Development 129, 5151-5160 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1863

Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis

Dipa Natarajan, Camelia Marcos-Gutierrez*, Vassilis Pachnis‡ and Esther de Graaff†

Division of Molecular Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Present address: GlaxoSmithKline Biologicals, Rue de l'Institut 89, B-1330 Rixensart, Belgium
†Present addresses: Department of Clinical Genetics, Erasmus University, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
‡Author for correspondence (e-mail: vpachni@nimr.mrc.ac.uk)

Accepted 28 August 2002

SUMMARY

The majority of neurones and glia of the enteric nervous system (ENS) are derived from the vagal neural crest. Shortly after emigration from the neural tube, ENS progenitors invade the anterior foregut and, migrating in a rostrocaudal direction, colonise in an orderly fashion the rest of the foregut, the midgut and the hindgut. We provide evidence that activation of the receptor tyrosine kinase RET by glial cell line-derived neurotrophic factor (GDNF) is required for the directional migration of ENS progenitors towards and within the gut wall. We find that neural crest-derived cells present within foetal small intestine explants migrate towards an exogenous source of GDNF in a RET-dependent fashion. Consistent with an in vivo role of GDNF in the migration of ENS progenitors, we demonstrate that *Gdnf* is expressed at high levels in the gut of mouse embryos in a spatially and temporally regulated manner. Thus, during invasion of the foregut by vagal-derived neural crest cells, expression of *Gdnf* was restricted to the mesenchyme of the stomach, ahead of the invading NC cells. Twenty-four hours later and as the ENS progenitors were colonising the midgut, *Gdnf* expression was upregulated in a more posterior region – the caecum anlage. In further support of a role of endogenous GDNF in enteric neural crest cell migration, we find that in explant cultures GDNF produced by caecum is sufficient to attract NC cells residing in more anterior gut segments. In addition, two independently generated loss-of-function alleles of murine *Ret, Ret.k–* and *miRet*^{51}, result in characteristic defects of neural crest cell migration within the developing gut. Finally, we identify phosphatidylinositol-3 kinase and the mitogen-activated protein kinase signalling pathways as playing crucial roles in the migratory response of enteric neural crest cells to GDNF.

Key words: Neural crest, Cell migration, Enteric nervous system (ENS), Receptor tyrosine kinase (RTK), RET, Glial cell line-derived neurotrophic factor (GDNF), Mouse

INTRODUCTION

Neural crest (NC) cells are the prototypical migratory cells of vertebrate embryos. They emigrate from the dorsal neural tube and, by following stereotyped migratory routes, colonise specific embryonic sites where they generate a variety of cell types, including neurones and glia of the peripheral nervous system (PNS) (Le Douarin, 1999). The ENS, the subdivision of the PNS that controls the peristaltic and secretory activity of the gut wall, is derived mainly from the vagal NC at the level of somites 1-7 (Durbec et al., 1996; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). Shortly after delamination [embryonic day (E) 8.5-9.0 in the mouse], the progenitors of the ENS migrate ventrolaterally and colonise transiently the immediate postbranchial region ventrally to the dorsal aorta (Durbec et al., 1996). These pre-enteric NC cells (PENCCs) subsequently invade the foregut mesenchyme (called thereafter, enteric NC cells-ENCCs) and, migrating in a rostrocaudal direction, colonise the entire gut over a period of 4 days. Thus, by E10.5, ENCCs have reached the midgut loop, while by E11.5 they have colonised the entire small intestine up to the caecum, the characteristic swelling at the junction of small and large intestine. Finally, colonisation of the hindgut is completed by E13.5 (Durbec et al., 1996; Kapur et al., 1992; Young et al., 1998). As ENCCs migrate along the gut, a subset of them differentiates to neurones, as indicated by expression of neuronal markers and the outgrowth of axonal processes (Baetge and Gershon, 1989; Pham et al., 1991; Young et al., 1999). Although the correct migration of PENCCs and ENCCs is essential for the normal development and function of the ENS, the signals that control this process in vivo are largely unknown.

Several genes have been identified that play crucial roles in the development of the mammalian ENS. Among them is *Ret*, which encodes for the receptor tyrosine kinase (RTK) RET (Takahashi et al., 1988; Takahashi and Cooper, 1987). *Ret* is

induced in PENCCs when they first reach the dorsal aorta and continues to be expressed in ENCCs throughout their rostrocaudal migration within the foetal gut (Durbec et al., 1996; Pachnis et al., 1993; Tsuzuki et al., 1995). Mutations of *RET* in humans lead to absence of enteric ganglia from the distal colon (congenital megacolon, Hirschsprung's disease-HSCR) (Parisi and Kapur, 2000), whereas mice homozygous for a targeted mutation of *Ret* (*Ret.k–)* have complete intestinal aganglionosis (Durbec et al., 1996; Schuchardt et al., 1994). RET is the signalling component of multisubunit receptors for GDNF and the other members of its family, such as neurturin, artemin and persephin (Baloh et al., 2000; Saarma, 2000). The interaction between these signalling molecules and RET is mediated by glycosyl-phosphatidyl-inositol (GPI)-linked cell-surface glycoproteins, called GFRα1-4 (Airaksinen et al., 1999). A series of in vitro and in vivo studies has established a crucial role for GDNF, GFRα1 and RET for the survival and differentiation of enteric NC cells (Baloh et al., 2000; Taraviras and Pachnis, 1999). In addition, it has recently been reported that GDNF can function as a chemoattractant of ENS progenitors in vitro (Young et al., 2001). Despite these studies, the role of RET and its functional ligands in the migration of ENS progenitors and the patterning of enteric neurone processes in vivo remains unclear. We show that GDNF/RET signalling is required for the invasion of the foregut by PENCCs and the subsequent migration of ENCCs along the length of the bowel. These migratory processes are ultimately controlled by the spatial and temporal regulation of GDNF expression in the mesenchyme of the foetal gut.

MATERIALS AND METHODS

Animals

The generation of mice carrying the *Ret.k–*, *miRet*^{51} and *Gdnf-* alleles have been described previously (de Graaff et al., 2001; Moore et al., 1996; Schuchardt et al., 1994). Unless specific genotypes were a requirement, all explant cultures were performed with tissue dissected from Parkes (outbred) mouse embryos. The day of vaginal plug was considered to be E0.5.

Explant cultures

Explant cultures in collagen gel matrices have been described previously (Natarajan et al., 1999). Briefly, ~1-2 mm long segments from small intestine were dissected from appropriate stage embryos, placed in three-dimensional collagen gel matrices in four-well NUNC plates (Tessier-Lavigne et al., 1988) and cultured in a defined medium (optiMEM; Life Technologies UK) supplemented with L-glutamine (1 mM; Life Technologies UK) and antibiotic mixture (100 U/ml; Life Technologies UK) in an atmosphere of 5% CO_2. For the co-culture experiments, the distance between the intestinal segments and either COS-7 cells or caecum, was ~300 μm. Foetal gut conditioned medium (FGCM), GDNF and inhibitors were added to the culture medium. At the end of the culture period, explants were stained for Tuj1, PGP9.5 or RET.

To prepare FGCM, eight to ten guts were dissected from wild-type E13.5 mouse embryos (Parkes), washed twice with Ca^{2+}- and Mg^{2+}-free PBS and incubated with 1 mg/ml dispase/collagenase mixture (Sigma) for 15 minutes at room temperature. The tissue was washed twice with PBS, dissociated by pipetting and plated onto fibronectin-coated dishes (20 μg/ml) in OptiMEM. After 3 days, the cell culture medium was collected, centrifuged (84 ***g*** for 5 minutes) and filtered (0.2 μ filter). FGCM was used at a 1:1 dilution.

rGDNF (Promega) was used at 10 ng/ml. Phosphatidylinsolitol-3 Kinase [PI(3)K] inhibitor (LY294002, New England Biolabs) and MEK1 inhibitor (PD98059, New England Biolabs) were added to the co-cultures of small intestine explants with COS/GDNF cells at the indicated concentrations (Fig. 5B). To quantitate the migratory response of ENCCs to GDNF, explants were counterstained with DAPI and the nuclei present in the space between small intestine explants and COS cells were counted. Although we noticed a qualitatively similar response of enteric axons to GDNF, no attempt was made to quantitate this response.

The anti-GDNF blocking antibody (from R&D Systems) was used at a concentration of 10 μg/ml, as described previously (Hashino et al., 2001; Worley et al., 2000).

COS-GDNF cells were generated by transiently transfecting COS-7 cells (using Lipofectamine-Gibco BRL) with a mammalian expression vector (pcDNA3) in which a cDNA fragment encoding human GDNF (kindly provided by Dr M Saarma; Institute of Biotechnology, Helsinki) had been subcloned. COS (control) cells were generated by transfecting COS-7 cells with the empty vector. Small clumps of COS or COS/GDNF cells were formed by hanging drop cultures. More specifically, 24 hours after transfection, cells were collected using a cell scraper in 500 μl of complete medium. 30 μl droplets of the cell suspension were placed on the inside surface of the lid of a tissue culture plate and cultured overnight over complete medium.

In situ hybridisation

Whole-mount in situ hybridisation histochemistry was performed as previously described (Durbec et al., 1996). The riboprobe used for detecting *Ret* mRNA was generated from pmcRet7 (Durbec et al., 1996). To isolate cDNA clones for mouse *Gdnf*, we screened an E13.5 mouse embryo brain cDNA library under low stringency conditions using as a probe a 700 bp cDNA fragment that corresponds to the coding region of human *GDNF*. Several clones were identified, and a subset of them were mapped and sequenced. The cRNA probed used for the present experiments was derived from a 1.7 kb *Eco*RI fragment that corresponds to the 3′ untranslated region of the *Gdnf* mRNA (C. M.-G. and V. P., unpublished). The Sox10 cRNA was generated from a cDNA clone that was kindly provided by Dr M. Wegner (Institute of Biochemistry, Erlangen).

Immunostaining

For immunostaining, explants were fixed for 2 hours in 4% paraformaldehyde (made in 1×PBS) at 4°C. After washing twice in PBT (1×PBS+0.1% Triton X-100) they were incubated overnight (at 4°C) with blocking solution (PBT+ 1% BSA +0.15% glycine). Primary antibodies were diluted in blocking solution as follows: Tuj1 (mouse; BABCO) 1:1000, PGP9.5 (rabbit; Biogenesis) 1:400 and RET (goat; R&D Systems) 1:50. After washing several times with blocking solution, secondary antibodies [anti-mouse Alexa Fluor™ (Molecular Probes) 1:500; anti-rabbit FITC-conjugated (from Jackson Labs) 1:500; anti-goat FITC-conjugated (Jackson Labs) 1:500] were added in blocking solution for 6 hours at room temperature. After washing for three times with blocking solution explants were counterstained with DAPI and analysed in a Zeiss Axiophot microscope.

To detect apoptotic cells, explants were fixed in 4% paraformaldehyde, cryoprotected, embedded in OCT and sectioned at 12 μM. Sections were then processed for TUNEL staining according to the manufacturer's instructions.

RESULTS

GDNF is an attractant for PENCCs and ENCCs in vitro

To determine whether diffusible signalling molecules produced by the embryonic gut can influence the migration of ENCCs, we examined the effect of foetal gut-conditioned medium

(FGCM) on explants of proximal small intestine dissected from E11.0-11.5 mouse embryos and cultured for 24-36 hours in three-dimensional collagen gel matrices (Natarajan et al., 1999; Tessier-Lavigne et al., 1988). At the end of the culture period, the position of ENCCs and enteric neurones was assessed by immunostaining with antibodies to PGP9.5 or RET (Sidebotham et al., 2001), while the position of enteric axons was analysed mainly by staining for neurone-specific tubulin (TuJ1). In all control cultures (10/10), the vast majority of NC-derived cells and axons were confined to the explant tissue with only few entering the surrounding collagen matrix (Fig. 1A, parts a,b). By contrast, in the majority of explants cultured in the presence of 50% FGCM (8/10), a large number of ENCCs and enteric axons invaded the collagen matrix in a non-directional manner (Fig. 1A, parts c,d). This suggested that diffusible molecules produced by foetal gut mesenchyme can influence the migration of ENS progenitors and the axonal outgrowth of enteric neurones.

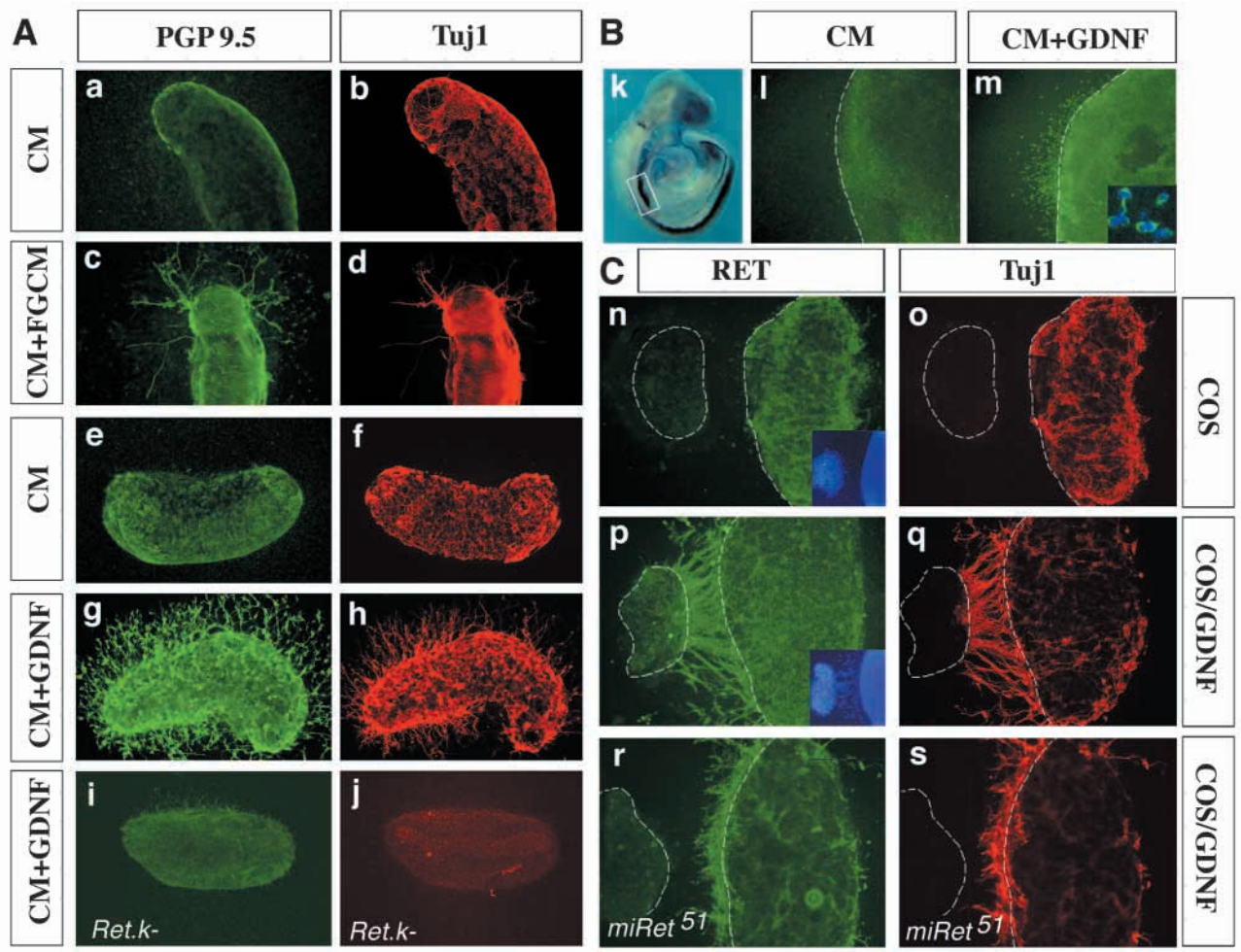

Fig. 1. Foetal gut-conditioned medium (FGCM) and GDNF promote invasion of collagen gels by ENCCs and enteric axons. (A) E11.0-11.5 proximal small intestine explants from wild-type (a-h) or *Ret.k*– (i,j) mouse embryos were cultured either in control medium (CM; a,b,e,f) or in the presence of 50% FGCM (c,d) or 10 ng/ml rGDNF (g-j) in three-dimensional collagen matrices. At the end of the culture period, explants were stained with PGP9.5 (a,c,e,g,i) and Tuj-1 (b,d,f,h,j) to visualise ENCCs and enteric axons. Both FGCM and rGDNF promote the invasion of collagen gels by NC-derived cells and axons (compare a,b with c,d and e,f with g,h). Explants derived from *Ret.k*– embryos are devoid of ENCCs or axons. (B) The PENCC-containing postbranchial region ventral to the dorsal aorta (boxed in part k; shows an E9.0 mouse embryo hybridised with a *Ret*-specific riboprobe) was dissected from wild-type E9.0 mouse embryos and cultured in control medium (CM; l) or in control medium supplemented with 10 ng/ml of rGDNF (CM+GDNF; m). At the end of the culture period, explants were stained for RET (green in l,m) and counterstained for DAPI (blue in inset of part m). rGDNF promotes the invasion of the collagen gel by explant-derived cells. The vast majority of emigrating cells express RET (inset in part m), indicating that they are of NC origin. Broken line indicates the boundary between the collagen gel matrix and the explant. (C) E11.0-11.5 proximal small intestine explants from wild-type (n-q) or *miRet*^{51} (r,s) embryos were co-cultured with either control COS-7 cells (COS; n,o) or COS-7 cells expressing GDNF (COS/GDNF; p-s). In every panel, the small intestine explant is on the right (left border highlighted by a broken line) and the COS-7 cells on the left (indicated by broken line). At the end of the culture period, explants were stained for RET (n,p,r) and Tuj1 (o,q,s) and counterstained with DAPI (inset in n,p). COS/GDNF cells induce cell and axonal migration always from the side of the explants facing the transfected cells (p,q). Insets in n,p show the area between explants and COS cells viewed with DAPI filter. The presence of a large number of nuclei specifically in the inset of p indicates that, in addition to axons, a large number of cells originating in the small intestine explant have invaded the collagen gel in response to GDNF. Explants from *Ret.k*^{51} homozygous embryos, cultured and processed in parallel to wild-type ones, showed reproducibly a weaker response.

Given the crucial role of GDNF and RET in the development of the mammalian ENS (Taraviras and Pachnis, 1999), we wished to examine whether purified GDNF could influence the migration of ENCCs. To test this, small intestine explants from E11.0-11.5 mouse embryos were cultured for 12-16 hours in the presence of recombinant (r) GDNF (10 ng/ml) and analysed as described before. As shown in Fig. 1A, parts e-h, the presence of rGDNF in the medium induces (*n*=37/40) profuse but non-directed migration of PGP9.5$^+$ cells and Tuj1-labelled axons away from the explant and into the collagen gel matrix. No PGP9.5- or Tuj1-positive cells or axons were detected in explant cultures (*n*=12) derived from homozygous *Ret.k–* embryos in which vagal NC fail to colonise the intestine (Fig. 1A, parts i,j), confirming that the responding cells were of NC origin. In addition to GDNF, neurturin (10 ng/ml) had a similar (albeit weaker) effect on cell and axonal migration from foetal small intestine explants (data not shown). Finally, netrin or endothelin 3 (ET3) failed to have any detectable effect on explants cultured under similar conditions (D. N. and V. P, unpublished; A. Barlow and V. P., unpublished).

To test whether GDNF can also induce a migratory response from pre-enteric ENS progenitors, the immediate postbranchial region containing RET-expressing PENCCs (boxed in Fig. 1B, part k), was dissected from E9.0-9.5 mouse embryos and cultured within collagen gel matrices in the absence or presence of rGDNF. In control medium, no RET+ cells left the postbranchial explant (*n*=4/4; Fig. 1B, part l). However, in the presence of 10 ng/ml of rGDNF, a large number of cells emigrated from the explant and invaded the collagen matrix (*n*=5/6; Fig. 1B, part m). Expression of *Ret* by the majority of responding cells indicates that they are of NC origin (inset in Fig. 1B, part m). Furthermore, no cell emigration was observed from explants derived from *Ret.k–* homozygous embryos (data not shown). These findings suggest that GDNF promotes the migration of ENS progenitors prior to their entry into the foregut.

To distinguish between a non-directional motogenic and a chemoattractive effect of GDNF, we examined the response of ENCCs to a localised source of GDNF. For this, E11.0-11.5 small intestine explants were co-cultured (for 12-16 hours) with a clump of COS-7 cells transfected with either a mammalian expression vector encoding GDNF (COS/GDNF cells) or the empty vector (COS cells). COS cells failed to induce cellular or axonal migration from such explants (*n*=40/40; Fig. 1C, parts n,o). By contrast, COS/GDNF cells promoted extensive ENCC and axonal emigration from the side of the explants facing the source of GDNF (*n*=55/55; Fig. 1C, parts p,q). The majority of cells and axons converged towards the GDNF-expressing COS cells irrespective of their orientation at the exit point (Fig. 1C, parts p,q).

We have previously described the generation (by targeted mutagenesis) of mice carrying a hypomorphic allele of *Ret*, which encodes only one of the RET isoforms (*miRet^{51}*) (de Graaff et al., 2001). Although newborn *miRet^{51}/miRet^{51}* animals have an apparently normal complement of enteric ganglia in the small intestine, they fail to develop enteric neurones in the colon (de Graaff et al., 2001). To examine whether the migratory response of ENCCs and enteric axons to GDNF depends on the signalling properties of RET, co-culture experiments were performed with small intestine explants derived from *miRet^{51}* homozygous embryos and either COS or COS/GDNF cells. Although the response of *miRet^{51}* explants was qualitatively similar to that of their wild-type counterparts (*n*=9/9), the number of cells and axons invading the collagen matrix and their distance from the explant over the same culture period were reduced (Fig. 1C, parts r,s; data not shown). Our studies so far extend those of Young et al. (Young et al., 2001) and suggest that GDNF can function as a chemoattractant that promotes the invasion of the foregut by PENCCs, the rostrocaudal migration of ENCCs and the outgrowth of enteric axons. Furthermore, our experiments indicate that these processes are dependent on the signalling properties of the RET RTK.

Stage and region specific expression of *Gdnf* in mouse foetal gut

To obtain further evidence for the role of GDNF as a chemoattractant of ENS progenitors in vivo, we used in situ hybridisation to analyse the expression of *Gdnf* in the gut of E8.5-13.5 mouse embryos. These stages encompass the entire migratory phase of PENCCs and ENCCs, and the early stages of enteric neurogenesis (Durbec et al., 1996; Kapur et al., 1992; Pham et al., 1991). In E8.5-9.5 embryos, *Gdnf* mRNA was expressed at high levels in the splachnic mesenchyme of the foregut (and particularly the stomach) and in the pharyngeal pouches (Fig. 2A,B,E). Twenty-four hours later (E10.5), the expression of *Gdnf* in the stomach appeared to be reduced, while a new well-defined domain of high expression was clearly evident more posteriorly, in the prospective caecum (Fig. 2; compare 2E with 2F). At this stage, relatively low level of *Gdnf* expression was detected throughout the small intestine. The appearance of the two domains of high *Gdnf* expression (i.e. stomach and caecum) preceded the arrival of RET-expressing NC cells at the corresponding regions. Thus, invasion of the foregut by PENCCs (white arrow in Fig. 2C) is taking place at E9.0-9.5 (at a stage when *Gdnf* is already expressed at high levels in the stomach anlage) (Fig. 2; compare 2B with 2C), while ENCCs arrive at the GDNF-expressing caecum at E11.0 (Fig. 2; compare 2F with 2G). These findings are consistent with a role of GDNF as an attractant of ENS progenitors. Furthermore, these data suggest that expression of *Gdnf* in the developing gastrointestinal tract is controlled independently of the invading ENS progenitors, an idea that is further supported by the normal pattern of *Gdnf* expression in the foregut of RET-deficient mouse embryos (Fig. 2D). During later stages of embryogenesis (E11.5-13.5), the posterior boundary of the caecal domain of *Gdnf* expression (arrow in Fig. 2H) extended progressively more caudally, until it spread throughout the entire length of the hindgut. This caudal extension of *Gdnf* expression coincided with the posterior migration of the front of ENCCs (arrow in Fig. 2I). At these stages, lower but easily detectable levels of GDNF mRNA were also present throughout the precaecal gastrointestinal tract.

Endogenous GDNF is likely to control the migration of ENS progenitors

To test whether GDNF produced by foetal gut can influence the migration of ENCCs, we co-cultured segments of proximal small intestine from E10.5 embryos (expressing relatively low levels of *Gdnf*), with caeca dissected from E11.0-11.5 mouse embryos (containing relatively high levels of GDNF). ENCCs

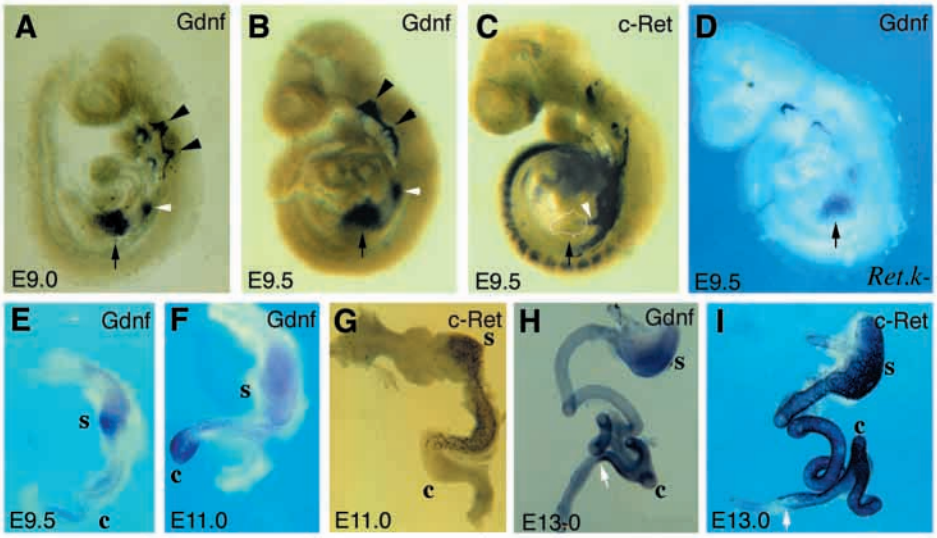

Fig. 2. Expression of *Gdnf* and *Ret* during mouse gut organogenesis. Wild-type (A-C), *Ret.k*– (D) embryos and gut preparations (E-I), were stained by whole-mount in situ hybridisation histochemistry using riboprobes specific for *Gdnf* (A,B,D,E,F,H) or *Ret* (C,G,I). In E9.0-9.5 embryos, *Gdnf* mRNA is detected in the splachnic mesenchyme of the stomach (indicated by arrow in all panels of the top row and by s in all panels of the bottom row) and in the branchial arches (black arrowheads in A,B). At this stage, RET-expressing ENS progenitors (white arrowhead in C) are starting to invade the GDNF-expressing region of the foregut (C; outline of the stomach is indicated by line and an arrow). (E) In the gastrointestinal tract of E9.5 embryos, expression of *Gdnf* is highest in the stomach region. However, in the gut of E10.5 embryos (F), the main site of *Gdnf* expression has shifted to the caecum (indicated by c). At this stage, the front of migrating NC cells was positioned rostrally to the high *Gdnf*-expressing caecum. At later stages, the domain of *Gdnf* expression in the hindgut extends posteriorly along with the wave of migrating NC cells (white arrows in H,I). Note the similar pattern of expression of *Gdnf* in RET-deficient mouse embryos (D). In addition to the gastrointestinal tract, *Gdnf* is also expressed in the pharyngeal pouches of the branchial arches (black arrowheads in A,B) and in a small region ventrally to the dorsal aortas (white arrowhead in A,B), the site where the progenitors of the superior cervical ganglia will first coalesce.

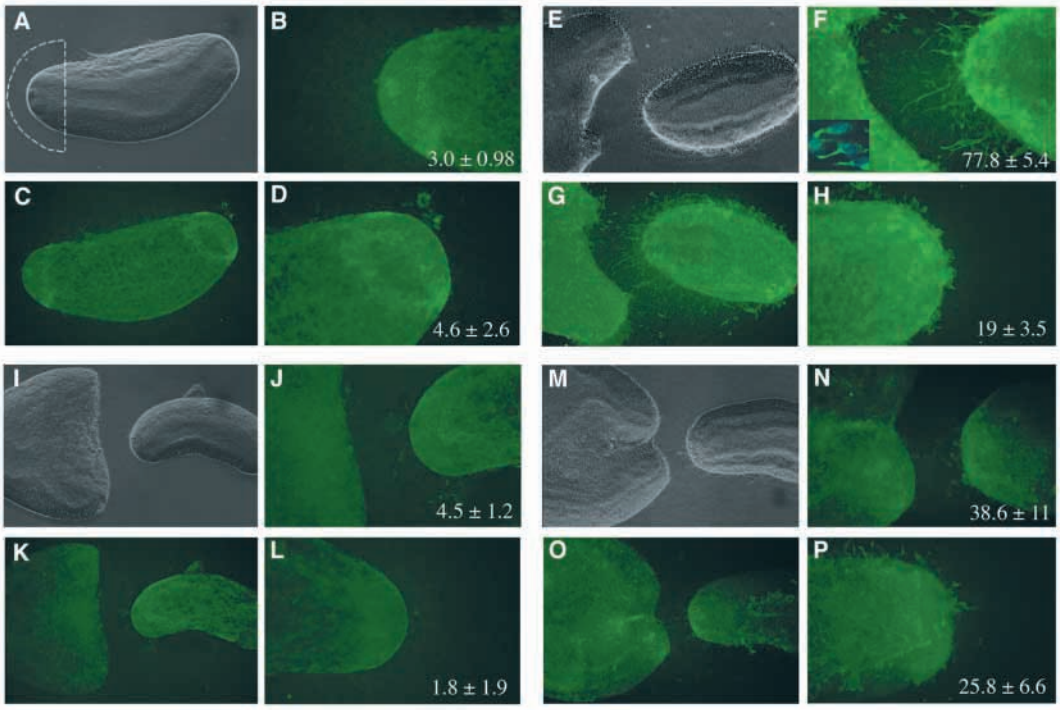

Fig. 3. Endogenous GDNF is a chemoattractant for ENC cells. Segments of small intestine from E10.5 wild-type embryos were cultured either alone (A-D) or with caecum from E11.0 wild-type (E-H,M-P) or GDNF-deficient (I-L) embryos. In M-P, explants were cultured in the presence of GDNF blocking antibodies. At the end of the culture period, explants were stained for RET and counterstained with DAPI. (A,E,I,M) Bright-field images of the explants; (C,G,K,O) corresponding fluorescent images; (B,D,F,H,J,L,N,P) higher magnification fluorescent images of the left (B,F,J,N) and right (D,H,L,P) ends of the explants. The position of the small intestine explants relative to the caecum was random and unrelated to the original rostrocaudal polarity of the tissue. To quantitate the effect of caecum under the various culture conditions, we counted the RET-expressing cells present outside the borders of the small intestine explant in the semicircles defined by the rounded ends of the explant (shown schematically by the broken line in A). The average number of cells recorded at each end of the small intestine explant is shown in the corresponding high magnification images. Note the large number of cells filling the space between small intestine and wild-type caecum cultured in control medium (F). The inset in this panel shows overlaid fluorescent images taken with the FITC and DAPI filters. The majority of cells emigrating from the small intestine explant express RET, indicating that they are of NC origin. The number of RET+ cells emigrating from small intestine towards GDNF-deficient caecum (J) or towards wild-type caecum in the presence of anti-GDNF blocking antibodies (N) is significantly reduced.

emigrating from the small intestine explants were identified at the end of the culture period by immunostaining for RET and counterstaining for DAPI. In small intestine explants cultured alone (n=26), a small but similar number of NC-derived cells emigrated from either end of the explant (Fig. 3A-D). However, in the case of co-culture experiments (n=38), an increased

number of RET-expressing cells emigrated from the small intestine explant. Interestingly, the majority of these cells emerged from the end of the small intestine closest to the caecum and filled the space between the two explants (Fig. 3E-H). To determine whether endogenous GDNF is responsible for the effect of caecum on ENCCs, similar co-culture experiments were performed with caeca isolated from mouse embryos homozygous for a null allele of *Gdnf* (*Gdnf*-) (Moore et al., 1996). As shown in Fig. 3I-L, GDNF-deficient caeca fail to induce cell emigration from wild-type small intestine explants (n=6/6). Finally, significantly reduced cell emigration was also observed from wild-type small intestine/caecum co-cultures in the presence of blocking anti-GDNF antibodies (n=13/13; Fig. 3M-P) (Hashino et al., 2001; Worley et al., 2000).

Mutations of *Ret* affect the rate of migration of ENS progenitors

Although reduced cell survival of PENCCs and ENCCs plays an important role in the generation of intestinal aganglionosis in RET-deficient mice (Taraviras et al., 1999), the contribution of a migratory defect to this phenotype has not been examined directly. To determine whether RET signalling is required in vivo for the invasion of the foregut by PENCCs, we compared the distribution of early ENS progenitors in the foregut of wild-type and RET-deficient (*Ret.k*–) E.9.5 mouse embryos using *Sox10* as an independent marker of early NC cells (Britsch et al., 2001; Southard-Smith et al., 1998). In addition to Sox10-expressing cells present in sensory and autonomic ganglia (arrows in Fig. 4A,B), a large number of positive ENS progenitors were detected in the postbranchial region (black arrowhead in Fig. 4A) and the foregut mesenchyme (white arrowhead in Fig. 4A) of wild-type embryos. By contrast, no Sox10+ cells were detected within the foregut of *Ret.k*– embryos, despite the presence of PENCCs in the postbranchial region (black arrowhead in Fig. 4B) and the apparently normal expression of *Sox10* in all other regions of the PNS (compare Fig. 4A with Fig. 4B).

We next compared the position of ENCCs within the gut of E10.5 wild-type and *miRet*^{51} homozygous embryos (de Graaff et al., 2001) using in situ hybridisation with Sox10-specific riboprobes. In wild-type embryos, the front of migrating ENS progenitors had reached the midgut loop (halfway between the caudal end of the pancreas and the caecum; black arrow in Fig. 4C). However, in similar stage *miRet*^{51} embryos, the front of migrating ENCCs was located more anteriorly, just beyond the pancreatic anlage (black arrow in Fig. 4D). In addition, we observed that the total number of Sox10-expressing cells was reduced in *miRet*^{51} homozygous embryos. Thus, normal RET signalling is likely to be necessary for the initial invasion and subsequent rostrocaudal migration of ENS progenitors within the gut of mouse embryos.

Mitogen activated protein (MAP) kinase and PI(3) kinase activation are required for the GDNF-induced migratory response of ENCCs

Previous studies have identified a number of intracellular signalling pathways that are activated upon binding of the GDNF/GFRα-1 complex to the extracellular domain of RET. Thus, activation of RET expressed by established cells lines and primary cell cultures, promotes cell survival and differentiation via activation of the MAP kinase and the PI(3) kinase pathways (Besset et al., 2000; Chen et al., 2001; De Vita et al., 2000; Hayashi et al., 2000; van Weering and Bos, 1998). To determine the relative roles of these intracellular signalling systems in ENCC and axonal migration in the gut during embryogenesis, small intestine explants and COS/GDNF cells were co-cultured (within collagen gel matrices) in the presence of PD98059 or LY294002, specific inhibitors of the MEK1 and PI(3) kinase, respectively (Atwal et al., 2000). Both inhibitors reduced the number of ENCCs and axons migrating towards the GDNF-producing COS cells (Fig. 5A, parts a-f) and their effect was additive (Fig. 5C). The effect of the inhibitors is unlikely to result from elimination of ENCCs as TUNEL staining of similar explants cultured in the presence of LY29002 (30 μM) or PD98059 (60 μM) failed to induce a

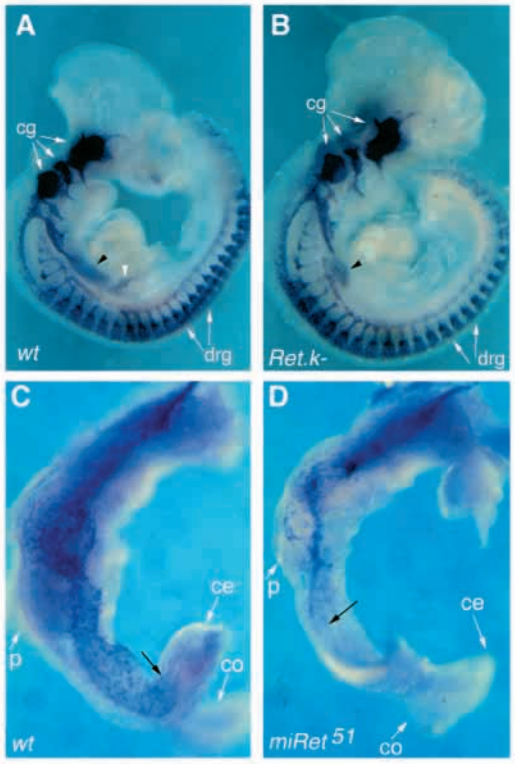

Fig. 4. Abnormal migration of ENS progenitor cells in embryos homozygous for mutant alleles of *Ret*. (A-D) E9.5 wild-type (A) and *Ret.k*– (B) embryos and guts dissected from E10.5 wild-type (C) and *miRet*^{51} (D) embryos were hybridised as whole-mount preparations with a Sox10 cRNA probe. In E9.5 wild-type embryos (A), Sox10+ ENS progenitors were detected in the postbranchial region (black arrowhead) and in the foregut (white arrowhead). By contrast, in similar stage *Ret.k*– embryos (shown in B), no Sox10+ cells were present in the foregut despite the presence of such cells in the immediate postbranchial region (black arrowhead). At this stage, both wild-type and mutant embryos express high levels of *Sox10* in cranial ganglia (cg) and in dorsal root ganglia (drg). In the gut of wild-type E10.5 embryos (C), the front of migrating Sox10+ cells (black arrow) has passed the midpoint of the midgut (halfway between the caudal region of the pancreas and the caecum). By contrast, in the gut of *miRet*^{51} homozygous embryos (D), the front of the migrating cells (black arrow) was located more rostrally, in the beginning of the midgut (black arrow). p, pancreas; ce, caecum; co, colon (hindgut).

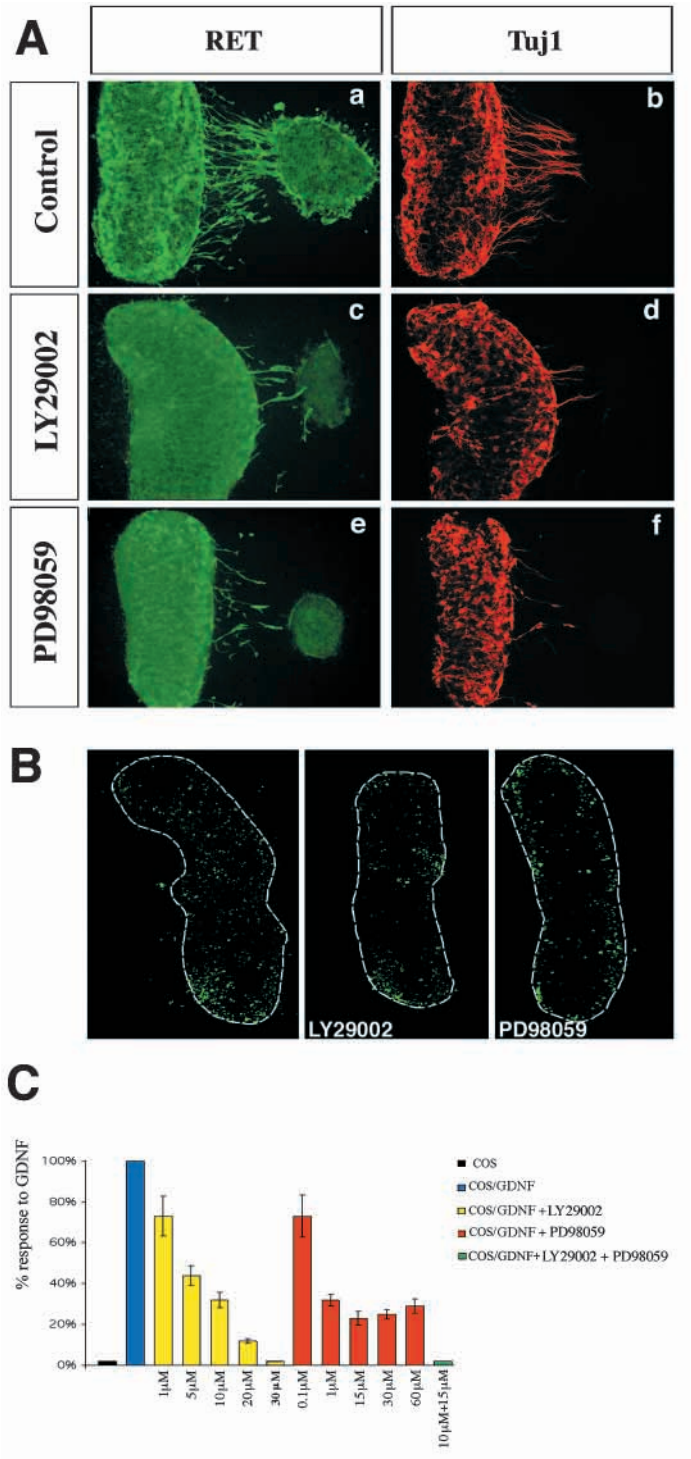

Fig. 5. Activation of the MAPK and PI(3)K signalling pathways is necessary for the chemoattractant response of ENCCs and enteric axons to GDNF. (A) Segments of proximal small intestine dissected from E11.0 mouse embryos were co-cultured with a clump of COS/GDNF cells (shown to the right of small intestine explants in a,c,e), in either control medium (a,b), or in medium supplemented with PI(3)K inhibitor (LY294002; c,d) or MEK1 inhibitor (PD98059; e,f). At the end of the experiment, cultures were stained for RET (a,c,e) and Tuj1 (b,d,f) and counterstained for DAPI. Reduced cell and axonal emigration were observed in the presence of inhibitors. (B) To examine whether the presence of the inhibitors resulted in increased apoptotic death, similar small intestine explants were cultured in control medium (left panel), or medium supplemented with 30 μM LY29002 (middle panel) or 60 μM PD98059 (right panel). At the end of the culture period, sections from the explants were stained for TUNEL to identify apoptotic cells. Note that under the present culture conditions, neither LY29002 nor PD98059 increases significantly the number of TUNEL+ nuclei. (C) To quantify the effect of increasing concentrations of PI(3)K and MEK1 inhibitors on ENCC migration, the number of RET-expressing cells present between the small intestine segment and COS/GDNF cells was counted. The response of explants cultured in control medium was considered as 100%. Increasing concentrations of PI(3)K inhibitor result in complete abrogation of the response. By contrast, a residual but significant response was observed even at high concentrations of MEK1 inhibitor. Nine explants were analysed for all concentrations tested for each inhibitor. Four explants were analysed in the experiments where the PI(3)K and MEK1 inhibitors were combined. The concentrations of LY294002 and PD98059 used for all explants shown in this figure were 10 μM and 30 μM, respectively.

significant change in the number of apoptotic nuclei (Fig. 5B and data not shown). Interestingly, increasing concentrations of the PI(3)K inhibitor LY294002 resulted in complete abrogation of the ENCC response to GDNF (Fig. 5C). By contrast, the response of ENCCs observed at low concentration (1 μM) of the MEK1 inhibitor PD98059 (approx. 30% relative to control), remained essentially unchanged even at 60-fold higher concentration (Fig. 5C). These findings suggest that activation of both the MAPK and PI(3)K signalling pathways in response to GDNF signalling is required for the normal migration of ENCCs and the correct patterning of enteric neurone projections. Furthermore, PI(3)K appears to have a crucial function in ENCC migration that cannot be compensated for by MAPK.

DISCUSSION

Correct guidance of NC cells and axons of peripheral neurones is essential for the normal development of the PNS and the connective and skeletal tissues of the head (Trainor and Krumlauf, 2000). Among the molecules that play an important role in NC cell migration and axonal pathfinding are members of the RTK superfamily. For example, the complementary expression of Eph receptors and their ephrin ligands provides repulsive signals that restrict NC cell migration to specific pathways within the mesenchyme of the head and the trunk (Wilkinson, 2001). In addition, activation of TrkB and TrkC (by the neurotrophins BDNF and NT-3, respectively) provides trophic activity that controls the stereotyped growth of the trigeminal sensory axons into the maxillary and mandibular processes (O'Connor and Tessier-Lavigne, 1999). Notwithstanding these studies, the potential role of RTK signalling in guiding NC cells to their destination sites in the embryo is usually masked by the effect of these molecules on cell survival. Indeed two, non-exclusive, models have been proposed to explain the mechanism(s) by which the final destination sites influence NC cell pathfinding and migration. The first model proposes that target areas release diffusible signalling molecules that chemoattract specific NC cells that happen to express the cognate RTK receptors. The second model suggests that RTKs are not implicated in the choice of

migratory pathways by NC cells, but instead mediate selective survival once the cells colonise sites that express the appropriate ligand(s) (Wehrle-Haller and Weston, 1997). By performing explant cultures and gene expression analysis in wild-type and mutant embryos over a broad range of developmental stages, we have shown that GDNF is likely to function as a chemoattractant of ENS progenitors in vivo. Such a role of GDNF is likely to depend on the spatial and temporal regulation of *Gdnf* expression along the developing bowel. We suggest that, at early stages of colonisation of the gut by NC cells, GDNF produced by the splachnic mesenchyme of the stomach attracts the RET-expressing PENCCs into the foregut; the ensuing upregulation of *Gdnf* in the caecum then maintains the migratory momentum of ENCCs and ensures the successful colonisation of the entire small intestine. Despite the evidence implicating GDNF in the migration of PENCCs and ENCCs, it is currently unclear whether the spatiotemporal distribution of GDNF mRNA is the sole determinant of the directional migration of ENS progenitors within the foetal gut. It is possible that additional NC-intrinsic mechanisms or gut mesenchyme-dependent signals could influence the rostrocaudal migration of ENS progenitors either directly or by modulating their response to GDNF gradients forming during gut organogenesis.

Our analysis of *Gdnf* expression is consistent with (and indeed extends) the studies of Young et al., who have previously reported high levels of GDNF mRNA in the caecum of E11.5-13.5 mouse embryos (Young et al., 2001). However, these authors did not report expression of *Gdnf* in the foregut, possibly because the earliest stage they analysed was E10.5. In addition, these authors suggested that the intense GDNF signal observed in the caecum, is likely to result from the increased thickness of this region of the gastrointestinal tract. Although after a certain point of embryogenesis, the mesenchymal walls of the caecum and (in particular) the stomach are thicker relative to other parts of the gut, we believe that the stronger GDNF signal observed in these regions is likely to reflect increased levels of *Gdnf* expression. This is based on the observation that the stomach- and caecum-specific upregulation of *Gdnf* is observed prior to these regions acquiring distinct anatomical characteristics that distinguish them from the rest of the gut (Fig. 2; D. N., C. M.-G., V. P. and E. d. G., unpublished). Indeed, high levels of GDNF mRNA are detected in the precursors of the splachnic mesenchyme of the stomach at a stage (E8.0-8.5) when they are still part of the lateral plate mesoderm (data not shown). In addition, analysis of sections of the foetal gut preparations shown in Fig. 2, indicates that mesenchymal cells in the stomach and caecum regions express higher levels of GDNF mRNA (data not shown).

The temporal and spatial distribution of GDNF in the developing gut and its capacity to function as a chemoattractant could provide a mechanism for the ordered colonisation of the oesophagus, stomach and small intestine. However, it is unclear whether a similar mechanism is responsible for the colonisation of the postcaecal region of the gut; our studies so far have failed to identify additional regions of GDNF expression that could function as chemoattraction centres for the colonisation of the hindgut. Instead, it appears that during hindgut colonisation by ENS progenitors, the caecal domain of GDNF expression extends posteriorly alongside the front of migrating cells. This suggests a permissive, rather than an instructive role for GDNF in the postcaecal region of the gastrointestinal tract. It is therefore likely that the mechanisms underlying the colonisation of the hindgut by ENS progenitors appear to be distinct from those operating in the fore- and midgut.

Activation of RTKs by their cognate ligands is likely to represent a general mechanism adopted by vertebrate embryos for the guidance of NC cells to their final destination sites. In addition to the evidence presented here, analysis of mice homozygous for a targeted mutation in neurofibromin (*Nf1*) has suggested that activation of the KIT RTK by stem-cell factor (SCF or mast cell growth factor-MGF) is required for the directed migration of melanocyte precursors in the lateral migratory pathway (Wehrle-Haller et al., 2001). It is likely therefore, that expression of distinct RTKs by specific groups of migrating NC cells and the complementary expression of their ligand(s) at the target site, creates a 'chemoattraction code' that is used to allocate subpopulations of NC cells to various locations within vertebrate embryos.

Phenotypic analysis of mice homozygous for *miRet*^{51} has shown that these animals have an apparently normal complement of enteric ganglia in the foregut and small intestine but lack enteric neurones and glia from the majority of the colon (de Graaff et al., 2001). This situation mimics the phenotype of congenital megacolon (HSCR) encountered in individuals with loss-of-function mutations of *RET* (Parisi and Kapur, 2000). The restriction of aganglionosis in the terminal part of the gut could suggest that full activity of RET signalling is required only during colonisation of the hindgut, and that hypomorphic alleles of *Ret* do not affect the pattern of migration of ENCCs in the foregut and midgut. However, our analysis of *miRet*^{51} homozygous embryos (Fig. 1C, parts r,s; Fig. 4C,D) has suggested that both the rate of migration and the number of ENCCs is already significantly reduced at E10.5 (when the midgut has only been partially colonised), suggesting that normal levels of RET signalling are required throughout the colonisation of the mammalian gut. In light of this, it is remarkable that newborn *miRet*^{51} animals appear to have normal enteric plexi in the foregut and small intestine, indicating a certain degree of recovery at later stages of embryogenesis. However, such compensatory mechanisms fail to restore a normal ENS in the colon of *miRet*^{51} animals, providing further support for the idea that the successful colonisation of the gut by ENS progenitors is determined by the interaction between NC-intrinsic mechanisms and region-specific mesenchymal signalling factors (de Graaff et al., 2001).

The normal patterning of enteric axonal projections is crucial for the coordinated peristaltic activity of the gut and the rostrocaudal movement of its contents (Gershon et al., 1994). Thus, rostrally projecting excitatory cholinergic neurones are responsible for contracting the musculature orally to a bolus, while inhibitory NOS-containing neurones project caudally and relax the musculature ahead of it (Brookes, 2001). So far, very little information is available on the mechanisms that control the growth and polarity of enteric axons and the extent to which such mechanisms are related to the directional migration of ENS progenitors within the foetal gut. However, the ability of GDNF to chemoattract neuronal processes present in the gut of E11.5 mouse embryos, suggests that at least a subpopulation of early enteric neurones is likely to respond to signals that are similar to those controlling the rostrocaudal migration of ENC cells. Supporting this

suggestion is the finding that the majority of axonal processes of the early-born catecholaminergic neurones present in the small intestine of E11.5 mouse embryos project caudally – towards the high GDNF expressing caecum. To what extent the polarity of enteric axons is controlled directly by GDNF or via the effect of this molecule on the migration of ENC cells is currently unclear.

Binding of GDNF to GFRalpha1 and RET results in the concerted activation of the MAP kinase and the PI(3) kinase intracellular signalling pathways which are key biological effectors of the receptor (Besset et al., 2000; Chen et al., 2001; De Vita et al., 2000; Hayashi et al., 2000; van Weering and Bos, 1998). Crucial for the activation of these signalling pathways is the recruitment of adaptor and signalling proteins at a multidocking site forming at tyrosine 1062 (Y^{1062}). In vitro and in vivo mutagenesis of Y^{1062} results in abrogation of the transforming activity of RET and failure of renal and ENS development during embryogenesis (Besset et al., 2000; Ishiguro et al., 1999; Segouffin-Cariou and Billaud, 2000) (S. Bogni, E. d. G. and V. P., unpublished). Using specific inhibitors of MAPK and PI(3)K, we observed that the normal response of RET-expressing ENS progenitors and enteric axons to a localised source of GDNF requires the activation of both biochemical pathways. The effect of these inhibitors is unlikely to be related to their potential role on cell survival. This suggestion is based on the relatively short duration of the assay (less than 12 hours) and the failure to observe during this period apoptotic cell death in explants cultured in the presence of inhibitors (D. N., C. M.-G., V. P. and E. d. G., unpublished). Despite the requirement of both signalling pathways for optimal response to GDNF, we find that activation of PI(3)K is the most crucial signalling event for the migration of ENS progenitors, and its role could not be compensated for by activation of other signalling pathways. This is based on the observation that efficient inhibition of the PI(3)K signalling pathway completely abolishes the response of ENCCs to GDNF, while a significant degree of chemotactic response was maintained even under conditions of efficient inhibition of the MAPK pathway. The mechanism by which PI(3)K activation results in asymmetric cytoskeletal rearrangements and migration are currently unknown. However, our findings are consistent with previous reports demonstrating that activation of the PI(3)K signalling pathway is necessary for lamellipodia formation by cultured cells as a response to GDNF (van Weering and Bos, 1997). Furthermore, our data are consistent with the essential role of PI(3)K signalling in the directional response of growth cones to chemotactic stimuli in vitro (Song and Poo, 2001).

Asymmetric expression of key guidance cues or the uneven activation of receptors on the cell surface, are necessary for the generation of directional cell migration. For example, in *Drosophila melanogaster*, homogeneous activation of RTKs by ligand overexpression or by mutations that render these receptors ligand independent and constitutively active, result in cell migration defects (Duchek and Rorth, 2001; Duchek et al., 2001). In that respect, it is interesting that germline mutations that lead to the constitutive activation of RET and the cancer syndrome multiple endocrine neoplasia type 2A (MEN2A), sometimes (15% of the cases) result in congenital megacolon (HSCR) (Decker et al., 1998; Mulligan et al., 1994). Although in some cases, HSCR could be due to the reduced expression or inefficient translocation of the mutant receptor to the cell membrane (Takahashi, 2001), our data provide an alternative explanation for the aganglionic phenotype encountered in a subset of MEN2A mutations. According to this view, the constitutive activation of RET on the cell surface of ENCCs prevents them from detecting or correctly interpreting the surrounding GDNF gradients, resulting occasionally in inefficient cell migration. Indeed, given our findings and those of Young et al. (Young et al., 2001), it is unclear why aganglionosis is not encountered more frequently in individuals with MEN2A patients. A potential explanation for this discrepancy is based on the capacity of GDNF to 'activate' MEN2A RET expressed on the cell surface (Mograbi et al., 2001) and thus generate asymmetric responses that are presumably sufficient for directional cell migration.

Several studies have established that members of the GDNF family of signalling molecules can promote survival and differentiation of various neuroectodermal derivatives, including the progenitors of the ENS (Baloh et al., 2000; Taraviras and Pachnis, 1999). The effect of GDNF and RET signalling in cell and axonal migration in vitro (Young et al., 2001) (this study) and in vivo (this study), adds to the spectrum of activities of *Ret* during development of the mammalian nervous system. Understanding the molecular mechanisms by which RET can regulate the activation of distinct but overlapping developmental processes that are crucial for mammalian ENS histogenesis, constitutes one of the main challenges in this field.

We thank M. Wegner and M. Saarma for probes and materials, Mary Dawson for animal husbandry and Dr Iris Salecker and all members of our laboratory for thoughtful comments on the manuscript. E. d. G. was a recipient of an EMBO fellowship (ALTF 260-1996). This work was also supported by the Medical Research Council and the NIH.

REFERENCES

Airaksinen, M. S., Titievsky, A. and Saarma, M. (1999). GDNF family neurotrophic factor signaling: four masters, one servant? *Mol. Cell. Neurosci.* **13**, 313-325.

Atwal, J. K., Massie, B., Miller, F. D. and Kaplan, D. R. (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron* **27**, 265-277.

Baetge, G. and Gershon, M. D. (1989). Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. *Dev. Biol.* **132**, 189-211.

Baloh, R. H., Enomoto, H., Johnson, E. M., Jr and Milbrandt, J. (2000). The GDNF family ligands and receptors – implications for neural development. *Curr. Opin. Neurobiol.* **10**, 103-110.

Besset, V., Scott, R. P. and Ibanez, C. F. (2000). Signaling complexes and protein-protein interactions involved in the activation of the ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. *J. Biol. Chem.* **275**, 39159-39166.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78.

Brookes, S. J. (2001). Classes of enteric nerve cells in the guinea-pig small intestine. *Anat. Rec.* **262**, 58-70.

Chen, Z., Chai, Y., Cao, L., Huang, A., Cui, R., Lu, C. and He, C. (2001). Glial cell line-derived neurotrophic factor promotes survival and induces differentiation through the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathway respectively in PC12 cells. *Neuroscience* **104**, 593-598.

de Graaff, E., Srinivas, S., Kilkenny, C., D'Agati, V., Mankoo, B. S., Costantini, F. and Pachnis, V. (2001). Differential activities of the RET

tyrosine kinase receptor isoforms during mammalian embryogenesis. *Genes Dev.* **15**, 2433-2444.

De Vita, G., Melillo, R. M., Carlomagno, F., Visconti, R., Castellone, M. D., Bellacosa, A., Billaud, M., Fusco, A., Tsichlis, P. N. and Santoro, M. (2000). Tyrosine 1062 of RET-MEN2A mediates activation of Akt (protein kinase B) and mitogen-activated protein kinase pathways leading to PC12 cell survival. *Cancer Res.* **60**, 3727-3731.

Decker, R. A., Peacock, M. L. and Watson, P. (1998). Hirschsprung disease in MEN 2A: increased spectrum of RET exon 10 genotypes and strong genotype-phenotype correlation. *Hum. Mol. Genet.* **7**, 129-134.

Duchek, P. and Rorth, P. (2001). Guidance of cell migration by EGF receptor signaling during Drosophila oogenesis. *Science* **291**, 131-133.

Duchek, P., Somogyi, K., Jekely, G., Beccari, S. and Rorth, P. (2001). Guidance of cell migration by the drosophila pdgf/vegf receptor. *Cell* **107**, 17-26.

Durbec, P. L., Larsson-Blomberg, L. B., Schuchardt, A., Costantini, F. and Pachnis, V. (1996). Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development* **122**, 349-358.

Gershon, M. D., Kirchgessner, A. L. and Wade, P. R. (1994). Functional anatomy of the enteric nervous system. In *Physiology of the Gastrointestinal Tract*, Vol. 1 (ed. L. R.Johnson), pp. 381-422. New York: Raven Press.

Hashino, E., Shero, M., Junghans, D., Rohrer, H., Milbrandt, J. and Johnson, E. M., Jr (2001). GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. *Development* **128**, 3773-3782.

Hayashi, H., Ichihara, M., Iwashita, T., Murakami, H., Shimono, Y., Kawai, K., Kurokawa, K., Murakumo, Y., Imai, T., Funahashi, H., Nakao, A. and Takahashi, M. (2000). Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor. *Oncogene* **19**, 4469-4475.

Ishiguro, Y., Iwashita, T., Murakami, H., Asai, N., Iida, K., Goto, H., Hayakawa, T. and Takahashi, M. (1999). The role of amino acids surrounding tyrosine 1062 in ret in specific binding of the shc phosphotyrosine-binding domain. *Endocrinology* **140**, 3992-3998.

Kapur, R. P., Yost, C. and Palmiter, R. D. (1992). A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice. *Development* **116**, 167-175.

Le Douarin, N. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge: Cambridge University Press.

Le Douarin, N. M. and Teillet, M. A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J. Embryol. Exp. Morphol.* **30**, 31-48.

Mograbi, B., Bocciardi, R., Bourget, I., Juhel, T., Farahi-Far, D., Romeo, G., Ceccherini, I. and Rossi, B. (2001). The sensitivity of activated Cys Ret mutants to glial cell line-derived neurotrophic factor is mandatory to rescue neuroectodermic cells from apoptosis. *Mol. Cell. Biol.* **21**, 6719-6730.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.

Mulligan, L. M., Eng, C., Attie, T., Lyonnet, S., Marsh, D. J., Hyland, V. J., Robinson, B. G., Frilling, A., Verellen-Dumoulin, C., Safar, A. et al. (1994). Diverse phenotypes associated with exon 10 mutations of the RET proto-oncogene. *Hum. Mol. Genet.* **3**, 2163-2167.

Natarajan, D., Grigoriou, M., Marcos-Gutierrez, C. V., Atkins, C. and Pachnis, V. (1999). Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture. *Development* **126**, 157-168.

O'Connor, R. and Tessier-Lavigne, M. (1999). Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. *Neuron* **24**, 165-178.

Pachnis, V., Mankoo, B. and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119**, 1005-1017.

Parisi, M. A. and Kapur, R. P. (2000). Genetics of Hirschsprung disease. *Curr. Opin. Pediatr.* **12**, 610-617.

Pham, T. D., Gershon, M. D. and Rothman, T. P. (1991). Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. *J. Comp. Neurol.* **314**, 789-798.

Saarma, M. (2000). GDNF – a stranger in the TGF-beta superfamily? *Eur. J. Biochem.* **267**, 6968-6971.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380-383.

Segouffin-Cariou, C. and Billaud, M. (2000). Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. *J. Biol. Chem.* **275**, 3568-3576.

Sidebotham, E. L., Woodward, M. N., Kenny, S. E., Lloyd, D. A., Vaillant, C. R. and Edgar, D. H. (2001). Assessment of protein gene product 9.5 as a marker of neural crest-derived precursor cells in the developing enteric nervous system. *Pediatr. Surg. Int.* **17**, 304-307.

Song, H. and Poo, M. (2001). The cell biology of neuronal navigation. *Nat. Cell Biol.* **3**, E81-E88.

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat. Genet.* **18**, 60-64.

Takahashi, M. (2001). The GDNF/RET signaling pathway and human diseases. *Cytokine Growth Factor Rev.* **12**, 361-373.

Takahashi, M. and Cooper, G. M. (1987). ret transforming gene encodes a fusion protein homologous to tyrosine kinases. *Mol. Cell. Biol.* **7**, 1378-1385.

Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. (1988). Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene* **3**, 571-578.

Taraviras, S. and Pachnis, V. (1999). Development of the mammalian enteric nervous system. *Curr. Opin. Genet. Dev.* **9**, 321-327.

Taraviras, S., Marcos-Gutierrez, C. V., Durbec, P., Jani, H., Grigoriou, M., Sukumaran, M., Wang, L. C., Hynes, M., Raisman, G. and Pachnis, V. (1999). Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development* **126**, 2785-2797.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G., Dodd, J. and Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775-778.

Trainor, P. A. and Krumlauf, R. (2000). Patterning the cranial neural crest: hindbrain segmentation and Hox gene plasticity. *Nat. Rev. Neurosci.* **1**, 116-124.

Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. and Asai, J. (1995). Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. *Oncogene* **10**, 191-198.

van Weering, D. H. and Bos, J. L. (1997). Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation. *J. Biol. Chem.* **272**, 249-254.

van Weering, D. H. and Bos, J. L. (1998). Signal transduction by the receptor tyrosine kinase Ret. *Recent Results Cancer Res.* **154**, 271-281.

Wehrle-Haller, B. and Weston, J. A. (1997). Receptor tyrosine kinase-dependent neural crest migration in response to differentially localized growth factors. *BioEssays* **19**, 337-345.

Wehrle-Haller, B., Meller, M. and Weston, J. A. (2001). Analysis of melanocyte precursors in Nf1 mutants reveals that MGF/KIT signaling promotes directed cell migration independent of its function in cell survival. *Dev. Biol.* **232**, 471-483.

Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat. Rev. Neurosci.* **2**, 155-164.

Worley, D. S., Pisano, J. M., Choi, E. D., Walus, L., Hession, C. A., Cate, R. L., Sanicola, M. and Birren, S. J. (2000). Developmental regulation of GDNF response and receptor expression in the enteric nervous system. *Development* **127**, 4383-4393.

Yntema, C. L. and Hammond, W. S. (1954). The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J. Comp. Neurol.* **101**, 515-542.

Young, H. M., Hearn, C. J., Ciampoli, D., Southwell, B. R., Brunet, J. and Newgreen, D. F. (1998). A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of phox2b, ret, and p75 and by explants grown under the kidney capsule or in organ culture. *Dev. Biol.* **202**, 67-84.

Young, H. M., Ciampoli, D., Hsuan, J. and Canty, A. J. (1999). Expression of Ret-, p75(NTR)-, Phox2a-, Phox2b-, and tyrosine hydroxylase-immunoreactivity by undifferentiated neural crest-derived cells and different classes of enteric neurons in the embryonic mouse gut. *Dev. Dyn.* **216**, 137-152.

Young, H. M., Hearn, C. J., Farlie, P. G., Canty, A. J., Thomas, P. Q. and Newgreen, D. F. (2001). GDNF Is a Chemoattractant for Enteric Neural Cells. *Dev. Biol.* **229**, 503-516.