased in numbers (Fig. 6B,C). Furthermore, immunofluoresence using neuron-specific (neuron-specific class III b-tubulin/Tuj1, or neuron-specific enolase/NSE) or glial-specific (S-100 or glial fibrillary acidic protein/GFAP) antibodies at the end of the culture period, indicated that the immunoselected cells were capable of differentiating into neurons and glia (Fig. 6B,C). Although in these experiments no accurate quantitative measurements were made, our results are consistent with the hypothesis that GDNF and NTN promote the survival, proliferation and differentiation of mammalian enteric NC cells.

In order to minimise cell-cell interactions that are likely to occur in high density cultures and mask any direct effects of GDNF and NTN, we established low density cultures of RET+ fetal gut cells. Although the overall viability of these cultures was limited compared to their high density counterparts (3-4 days), they were used in all subsequent experiments in order to obtain more accurate quantitative data. Throughout the culture period, two morphologically distinct types of cells were identified and their absolute and relative numbers depended on the culture conditions (see below). The first population

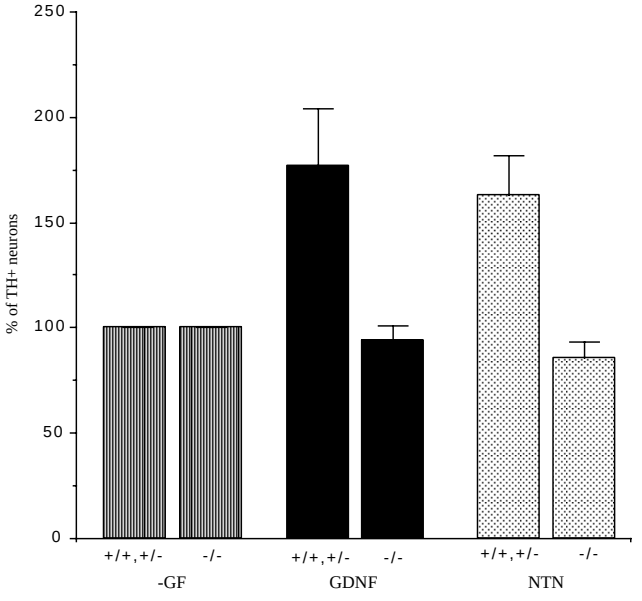

Fig. 5. Quantification of the effect of GDNF and NTN on TH-expressing ventral midbrain neurons isolated from wild-type and heterozygous (+/+, +/–) or mutant (–/–) E13.5 mouse embryos. % of TH+ neurons indicates the percentage of TH-expressing neurons surviving in the presence of GDNF and NTN relative to the number of TH+ neurons present in control cultures (i.e. no neurotrophic factors) of the same genotype.

consisted of neuronal cells, as indicated by their characteristic morphology and confirmed by immunostaining with Tuj1 or NSE antibodies (Fig. 6D-F). The remaining cells were more flat and round, negative for neuronal or glial markers and thus were likely to represent undifferentiated multipotential ENS progenitors (Fig. 6D-F). The failure to detect glial cells in the low density cultures is likely to be due to insufficient time for glial cell differentiation, as large numbers of mature glial cells were present in high-density cultures, which survive for longer periods (see above). In the absence of GDNF and NTN, the number of neuronal and non-neuronal cells in low density RET+ cell cultures established from the gut of E12.5-13.5 rat embryos ('early cultures'), decreased progressively relative to the number of cells originally plated and by the end of the third day in culture, no live cells were present (Fig. 7top, –GF). Interestingly, we consistently observed that the non-neuronal cells were more sensitive to the absence of neurotrophic factors since the majority of these cells had died by 24-30 hours after plating. In contrast, neuronal cells were relatively more resistant, requiring an additional 24-48 hours to be eliminated (Fig. 7top, –GF). A dramatically different response was recorded for both neuronal and non-neuronal cells in parallel cultures containing GDNF or NTN. 50 ng/ml of either neurotrophic factor prevented to a large extent the death of non-neuronal cells, the number of which decreased only slightly during the 3-day culture period (Fig. 7top, GDNF, NTN). In addition, GDNF or NTN resulted in a dramatic increase of neurons relative to the number present in the cultures shortly after plating (Fig. 7top, GDNF, NTN). These neurons were usually clustered into groups of 4-8 cells and had long axonal projections interconnecting the various neuronal islands (Fig. 6E,F). The rescue of the RET+ cells and the increase in their number suggest that GDNF and NTN promote the survival and proliferation of this group of NC-derived cells. The role of these neurotrophic factors as mitogens is further supported by BrdU incorporation experiments. In the absence of neurotrophic factors, only 1% of neurons incorporated BrdU, while in the presence of GDNF and NTN, 40% and 36% of neurons respectively incorporated the nucleotide analogue (data not shown). Finally, the increase in the number of neurons observed in the presence of GDNF or NTN are consistent with the hypothesis that these molecules promote the differentiation of enteric NC towards the neuronal phenotype.

Stage-specific effects of GDNF and NTN on ENS cultures of mammalian embryos

Low-density cultures of RET+ cells established from the gut of later stage embryos (E14.5-15.5; 'late' cultures) showed a qualitatively similar response to GDNF and NTN, although some notable differences were also observed. Thus, in the absence of growth factors, the relative number of mature neurons and non-neuronal cells present were decreased dramatically during the 3-day culture period (Fig. 7bottom, –GF). Again, the fall in the number of non-neuronal cells relative to postmitotic neurons was more precipitous, indicating a greater dependence of these cells to GDNF and NTN for in vitro survival. As in the case of the 'early' cultures, addition of 50 ng/ml of GDNF or NTN resulted in the rescue of a significant fraction of the non-neuronal cells. However, the extent of rescue, albeit similar for the two molecules, was smaller compared to the 'early' cultures, since the number of undifferentiated cells present at the end of the culture period was only 25% of the original number of non-neuronal cells (Fig. 7bottom, GDNF, NTN). Finally, we reproducibly observed that both growth factors increased the number of postmitotic neurons during the culture period. Again though, this differentiation effect was weaker compared to that observed on cultures from younger embryos and, furthermore, NTN was less efficient compared to GDNF (Fig. 7bottom, GDNF, NTN). Overall, our in vitro studies suggest that GDNF and NTN promote the survival, proliferation and differentiation of enteric NC of mammalian embryos and that such effects are more pronounced during relatively early stages of ENS histogenesis.

NT-3 and CNTF have only minimal effects on 'early' RET+ cells

NT-3 has been shown to promote neuron and glial cell development in cultures of NC-1/HNK-1-expressing cells immunoselected from the gut of E14.5 rat embryos (Chalazonitis et al., 1994). This report, together with the findings presented here (indicating dramatic effects of GDNF and NTN on relatively early cultures of fetal ENS), suggest that NT-3 may function as a stage-specific survival and differentiation factor of enteric NC cells operating during relatively late stages of ENS development. A prediction of this hypothesis would be that the survival and differentiation effects of NT-3 on early ENS cultures would be relatively weak compared to those of GDNF or NTN. To test this hypothesis, the effects of GDNF, NTN and NT-3 were analysed in parallel on cultures of RET+ cells isolated from the gut of E13.5 rat embryos. While GDNF and NTN increased the proportion of neuronal cells and functioned as efficient survival factors for these cells, the effects of 50 ng/ml of NT-3 were only minimal (Fig. 8).

Targeted mutagenesis of the genes encoding the α or β components of the receptor for ciliary neurotrophic factor (CNTFRα or LIFRβ, respectively) leads to neonatal lethality, which is associated with lack of substance P (SP)- and nitric oxide synthase (NOS)-expressing motor neurons from the gut (DeChiara et al., 1995; Gershon, 1997). Although it is unlikely that CNTF is the endogenous ligand required for normal development of the enteric motor neurons (Gershon, 1997), we tested the effects of CNTF on cultures of E13.5 gut RET+ cells, as an indication of the biological activity of the CNTF receptor complex. As shown in Fig. 8, addition of 50 ng/ml of CNTF had no appreciable effects on the proportion of neuronal cells present in the cultures relative to control medium.

The *Ret.k*$^{-}$ mutation leads to increased programmed cell death in the gut of mouse embryos

The in vitro effects of GDNF and NTN on RET+ fetal gut cells suggest that the intestinal aganglionosis associated with mutations of *c-Ret* in mammals could be secondary to a proliferative defect, a differentiation defect or increased programmed cell death (apoptosis). To examine directly the role of RET on the survival of the enteric NC in vivo, we used TUNEL histochemistry to compare the extent of apoptosis in the gut of wild-type and mutant (*Ret.k*$^{-}$/*Ret.k*$^{-}$) embryos. Our experiments identified a large number of TUNEL+ cells in the foregut of E10.5 *Ret.k*$^{-}$/*Ret.k*$^{-}$ mouse embryos, while no apoptotic cells were present in the foregut of control littermates

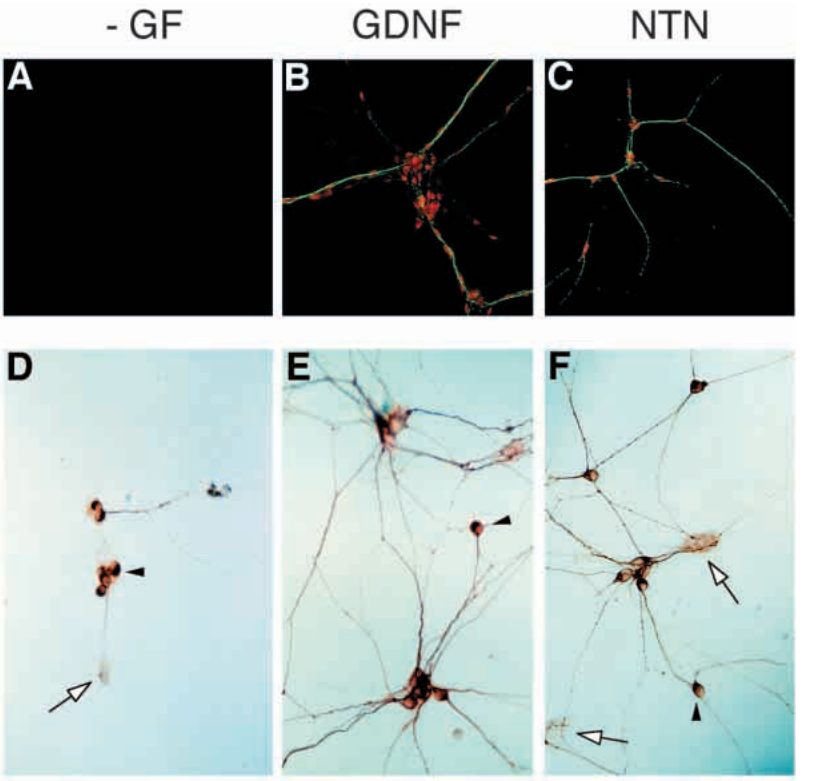

Fig. 6. Enteric NC survive and differentiate in the presence of GDNF or NTN. RET+ cells were isolated from the gut of rat embryos and plated in defined medium as high-density (top) or low-density (bottom) cultures. In the absence of neurotrophic factors all cells of high-density cultures died by the end of the third day, but in the presence of GDNF or NTN the cells survived for at least 10 days. Shown above are such high-density cultures that were fixed and double-labelled by immunofluoresence with Tuj1 (neuron-specific; green) and S100 (glia-specific; red) antibodies on the ninth day. No cells survived in the absence of neurotrophic factors (A), while large numbers of neurons and glia were detected in parallel cultures containing GDNF (B) or NTN (C). The overall viability of low-density cultures was reduced compared to their high-density counterparts and thus they were kept only for 3 days. Shown in the bottom panels are RET+ cells cultured in the absence of neurotrophic factors (–GF; D) or in the presence of GDNF (E) or NTN (F) and immunostained at the end of the second day with Tuj1 antibody. Note the dramatic effect of both factors on the extent of axonal outgrowth. Arrowheads indicate the Tuj1+ neurons while arrows indicate non-neuronal cells.

(Fig. 9A,B). Shortly after the invasion of the foregut mesenchyme by enteric NC cells (E9.0-9.5), a subset of them initiates expression of markers characteristic of the catecholaminergic phenotype, such as tyrosine hydroxylase (TH) (Baetge and Gershon, 1989; Baetge et al., 1990). We have previously suggested that the relatively small population of

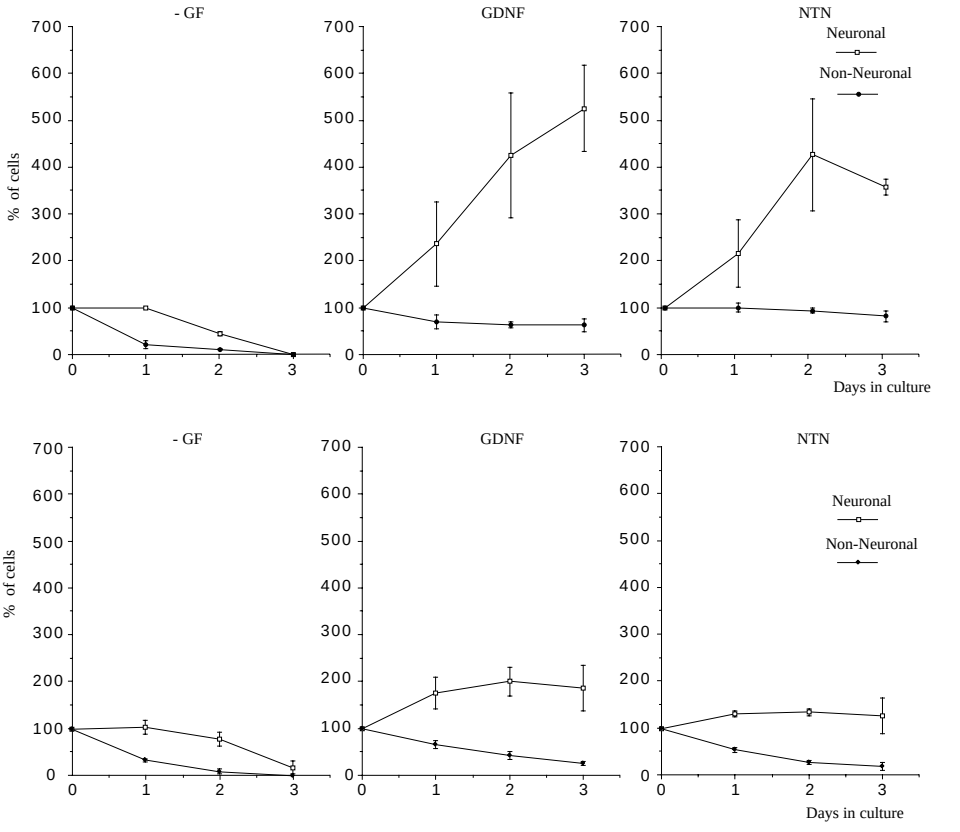

Fig. 7. Age-dependent response of enteric NC to GDNF and NTN in vitro. Low-density cultures were established from RET+ cells isolated from the gut of E12.5-13.5 (top) or E14.5-15.5 (bottom) rat embryos. Equal number of cells were plated on day 0 in multiple dishes in the absence of any neurotrophic factors (–GF) or in the presence of GDNF or NTN. 24, 48 and 72 hours post-plating, a subset of culture dishes was fixed and stained with Tuj1 (neuron-specific) antibody and the positive (neuronal) and the negative (non-neuronal) cells were counted. % of cells indicates the percentage of neuronal and non-neuronal cells at each time-point relative to the equivalent cell types present 6 hours post-plating (day 0).

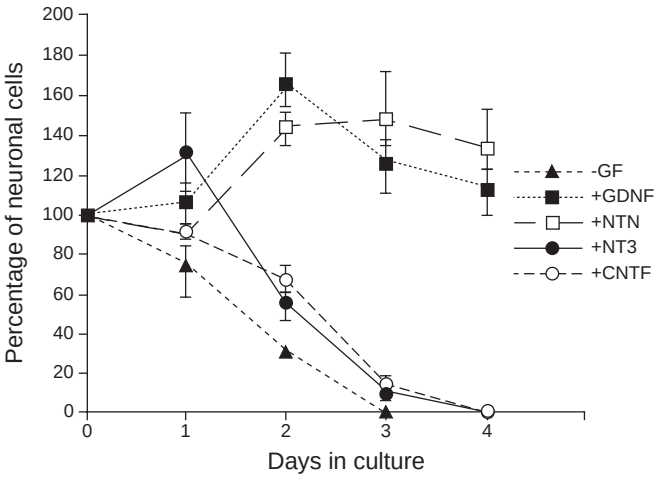

Fig. 8. NT-3 and CNTF have minimal effects on enteric NC isolated from E13.5 rat embryos. RET+ cells were isolated from the gut of E13.5 rat embryos and cultured for 4 days in the absence of any neurotrophic factors (–GF) or in the presence of GDNF, NTN, NT-3 or CNTF. Sister cultures were fixed at various time-points and immunostained with Tuj1 (neuron-specific) antibody. The percentage of neurons relative to those present on day 0, are indicated for each condition and at various time intervals post-plating.

neuronal and glial cells surviving in the foregut of *Ret.k*$^{-}$ homozygous embryos are derived from the TH+ transient catecholaminergic sublineage of the ENS (Durbec et al., 1996b). A prediction of this hypothesis is that the transient catecholaminergic cells of the ENS are affected to a lesser extent by the apoptotic effects of the *Ret.k*$^{-}$ mutation. To directly test this hypothesis, we performed double labelling of foregut sections from *Ret.k*$^{-}$/*Ret.k*$^{-}$ mouse embryos for TUNEL and TH immunostaining. As shown in Fig. 9C, TUNEL+ cells were negative for TH, suggesting that the absence of normal RET function affects primarily the TH-negative (non-catecholaminergic) population of ENS progenitors.

DISCUSSION

RET is necessary for signalling by GDNF and NTN

GDNF, NTN and PSP constitute a small subfamily of neurotrophic and urotropohic factors which, in conjunction with the GPI-linked GFRα coreceptors, are capable of activating the RET RTK. Despite the documented ability of these molecules to activate RET, several observations have raised the possibility that they could signal independently of this RTK (see Introduction). By comparing the effects of GDNF and NTN on primary neuronal cultures established from wild-type and RET-deficient (*Ret.k*$^{-}$) mouse embryos, we demonstrate that RET is required for signalling by both neurotrophic factors. Peripheral sensory (DRG) and CNS dopaminergic neurons derived from wild-type or heterozygous (*+/Ret.k*$^{-}$) mice respond to GDNF or NTN by increased survival and differentiation. However, parallel cultures established from RET-deficient animals failed to respond to both neurotrophic factors. Analysis of the expression pattern of *Gfrα-1* and *Gfrα-2* failed to detect any significant changes in mutant mice relative to wild-type controls. This, in addition to the short-term nature of our cultures, suggests that the absence of response of the mutant cells to GDNF and NTN is unlikely to reflect alterations in the level or pattern of expression of the GPI-linked obligatory coreceptors. Therefore, the in vitro studies presented here, together with the phenotypic analysis of *c-Ret*, *Gfrα-1* and *Gdnf* mutant mice, indicate that RET, in addition to being sufficient (in conjunction with the GFRα coreceptors), is also necessary for signalling by both GDNF and NTN in vivo. However, at this point we cannot exclude the possibility that in a small subpopulation of peripheral or CNS neurons or non-neuronal cell types and/or for responses other than the ones recorded here (i.e. cell survival and differentiation), GDNF and NTN might be signalling independently of RET.

A normal complement of VMDNs and DRG sensory neurons has been described in mice carrying loss-of-function mutations in *c-Ret*, *Gdnf* and *Gfrα-1* ((Marcos and Pachnis, 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; M. S. and V. P., unpublished). These observations, together with the data indicating that RET is required for signalling by GDNF and NTN, suggest strongly that all components of this signalling pathway are dispensable for normal development of midbrain catecholaminergic and peripheral DRG sensory neurons. However, the evolutionary conservation of *c-Ret* expression (and GDNF response) by these cells in different vertebrates (Avantaggiato et al., 1994; Marcos-Gutierrez et al., 1997; Pachnis et al., 1993; Robertson and Mason, 1995; Schuchardt et al., 1995), suggests an

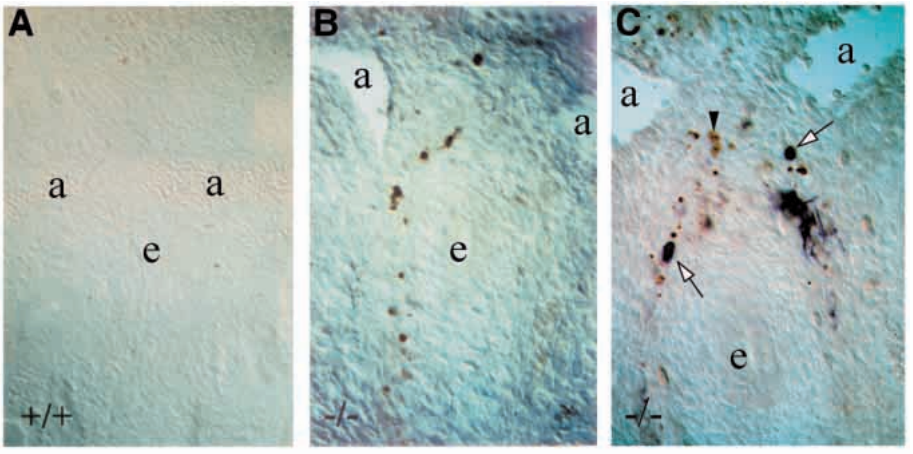

Fig. 9. Programmed cell death of enteric NC in the foregut of RET-deficient embryos. Sections through the foregut of wild-type (+/+; A) and mutant (–/–; B) embryos were processed for TUNEL histochemistry. Cells in B with dark-stained nuclei represent NC-derived cells undergoing programmed cell death in the foregut of mutant embryos. (C) A section serial to the one shown in B was double labelled for TUNEL histochemistry (dark purple; arrows) and TH immunohistochemistry (brown; arrowheads). Cells positive for TUNEL were negative for TH. a, dorsal aoarta; e, endoderm of the esophagus.

important biological function for this signalling pathway. Such a role is likely to manifest itself during postnatal stages and to be related to the survival and support of normal cell function in adult animals. Although the available targeted mutations in the loci encoding components of the RET signalling pathway lead invariably to neonatal death, the generation of stage-specific mutations should address their potential role at postnatal stages.

The role of the RET RTK in the development of the mammalian ENS

Although several genetic studies have established the critical role of GDNF, GFRα-1 and RET in the development of the mammalian ENS (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994), the mechanism of action of this signalling pathway in vivo is less well understood. Data presented here and in other studies are consistent with the hypothesis that GDNF and NTN promote the survival of enteric neurons as well as the survival, proliferation and differentiation of ENS progenitors present in the mammalian gut. This suggestion is based both on in vitro and in vivo experiments. Cultures of RET+ cells purified from the gut of rat embryos failed to survive or differentiate in the absence of neurotrophic factors. However, in the presence of GDNF or NTN, they survived efficiently, increased in numbers and differentiated into neurons and (in the case of the high density cultures) glial cells. These results are in agreement with and extend the findings of other groups. Thus, Hearn et al. (1998) have shown that GDNF specifically increases the uptake of BrdU from neural crest cells immunoselected from the gut of quail embryos. Also, Heuckeroth and colleagues have suggested that GDNF and NTN promote the proliferation of mammalian ENS progenitors and their differentiation to neurons and glia (Heuckeroth et al., 1998). However, in this study dissociated cultures of total fetal gut were used (containing a mixture of neural crest- and non-neural-crest-derived cells), thus precluding the establishment of any direct effects of GDNF or NTN on ENS cells. Finally, Chalazonitis and colleagues have shown that cultures of $p75^{NTR}$-expressing ($p75^{NTR}$ +) neural crest cells immunoselected from rat embryo gut responded to GDNF by increased survival, proliferation and differentiation (Chalazonitis et al., 1998).

That these in vitro results represent genuine in vivo functions of the RET signalling pathway is supported by our in vivo findings. Thus, a large number of cells undergoing apoptosis was observed in the foregut of RET-deficient embryos. Several lines of evidence suggest that these apoptotic cells represent the RET-dependent enteric NC. First, expression of *c-Ret* in the gut of mammalian embryos is restricted to NC-derived cells, in which it functions cell-autonomously (Avantaggiato et al., 1994; Durbec et al., 1996b; Natarajan et al., 1999; Pachnis et al., 1993; Young et al., 1998). Second, the arrangement of the TUNEL+ cells in the foregut of mutant (*Ret.k*$^{-}$*/Ret.k*$^{-}$) embryos (Fig. 9) is identical to that of migrating RET+ NC-derived cells in their wild-type counterparts (Pachnis et al., 1993). Third, the TUNEL+ cells were confined to the areas of foregut mesoderm that contain the NC-derived TH+ cells (Fig. 9) (Baetge and Gershon, 1989).

The RET+ cells immunoselected and studied here are likely to represent multipotential progenitors of the mammalian ENS. It has been shown previously that a fraction of RET+ cells isolated from the gut of E14.5 rat embryos is capable of generating, under clonal culture conditions, both neuronal and non-neuronal progeny (Lo and Anderson, 1995; Lo et al., 1997). We have extended these studies by using an organ culture system of mouse fetal gut in which the ENS develops under conditions that preserve the three-dimensional organisation of the bowel and thus maintain normal interactions between its cell populations. Grafting of a small number or single RET+ cells purified from the gut of E11.5 mouse embryos into the gut wall in organ culture resulted invariably in the generation of large numbers of progeny that had differentiated into neurons and glia (Natarajan et al., 1999). Finally, the RET+ cell population studied here is positive for nestin, a marker of neuroectoderm-derived precursor cells (D. Natarajan, unpublished observations). Based on this evidence, we suggest that GDNF and NTN are capable of acting on multipotential progenitors of the mammalian ENS to promote their survival, proliferation and differentiation.

The effects of GDNF and NTN on ENS progenitors is stage-specific. Both our study and that of Chalazonitis et al. (1998), indicate that two phenotypically defined populations of neural crest-derived cells of rat embryo gut (RET+ and $p75^{NTR}$+, respectively) responded to GDNF (both studies) and NTN (present study) when isolated from relatively early stages of embryogenesis (E12.5-13.5). However, phenotypically similar cell populations isolated from the bowel of later-stage embryos (E14.5-15.5) had a drastically reduced response to both neurotrophic factors (this study; Chalazonitis et al., 1998). Taken together, these findings suggest that the population of NC-derivatives responsive to GDNF and NTN is abundant during the early stages of ENS development but diminishes significantly during later stages. That these findings do not represent an artifact of the cell culture conditions is strongly suggested by similar findings obtained using the organ culture system we have described recently (Natarajan et al., 1999). Thus, despite the extensive proliferation of E11.5 RET+ enteric crest isolated from mouse embryos when introduced into the gut wall in organ culture, a similar population of enteric crest isolated from the gut of later stage embryos (E16.5) showed a dramatically reduced proliferative capacity and developmental potential (D. Natarajan and V. P., unpublished data). The stage-specific response of various neuronal lineages to neurotrophic factors is a well-established phenomenon (Davies, 1997). Our experiments do not provide any direct evidence that the reduction in responsiveness to GDNF and NTN by ENS progenitors and neurons is associated with a concomitant acquisition of competence to respond to other neurotrophic factors. However, NT-3, a neurotrophin that has no effect on 'early' RET+ ENS progenitors (this study; Chalazonitis et al., 1998), promotes the generation of neurons and glia in NC-derived cell cultures established from the gut of E14.5 rat embryos (Chalazonitis et al., 1998, 1994). This is consistent with expression studies indicating the presence of high levels of trkC mRNA (encoding the NT-3 receptor) in the ENS of later stage mammalian embryos (Tessarollo et al., 1993) and the increase in the relative abundance of TrkC-expressing enteric crest cells in response to GDNF in vitro (Chalazonitis et al., 1998). It is therefore likely that GDNF/NTN and NT-3 are components of two signalling systems that operate sequentially during embryogenesis to promote neurogenesis in

the mammalian gut. Although the mechanism(s) responsible for the reduction of GDNF and NTN responsiveness is currently unknown, it is unlikely that is related to downregulation of *c-Ret* expression. This suggestion is supported by the fact that both the responsive 'early' and the relatively unresponsive 'late' ENS cells studied here, were purified on the basis of expression of the RET RTK on their cell surface. Furthermore, expression studies have shown that high levels of c-Ret mRNA and protein are present on a large number of ENS cells throughout mammalian embryogenesis (Marcos and Pachnis, 1996; Martucciello et al., 1995; Pachnis et al., 1993; Tam et al., 1996; Young et al., 1998). It is therefore possible that loss of responsiveness to GDNF and NTN may be due to downregulation of the GPI-linked obligatory coreceptors or other critical components of the signal transduction machinery. The identification of the components of the RET signalling pathway that function as rate-limiting steps in the response of NC-derived cells to GDNF and NTN is of considerable interest and warrants further experimentation.

Whatever the molecular mechanisms underlying the stage-specific control of the response of ENS progenitors to GDNF and NTN, it is likely to have a critical role in the regulation of cell number and differentiation during development of the mammalian ENS. Using the products of several endogenous or reporter genes as molecular markers (RET, SOX10, MASH1, TH, PhOX2B, DBH-LacZ), we and others have identified a population of NC cells that invade the foregut mesenchyme and form the starting population of enteric NC, which subsequently generates the majority of the cells of the vertebrate ENS (Baetge and Gershon, 1989; Blaugrund et al., 1996; Durbec et al., 1996b; Kapur et al., 1992; Pattyn et al., 1997; Southard-Smith et al., 1998; Young et al., 1998). Given the staggeringly large number of cells present in the mature ENS of adult animals, it is obvious that this starting population of enteric crest cells needs to expand considerably in order to generate the necessary numbers of neuronal and glial progenitors that are capable of colonising successfully the entire bowel. This is likely to be controlled by gut mesenchyme-derived signals, which promote the proliferation of enteric crest cells and their continuous differentiation (Gershon, 1998; Le Douarin and Dulac, 1992). Data from this and other studies suggest that GDNF and NTN are likely to represent the factors that promote the initial expansion and subsequent differentiation of the enteric NC. Such a role is likely to be corroborated by other signalling systems. For example, it has been suggested that one of the functions of endothelin-3 (ET-3) and its receptor (EDNRB), are to prevent the early differentiation of enteric crest in order for the proliferative signals to have their maximum effect (Hearn et al., 1998). As the population of enteric crest expands and significant numbers of progenitor and differentiated cells accumulate in the gut wall (under the combined influence of GDNF, NTN and ET-3), the rate of cell number increase and differentiation must be reduced in order for the correct numbers of mature cell types to aggregate and form functional ganglia. Although this is likely to result partly from a reduction in the amounts of neurotrophic factors produced by the gut wall (such as GDNF), our data suggest that it is also dependent on the reduced responsiveness of ENS progenitors to the activation of the RET signalling pathway.

Various aspects of this model are consistent with genetic studies. Thus, several mutations have been described in the components of the RET signalling pathway loci which result in reduced signalling by the RET RTK (Angrist et al., 1996; Doray et al., 1998; Ivanchuk et al., 1996; Robertson et al., 1997; Wartiovaara et al., 1998). Such mutations often result in congenital megacolon, a condition characterised by the absence of enteric ganglia from varying lengths of the terminal colon. It is likely that reduced signalling by RET in response to GDNF or NTN results in inadequate expansion of the original enteric crest cell population, which is now unable to cope with the demanding task of colonising successfully the growing fetal bowel along its entire length.

The requirement of normal *c-Ret* function for the survival of the RET-dependent enteric NC precludes the study of the in vivo role(s) of the RET RTK during later stages of ENS histogenesis. However, the in vitro studies presented here (and similar studies from other groups) suggest that, in addition to promoting cell survival, RET is also likely to promote proliferation and differentiation of NC-derived cells in the gut of mammalian embryos. Also, our in vitro studies suggest that, in addition to GDNF, NTN is very likely to have a direct role(s) in ENS neurogenesis. Although the nature of the in vivo function of NTN in the development of the mammalian ENS is currently unclear, it is likely to be related the survival, proliferation or differentiation of ENS progenitors or other aspects of neurogenesis in the gut. To address these issues, further genetic, in vivo and in vitro studies are necessary.

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Note added in proof

While our manuscript was under review, two papers appeared which describe the phenotypes of mice deficient in NTN and its receptor GFRa-2 (Rossi et al. (1999) *Neuron* **22**, 243-253 and Heuckeroth et al. (1999) *Neuron* **22**, 253-263). The findings reported in these papers, together with our preliminary analysis of the CNS and parasympathetic ganglia phenotype reported by Marcos and Pachnis (1996) further support our conclusion that RET is required for signalling by NTN.

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