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Common origin and developmental dependence on *c-ret* of subsets of enteric and sympathetic neuroblasts

Pascale L. Durbec[1], Lena B. Larsson-Blomberg[1], Anita Schuchardt[2,*], Frank Costantini[2] and Vassilis Pachnis[1,†]

[1]Division of Developmental Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[2]Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

*Present address: Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA
†Author for correspondence

SUMMARY

***c-ret* encodes a tyrosine kinase receptor that is necessary for normal development of the mammalian enteric nervous system. Germline mutations in *c-ret* lead to congenital megacolon in humans, while a loss-of-function allele (*ret.k*$^-$) causes intestinal aganglionosis in mice. Here we examine in detail the function of *c-ret* during neurogenesis, as well as the lineage relationships among cell populations in the enteric nervous system and the sympathetic nervous system that are dependent on *c-ret* function. We report that, while the intestine of newborn *ret.k*$^-$ mice is devoid of enteric ganglia, the esophagus and stomach are only partially affected; furthermore, the superior cervical ganglion is absent, while more posterior sympathetic ganglia and the adrenal medulla are unaffected. Analysis of mutant embryos shows that the superior cervical ganglion anlage is present at E10.5, but absent by E12.5, suggesting that *c-ret* is required for the survival or proliferation of sympathetic neuroblasts. In situ hybridization studies, as well as direct labelling of cells with DiI, indicate that a common pool of neural crest cells derived from the postotic hindbrain normally gives rise to most of the enteric nervous system and the superior cervical ganglion, and is uniquely dependent on *c-ret* function for normal development. We term this the sympathoenteric lineage. In contrast, a distinct sympathoadrenal lineage derived from trunk neural crest forms the more posterior sympathetic ganglia, and also contributes to the foregut enteric nervous system. Overall, our studies reveal previously unknown complexities of cell lineage and genetic control mechanisms in the developing mammalian peripheral nervous system.**

Key words: c-ret, receptor tyrosine kinase, enteric nervous system, superior cervical ganglion

INTRODUCTION

The enteric nervous system (ENS) is the most complex subdivision of the peripheral nervous system (PNS). It contains a large number of neurons organised in intricate neuronal circuits that control the movement, blood flow and secretions of the alimentary canal (Furness and Costa, 1987). The majority of the ENS precursors are derived from the vagal neural crest, which includes neural crest of cranial origin (postotic hindbrain corresponding to somites 1-5, anterior vagal crest), as well as neural crest of anterior trunk origin (corresponding to somites 6-7, posterior vagal crest) (Le Douarin and Teillet, 1973; Burke et al., 1995). Although the relative contributions of the anterior and posterior vagal neural crest to the development of the ENS have not been examined in detail, ablation of the entire vagal crest region in chick embryos leads to severe intestinal aganglionosis (Yntema and Hammond, 1954; Peters van der Sanden et al., 1993a). An additional source of neurons and glia of the avian and mammalian ENS is the sacral neural crest, which, originating posterior to somite 28, contributes to the formation of the postumbilical ENS (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Pomeranz et al., 1991; Pomeranz and Gershon, 1990; Serbedzija et al., 1991).

The ENS is closely related to the sympathetic nervous system. Phenotypic analysis of sympathetic and enteric neuroblasts in mammalian embryos has demonstrated that they coexpress several molecular markers. For example, in both the rat and mouse embryo, the migrating enteric neuroblasts express neurofilament, the low affinity neurotrophin receptor ($p75^{LNGFR}$) and enzymes of the catecholamine biosynthetic pathway (such as tyrosine hydroxylase-TH, dopamine-β-hydroxylase-DβH) as well as other markers of the sympathoadrenal lineage (Baetge et al., 1990; Baetge and Gershon, 1989). These findings led to the suggestion that the transiently catecholaminergic neuroblasts of the ENS are lineally related to the precursors of the sympathoadrenal (SA) lineage (Carnahan et al., 1991). However, the exact lineage relationship of the enteric and sympathetic precursors has not been firmly established in the vertebrate embryo.

Genetic studies have identified several loci and molecules that play a critical role in the development of the ENS. For example, spontaneous or targeted mutations of three loci,

lethal spotted (*ls*, encoding endothelin-3), *piebald lethal* (s^l, encoding endothelin receptor B) and *Dominant megacolon (Dom)*, lead to aganglionosis in the distal colon (Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994; Lane and Liu, 1984). We have recently reported that the *c-ret* proto-oncogene, encoding a member of the receptor tyrosine kinase (RTK) superfamily, is necessary for normal development of the mammalian ENS; targeted mutagenesis of the *c-ret* locus leads to absence of enteric ganglia from the intestine of newborn homozygous mice (Schuchardt et al., 1994). These findings are consistent with the identification of germline mutations in the human *c-RET* locus that lead to congenital megacolon, characterised by absence of enteric neurons from the distal part of the colon (Edery et al., 1994; Romeo et al., 1994). Although the role of the ret receptor in the development of the ENS has been firmly established, the stage at which the receptor functions, its mechanism of action and the particular groups of cells affected by mutations in the *c-ret* locus, remain unknown. Also, these studies did not address the potential effect of such mutations on other parts of the PNS that express high levels of c-ret mRNA (Pachnis et al, 1993).

Here we examine the role of the ret RTK during ENS neurogenesis and report that inactivation of *c-ret* prevents the successful colonization of the midgut and hindgut by enteric neuroblasts. However, the foregut ENS is only partially affected. Our analysis also shows that inactivation of *c-ret* eliminates the superior cervical ganglion (SCG), without affecting the other sympathetic ganglia or the adrenal medulla. Using in situ hybridization studies and lineage analysis, we demonstrate that the ret-dependent ENS and SCG precursors share a common origin from the neural crest of the postotic hindbrain, while the ret-independent foregut neurons are likely to be derived, along with the remainder of the sympathetic chain, from trunk level neural crest. Our findings suggest that two genetically distinct lineages contribute to the formation of the ENS and the sympathetic ganglia: a ret-dependent lineage that gives rise to the bulk of the ENS and the SCG and a ret-independent sympathoadrenal lineage that gives rise to the rest of the sympathetic ganglia and the foregut ENS.

MATERIALS AND METHODS

Animals

The *ret.k*$^-$ mutation was 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 morning of the vaginal plug was considered as E0.5.

Histology and immunostaining on sections

For histology, embryos were fixed in 4% paraformaldehyde (in PBS) at 4°C for 12-16 hours, dehydrated and embedded in paraffin. Sections (8 μm) were cut and stained with haematoxylin and eosin.

For the immunofluorescence experiments of Fig. 1, embryonic intestines were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 2 hours. Frozen 10 μm sections were mounted onto gelatin-coated slides and washed twice with PBS. The neurons and the glial cells in the enteric ganglia were identified with rabbit antibodies against peripherin (kindly provided by Dr Ronald

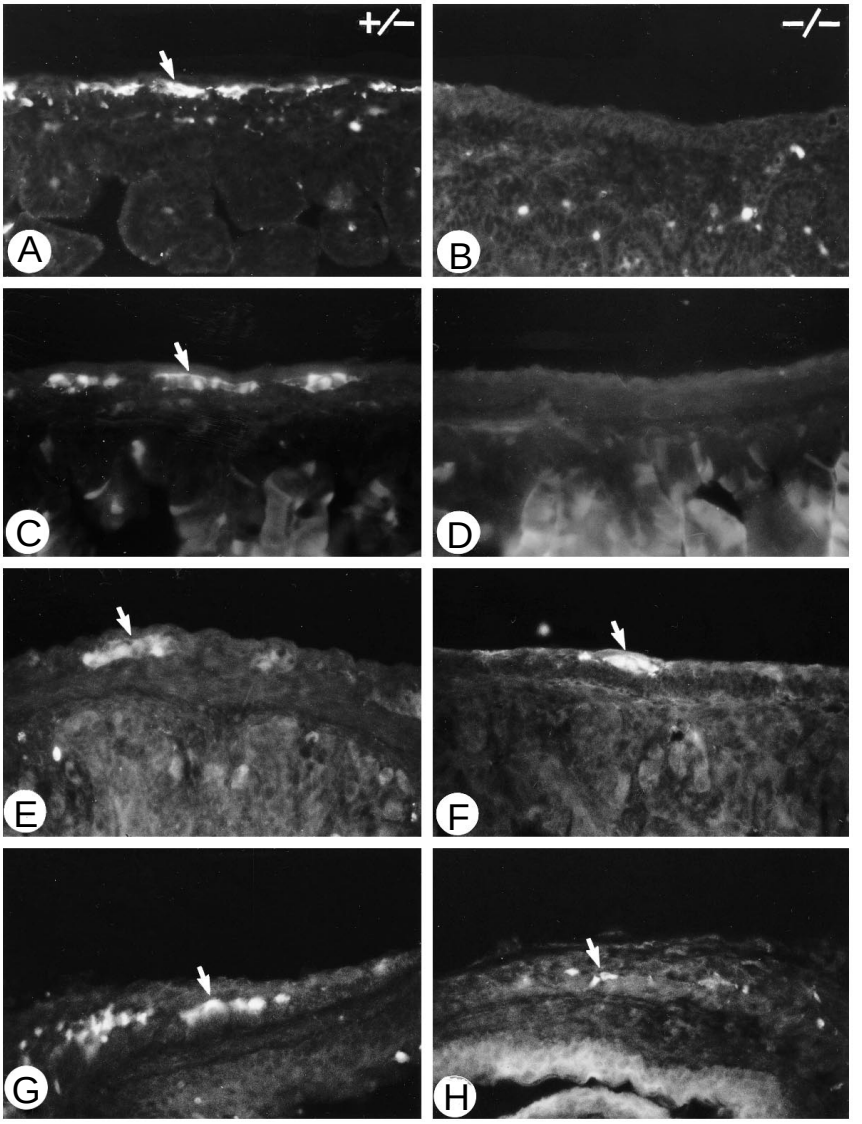

Fig. 1. Enteric ganglia fail to form in the intestine (midgut and hindgut), but are present in the foregut of homozygous *ret.k*$^-$ embryos. Immunofluorescence micrographs of sections from the gastrointestinal tract of heterozygous (+/–, on the left) and homozygous *ret.k*$^-$ mutant (–/–, on the right) embryos, immunostained with antibodies against peripherin (A,B,E,F) and GFAP (C,D,G,H). Sections A-D are from the small intestine of E15.5 (A,B), or E17.5 (C,D) embryos. Sections E-H are from the lower esophagus of E17.5 embryos. Enteric neurons and glia cells are present along the entire length of the gastrointestinal tract of heterozygous embryos (A,C,E,G). In homozygous *ret.k*$^-$ embryos, neurons and glia cells were absent from the intestine (B,D), but were present (albeit in smaller numbers) in the foregut (F,H).

Liem, Columbia University), neuron-specific enolase (NSE; Polysciences Inc.), S-100 (DAKOPATTS) and GFAP (Sigma Immunochemicals). Sections were incubated in primary antibodies overnight at 4°C. After washing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin (a gift from R. Morris) for 2 hours at 22°C, and finally washed twice with PBS. Sections were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. With each antibody, at least five heterozygous and five homozygous mutant embryos were analyzed.

For the immunohistochemistry experiments of Fig. 3, the sections were treated as described in the previous paragraph, except that horseradish peroxidase-conjugated secondary antibodies were used to visualise the signal.

Whole-mount immunohistochemistry

For whole-mount immunohistochemistry, embryos or tissues were dissected in PBS, fixed in 4% paraformaldehyde (in PBS) at 4°C for 1-2 hours. After two washes in PBS, they were incubated overnight in 0.1% H_2O_2 in PBT (PBS+1% triton X-100), washed again in PBT and incubated for several hours in PBT containing 2 mg/ml bovine serum albumin and 10% sheep serum. Incubation with anti-TH polyclonal antibodies (Affinity; 1:100) or anti-NF monoclonal antibody (Amersham; clone RPN 1104) was performed for 1-4 days at 4°C in PBT+10% sheep serum. After extensive washes in PBT, the tissue was incubated with the horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (DAKOPATTS) for 12-16 hours and colour was developed by incubating the tissue in DAB/H_2O_2.

Whole-mount in situ hybridization

The whole-mount in situ hybridization was performed essentially as described in Wilkinson (1992). For sections of the whole-mount preparations, embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C, washed three times in PBS and cleared in PBS-1% Triton X-100 for several hours. Embryos were then equilibrated with 30% sucrose in PBS, embedded in OCT and cryosections were cut at 25-30 μm.

Mouse embryo culture and DiI labelling of neural crest

Mouse embryo culture and DiI labelling of the postotic hindbrain neural crest were performed essentially as described in Beddington, (1994) and Serbedzija et al. (1992). Briefly, E8.5 mouse embryos (Parkes) were dissected (taking care not to damage the extraembryonic membranes) and collected in DMEM containing 10% fetal calf serum. Embryos containing 10-12 somites were used for the labelling of the vagal neural crest while embryos containing 12-15 somites were used for the labelling of the trunk neural crest. Labelling of the neural crest was achieved by applying a 0.05% solution of DiI in 0.3 M sucrose at the dorsal aspect of the neural tube at the appropriate anteroposterior level. Embryos were then transferred to fresh DMEM medium containing 50% rat serum and incubated in rolling bottles for 48 hours at 37°C. Embryos were maintained in 5% CO_2 and 5% O_2 during the first 24 hours, 20% O_2 during the next 12 hours and 40% O_2 during the last 12 hours. At the end of the incubation period, embryos were fixed in 4% paraformaldehyde (in PBS) at 4°C overnight. In the vagal neural crest labelling, embryos were embedded in OCT compound and cryosections were cut at 30 μm (Fig. 6A-C). In the trunk neural crest labelling, the foregut was dissected from fixed embryos and flat-mounted (Fig. 6F,G). Sections and flat-mount preparations were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. Control experiments using several molecular markers established that the enteric and autonomic nervous system of in vitro incubated embryos mature similarly to those developing in utero (P. D and V. P., unpublished observations). We obtained identical results with all eight embryos that we included in the vagal neural crest labelling and the seven embryos used for the trunk neural crest labelling, although the intensity of fluorescent labelling was variable.

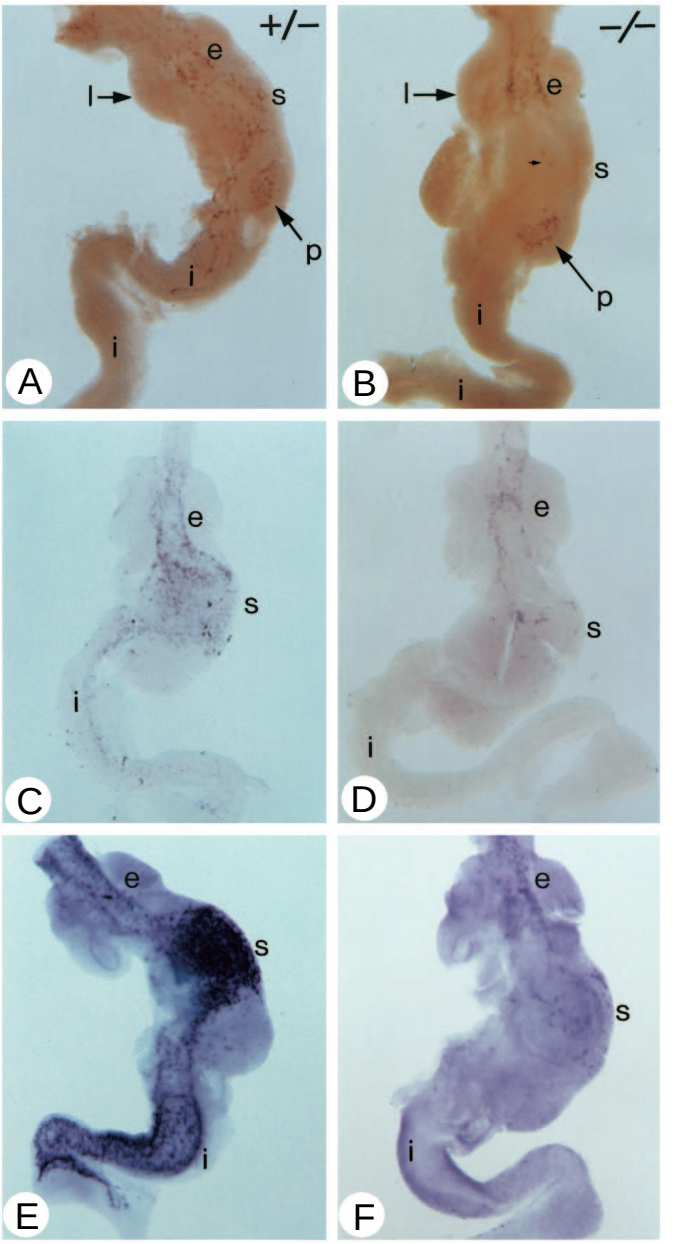

Fig. 2. Enteric neuroblasts fail to populate the midgut and hindgut of homozygous *ret.k*$^{-}$ embryos. Whole-mount preparations of the gut of E10.5 (A,B) or E11.5 (C-F) embryos, immunostained with a TH-specific antiserum (A,B), or hybridized with a MASH-1 (C,D) or c-ret cRNA probe (E,F). In heterozygous embryos (+/–, on the left), enteric neuroblasts have populated the rudiments of the esophagus (e), stomach (s) and intestine (i). In mutant embryos (–/–, on the right), enteric neuroblasts are present in the esophagus and to a smaller extent in the stomach, but are absent from the intestine. The small arrow in B indicates a small group of cells in the stomach of an E10.5 mutant embryo that express TH. (D,F) MASH-1- and c-ret-expressing cells are clearly evident in the stomach of E11.5 mutant embryos. p and l indicate the anlage of the pancreas and the lung respectively.

RESULTS

Effects of the *ret.k*⁻ mutation on the development of the ENS

To understand further the role of the ret RTK in the development of the ENS, we wished to determine the stage of ENS neurogenesis during which ret signalling is required. For this, the development of the ENS was studied in the gut of wild-type (+/+), heterozygous (+/*ret.k*⁻) and homozygous mutant (*ret.k*⁻/*ret.k*⁻) embryos with a variety of molecular markers. Initially, we examined the formation of enteric ganglia in the gastrointestinal tract of embryos between E13.5 and birth, using polyclonal antisera against neuronal markers, such as peripherin and neuron-specific enolase (NSE), and glial markers, such as glial fibrillary acidic protein (GFAP) and S-100 (Bishop et al., 1985; Troy et al., 1990). As expected, large numbers of neurons and glial cells were present along the entire length of the gastrointestinal tract of +/+ and +/*ret.k*⁻ embryos (Fig. 1). However, in *ret.k*⁻/*ret.k*⁻ embryos, both neurons and glia were absent from the distal stomach, duodenum, small and large intestine (Fig. 1B,D, and data not shown). Thus, the observed intestinal aganglionosis in *ret.k*⁻ mice could result from a defect of the early ENS progenitor(s), prior to its divergence into neuronal and glial cell phenotypes (Gershon and Rothman, 1991). Alternatively, normal *c-ret* function might be necessary for normal development of the neuronal progenitors of the ENS, which would in turn influence the differentiation of glial cells.

Despite the absence of enteric ganglia from the small and large intestine, a large (albeit reduced compared to the wild-type) number of cells expressing neuronal or glial markers were present in the esophagus and the proximal stomach of *ret.k*⁻/*ret.k*⁻ mice and embryos (Fig. 1F,H, and data not shown). These cells are likely to form a functional neuronal network controlling the peristaltic movements of the esophagus, as indicated by the presence of milk in the stomach of ret-deficient newborn animals (Schuchardt et al., 1994). Thus, unlike the intestinal ENS, a ret-independent lineage of neural crest cells contributes to the formation of the foregut ENS in mouse embryos.

During migration through the foregut and midgut of mammalian embryos (E9.5-11.5 in mouse embryos), at least a subset of ENS precursors express neuronal markers, such as enzymes of the catecholamine biosynthetic pathway (TH, DβH, etc) and neurofilament (NF) (Baetge et al., 1990; Baetge and Gershon, 1989), indicating their commitment to neuroblasts. We used this transient catecholaminergic phenotype to follow the ENS precursors in mutant *ret.k*⁻ embryos. Although TH and NF immunoreactive cells were present in the gut of all the progeny of +/*ret.k*⁻ intercrosses, their number and location was dependent on the genotype of the embryos. In E10.5 +/+ and +/*ret.k*⁻ embryos, a large number of TH- and NF-expressing cells had already populated the rudiments of the esophagus, stomach and the proximal loop of the intestine. However, in *ret.k*⁻/*ret.k*⁻ embryos of similar stage, TH- and NF-positive cells were restricted to the esophagus, with only very few immunoreactive cells in the stomach and none in the intestine (shown in Fig. 2A,B are the results with TH antibodies). Interestingly, at this stage, TH-immunoreactive cells were present in similar numbers in the pancreatic rudiment of both +/+ (or +/*ret.k*⁻) and *ret.k*⁻/*ret.k*⁻ embryos (p in Fig 2A,B). The catecholaminergic cells of the pancreatic anlage are thought to represent the precursors to the endocrine cells of the islets of Langerhans and the lack of effect of the *ret.k*⁻ mutation is consistent with (and indeed provides further support to) the suggestion that these cells are of endodermal (as opposed to neural crest) origin (Alpert et al., 1988; Le Douarin, 1988). A similar distribution of enteric neuroblasts in the gastrointestinal tract of *ret.k*⁻ embryos was observed with an independent marker of the migrating ENS precursors, MASH-1 mRNA (Lo et al., 1991). As shown in Fig. 2C,D, in E11.5 wild-type embryos, MASH-1-expressing cells had populated most of the bowel while, in homozygous mutant embryos of similar stage, MASH-1-positive cells were restricted to the esophagus and the proximal stomach.

Absence of TH-, NF- and MASH-1-expressing cells from the midgut and hindgut of mutant embryos could be due to inability of the mutant ENS progenitors to colonise these segments of the bowel or, alternatively, to a failure of the ret-deficient neural crest cells to differentiate into enteric neuroblasts. Since c-ret mRNA is an early marker of the autonomic neural crest (Pachnis et al., 1993), prior to the expression of TH, NF or MASH-1 mRNA (P. D. and V. P., unpublished observations), we wished to use transcripts from the *ret.k*⁻ locus in order to follow the ret-deficient enteric neural crest. Preliminary experiments indicated the feasibility of this experiment, since the *ret.k*⁻ mutation, although reducing the levels, did not alter the cellular specificity of the mutant transcripts relative to the wild-type c-ret mRNA ((Schuchardt et al., 1994) and P. D. and V. P., unpublished data). We therefore used riboprobes corresponding to the 5′ half of the c-ret mRNA in whole-mount in situ hybridization experiments to follow the enteric crest in homozygous mutant embryos. In agreement with the other markers used (anti-TH and anti-NF antibodies and MASH-1 mRNA), mutant enteric crest cells were detected in the esophagus and the proximal stomach, but were absent from the rest of the gastrointestinal tract of *ret.k*⁻/*ret.k*⁻ embryos (Fig. 2E,F). These results indicate that the ret-deficient neural crest cells, although capable of migrating into the foregut mesenchyme and differentiating into postmitotic neurons and glial cells, are unable to colonise successfully the wall of the mid- and hindgut.

Effects of the *ret.k*⁻ mutation on the development of the sympathetic ganglia

Given the suggested close relationship between the enteric and sympathetic neuroblasts (Carnahan et al., 1991) and the expression of c-ret mRNA in the SA lineage (Pachnis et al., 1993; Anderson, 1994), it was of interest to examine in detail the development of the sympathetic ganglia in ret-deficient embryos. Histological analysis and immunohistochemistry using anti-TH antibodies, revealed that, in homozygous *ret.k*⁻ embryos, the majority of the sympathetic chain, from the cervical stellate ganglion to the most caudal paravertebral ganglia as well as the adrenal medulla, are apparently normal (Fig. 3C,D). However, the SCG, the most anterior ganglion of the sympathetic chain, was reproducibly absent from late mutant embryos and newborn animals (Fig. 3A,B and data not shown). Using TH and NF as markers for sympathoblasts and postmitotic sympathetic neurons, we failed to detect the SCG in *ret.k*⁻ homozygous mutant embryos as early as E12.5 (Fig. 3 E,F and data not shown), while at this stage all other ganglia

of the sympathetic chain, including the stellate ganglia (Fig. 3G,H), had formed normally. Occasionally, the stellate ganglia of *ret.k⁻/ret.k⁻* embryos were larger compared to their +/+ or +/*ret.k⁻* counterparts (Fig. 3G,H).

The absence of the SCG in homozygous mutant embryos could be due to inability of the ret-deficient neural crest to form its anlage or, alternatively, to the elimination of the neuroblasts of the already formed SCG anlage. To distinguish between these possibilities, we compared the early stages of gangliogenesis of the sympathetic chain between +/+ or +/*ret.k⁻* and *ret.k⁻/ret.k⁻* embryos. The earliest stage during mouse embryogenesis at which the sympathetic ganglia can be identified as discrete cellular formations is at E10-10.5. At this stage, the sympathetic neuroblasts form a continuous group of cells extending posterior to the Xth cranial ganglion, along the dorsolateral aspect of the dorsal aorta (Fig. 4). Using TH and Phox-2 mRNA (Valarche et al., 1993) as sympathoblast markers in E10.5 embryos, no difference was detected in the formation of the sympathetic chain, between +/+ or +/*ret.k⁻* and *ret.k⁻/ret.k⁻* embryos (Fig. 4). This included the most anterior part of the sympathetic chain, which presumably gives rise to the SCG (Rubin, 1985). These experiments suggest that the *ret.k⁻* mutation does not prevent the migration or differentiation of the progenitors of the SCG, as indicated by the formation of the SCG anlage, although normal *c-ret* function is necessary for subsequent stages of gangliogenesis.

c-ret mRNA expression in the precursors of the ENS and the SCG

Lineage studies with avian embryos have indicated that, while the majority of the ENS precursors in vertebrates are derived from cranial neural crest (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973), the precursors of the sympathetic ganglia originate in the more posterior neural crest of the trunk (Le Douarin and Teillet, 1973; Le Douarin, 1986). More specifically, studies on rat embryos have suggested that the precursors of the SCG are derived from the thoracic neural crest, migrating first ventrally and then anteriorly along the dorsal aorta (Rubin, 1985). In light of these studies, the developmental dependence of the ENS and the SCG on the ret RTK would represent a parallel function of this molecule in two topographically distinct cell lineages. An alternative hypothesis, however, would be that normal *c-ret* function is necessary in a common cell lineage that gives rise to both the ENS and the SCG. To address this issue, we first used c-ret mRNA as a molecular marker to follow the precursors of the ENS and the SCG in whole-mount in situ hybridization experiments. As

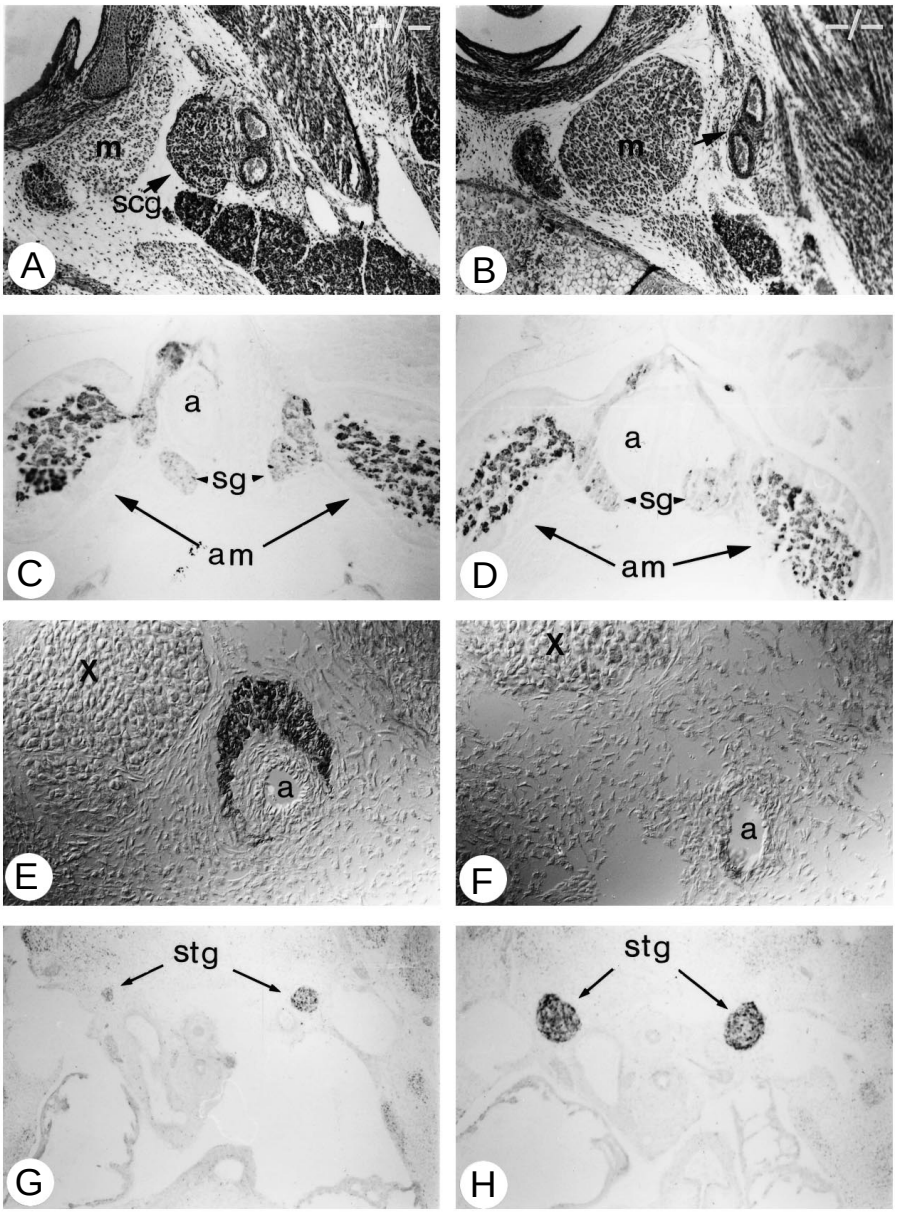

Fig. 3. Absence of the SCG from homozygous *ret.k⁻* embryos. (A,B) Transverse sections of E16.5 heterozygous (A) or homozygous mutant (B) embryos at the level of the bifurcation of the carotid artery. The SCG, present in heterozygous embryos, is absent from mutant embryos (arrows). (C,D) Transverse sections from E16.5 embryos at the level of the adrenal glands, stained with TH antisera. Both the paravertebral sympathetic ganglia (sg) in the vicinity of the dorsal aorta (a) and the adrenal medulla (am) are present in both heterozygous (C) and homozygous mutant (D) embryos. (E,F) Transverse sections of E12.5 embryos through the cervical level immunostained with anti-TH antibodies. Strongly immunoreactive SCG precursors were present around the dorsal aorta (a) of heterozygous embryos (E), while no TH-positive cells were observed in homozygous mutant (F) embryos. (G,H) The next major ganglia of the sympathetic chain, the stellate ganglia (stg), were present in both heterozygous (G) and homozygous mutant (H) embryos. The sections in E and F were viewed under Nomarski optics. m, muscle; X, nodose ganglion.

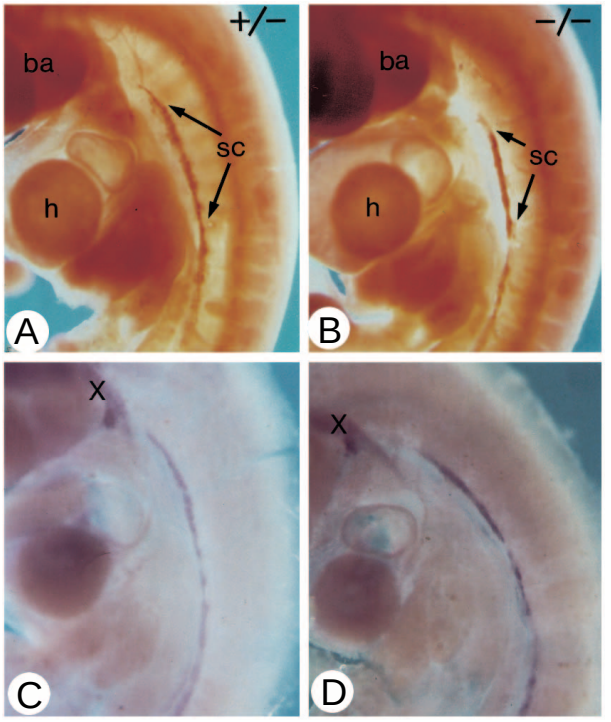

Fig. 4. The anlage of the sympathetic chain is indistinguishable between heterozygous and homozygous *ret.k*$^{-}$ embryos. In E10.5 mouse embryos, the sympathetic chain (sc), including at its most anterior end the anlage of the SCG, is a continuous group of cells extending caudally to the Xth cranial ganglion, along the dorsolateral aspect of the dorsal aorta. Heterozygous (A,C) and homozygous mutant (B,D) embryos were processed for whole-mount immunohistochemistry with anti-TH antibodies (A,B), or for whole-mount in situ hybridization with a Phox-2 cRNA probe (C,D). ba, branchial arches; h, heart; X, nodose ganglion.

shown in Fig. 5A, in wild-type E9.0 mouse embryos, a group of c-ret-positive cells is located ventral to and in close association with the cervical branches of dorsal aorta (arrowheads). During the subsequent 12 hours of embryogenesis, this population of ret-expressing cells expands and eventually splits into two subpopulations, a ventrally located one that invades the foregut mesenchyme (arrows in Fig. 5B,C,E) and a more dorsal group that remains in close contact with the ventral side of the dorsal aorta (arrowheads in Fig. 5B,C,E). It appears that these two subpopulations eventually migrate into their final destinations, i.e. the gut and the dorsal aspect of the cervical branches of the dorsal aorta, contributing to the formation of the ENS and the SCG respectively (Fig. 5D). These expression studies suggest that the precursors of the SCG and the ENS form transiently a common pool of c-ret-positive cells in the mesenchyme between the dorsal aorta and the foregut (Fig. 5E), and presumably share a common origin from the vagal neural crest, the main source of enteric neurons and glia in vertebrate embryos.

DiI labelling of the anterior vagal and trunk neural crest

To directly test this possibility, we used the fluorescent lineage tracer DiI, in conjunction with embryo culture, to trace the vagal neural crest of E8.5 mouse embryos (Serbedzija et al., 1992). Application of DiI to the dorsal aspect of the neural folds adjacent to the first four somites of an E8.5 embryo (10-

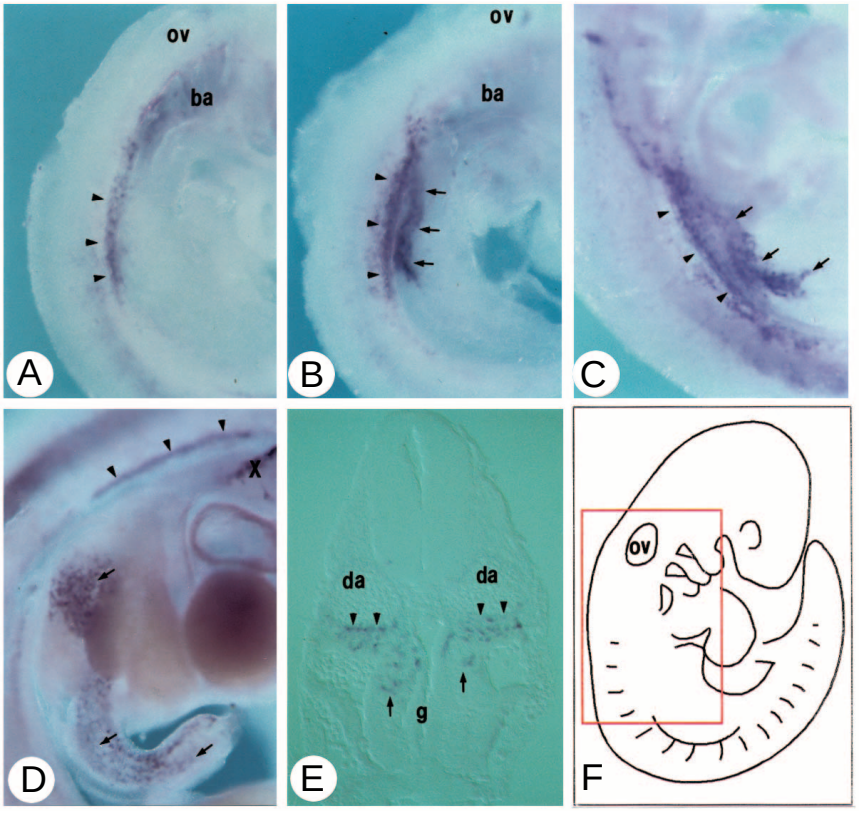

Fig. 5. Neural crest precursors of the anterior sympathetic ganglia and the ENS form a common pool of c-ret-expressing cells. Whole-mount in situ hybridization of E9.0-10.5 embryos with a c-ret cRNA probe. (F) A diagrammatic presentation of a mouse embryo at a stage close to the ones used in the experiments presented in this figure. The red box indicates the part of the embryo shown in more detail in A-D. In E9.0 embryos (A), a population of c-ret-positive cells (arrowheads), extends posterior to the branchial arches and ventrally to the dorsal aorta. In E9.5 embryos (B), the initially homogeneous population of c-ret-positive cells has split into two groups. One group (arrowheads) remains in close contact with the dorsal aorta, while the other (arrows) has migrated more ventrally, into the foregut mesenchyme. A section through this ret-positive group of cells is shown below in E. In E9.75 embryos (C), the relative position of the two c-ret subpopulations of cells remains unchanged, although the ventrally located cells (arrows) have migrated further into the foregut mesenchyme. In E10.5 embryos (D), the anlage of the SCG (arrowheads) has formed dorsally to the dorsal aorta, while the proximal loop of the gut has been filled with c-ret positive enteric neuroblasts (arrows). ba, branchial arches; da, dorsal aorta; g, gut; ov, otic vesicle.

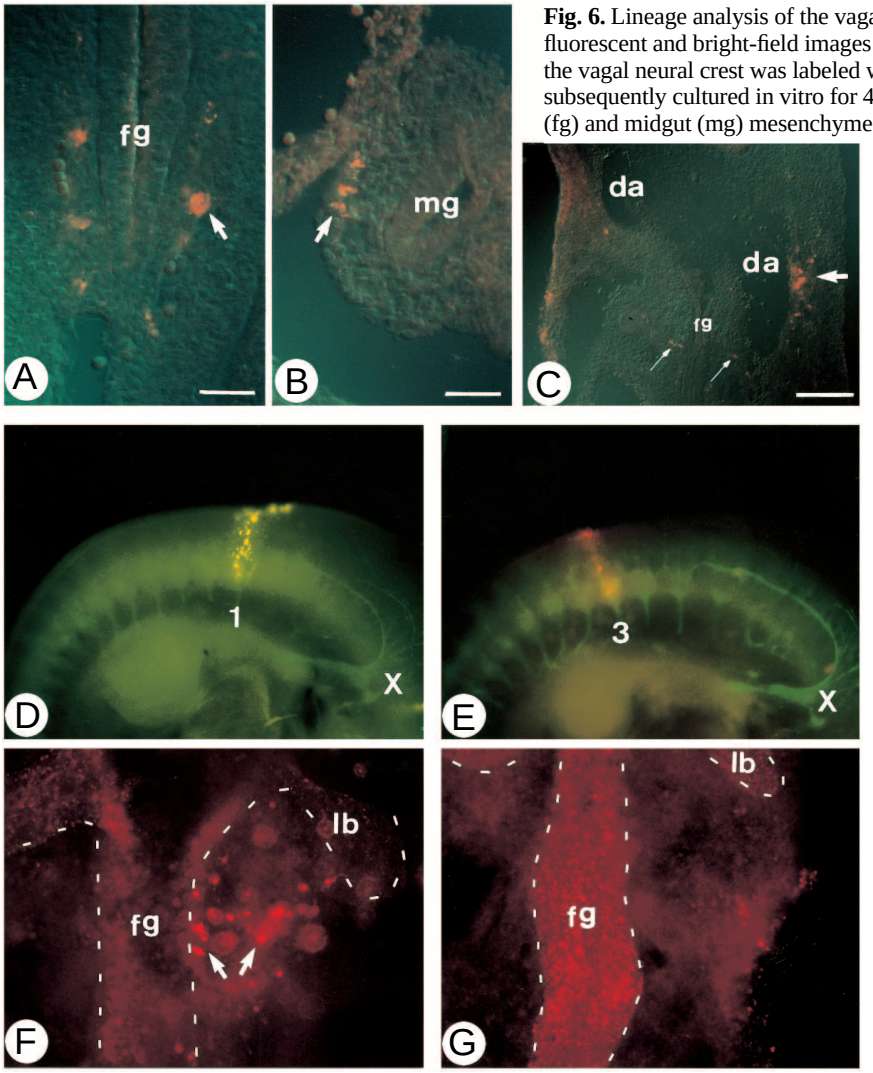

Fig. 6. Lineage analysis of the vagal and the trunk neural crest. (A-C) Superimposed fluorescent and bright-field images of transverse sections from a normal embryo of which the vagal neural crest was labeled with DiI at the 11-somite stage. This embryo was subsequently cultured in vitro for 48 hours. DiI-labeled cells were present in the foregut (fg) and midgut (mg) mesenchyme (arrows in A and B, respectively). (C) A group of fluorescent cells (thick arrow) is also present dorsolaterally to the cervical branches of the dorsal aorta (da). Thin arrows indicate the fluorescent cells in the foregut. (D,E) Fluorescent micrographs of whole-mount preparations of embryos that were labelled with DiI at the 15-somite stage and subsequently cultured for 48 hours. The neural crest of the embryo in D was labeled at the level of the 6th somite and its descendants populated the first dorsal root ganglion (1, yellow fluorescence), while the neural crest of the embryo in E was labeled at the level of the 8th somite and its descendants populated the third dorsal root ganglion (3, red fluorescence). Both embryos were also stained with anti-neurofilament antibodies (green fluorescence) which highlight the dorsal root ganglia (the first and the third are numbered in D and E, respectively) and the Xth cranial ganglion (X). (F,G), The foreguts of the embryos shown in D and E were dissected, flat-mounted and their fluorescent images are shown in F and G, respectively. The dotted line indicates the boundary between the endoderm of the foregut (fg) or the lung bud (lb) and the surrounding mesoderm. A large number of intensely fluorescent cells (arrows) are present in the mesoderm of the foregut of embryo that was labeled at the level of the 6th somite (F). No such cells are found in the mesoderm of the embryo that was labeled at the level of the 8th somite (G). We often observed that the cells of the foregut endoderm were fluorescent (as in G), most likely due to the fact that at the time of DiI application, they are directly in contact with the amniotic fluid which frequently contains "leaked" DiI. Scale bar in A,B, 87 μm; in C, 174 μm.

12 somites), resulted, 48 hours later, in fluorescent cells in the Xth (vagal) ganglion (not shown) and the foregut and midgut of the injected embryos (Fig. 6A,B), indicating that, as is the case for avian embryos, the mammalian postotic hindbrain is an important source of ENS precursors. More interestingly, a large number of intensely fluorescent cells was also present laterally to the anterior branches of the dorsal aorta (Fig. 6C), where the sympathoblasts for the SCG coalesce. No fluorescent cells were observed more posteriorly, along the common dorsal aorta, where the rest of the sympathetic chain ganglia form, consistent with several reports indicating that the majority of the sympathetic ganglia are derivatives of the trunk

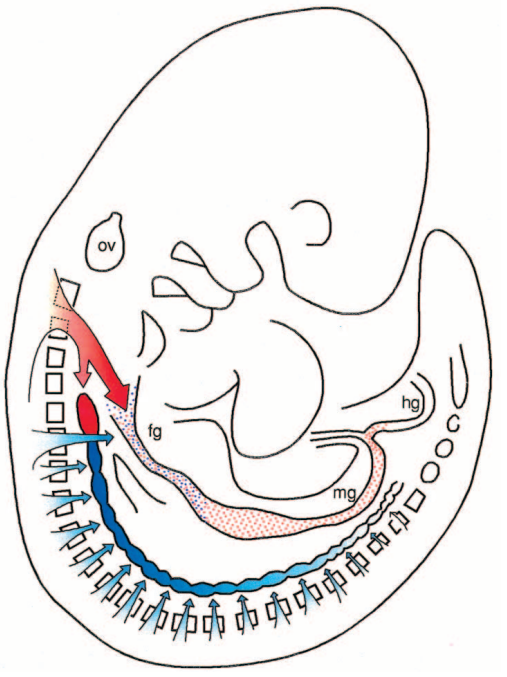

Fig. 7. Schematic presentation of the derivatives of the sympathoadrenal (SA) and the sympathoenteric (SE) lineages. The ret-dependent SE lineage (shown in red) originates in the vagal neural crest of the hindbrain (corresponding to somites 1-5) and migrates ventrally to populate the entire gut as well as the superior cervical ganglion (SCG). The ret-independent SE lineage (shown in blue), originates in the trunk neural crest (posterior to somite 6) and populates the foregut as well as the ganglia of the sympathetic chain posterior to the SCG. The foregut is therefore populated by both SE and SA derivatives.

neural crest (Le Douarin and Teillet, 1973; Le Douarin, 1986). Thus, our lineage studies confirm that (at least a subset of the) precursors of the ENS and SCG, share a common origin in the neural crest of the postotic hindbrain (anterior vagal crest).

As it appears that the sympathetic ganglia which are not affected by the *ret.k*$^-$ mutation are derivatives of the trunk neural crest, we reasoned that the enteric ganglia present in the foregut of *ret.k*$^-$*/ret.k*$^-$ embryos might also originate in the trunk neural crest. If this were the case, we would predict that, in wild-type embryos, a subpopulation of ENS progenitors originates in the neural crest of the trunk. To test this hypothesis directly, we used DiI to label in vitro neural crest cells emerging from the neuroepithelium that corresponds to the anterior trunk, and examined the gut for presence of fluorescent cells 48 hours later. Indeed, labelling of the neural crest at the level of somites 6-7, resulted reproducibly in the presence of fluorescent cells in dorsal root ganglia (characteristic trunk structures) and the foregut (Fig. 6D,F, and data not shown). However, labelling of the neural crest at the level of the 8th somite (which results in fluorescent labelling in the third dorsal root ganglion), or posterior, failed to label any cells in the gut of the cultured embryos (Fig. 6C,G, and data not shown). These findings indicate that, in mammalian embryos, a subset of the foregut ENS is derived from the anterior trunk neural crest.

DISCUSSION

The ret RTK is necessary for normal development of the mammalian ENS. Individuals heterozygous for loss-of-function mutations in the *c-RET* proto-oncogene develop congenital megacolon, due to absence of enteric ganglia from the terminal colon (Edery et al., 1994; Romeo et al., 1994), while mice homozygous for a targeted mutation of the *c-ret* locus *(ret.k*$^-$*)* die soon after birth and display intestinal aganglionosis and severe kidney dysplasia (Schuchardt et al., 1994). Here we characterise further the effects of the *ret.k*$^-$ mutation on the enteric and sympathetic nervous systems of embryos and newborn animals. In the ENS, we find that, despite the intestinal aganglionosis of homozygous *ret.k*$^-$ embryos, a subset of enteric ganglia of the foregut develop in the absence of a functional *ret* allele. Also, in the sympathetic chain, only the most anterior ganglia (the SCG) are dependent on *ret* function for their normal development. The phenotypic analysis of mutant embryos, combined with cell lineage studies on wild-type embryos, suggest strongly that the subset of enteric and sympathetic ganglia affected by the *ret.k*$^-$ mutation are derivatives of the anterior vagal neural crest, while those phenotypically similar structures not dependent on *ret* derive from the trunk neural crest. Overall, our studies reveal complexities of cell lineage and genetic control mechanisms in the developing mammalian PNS, which are not apparent from patterns of gene expression or other functional studies.

Despite the absence of enteric ganglia from the midgut and hindgut of *ret.k*$^-$ mice, a significant, albeit reduced, number of neurons and glial cells develop in the esophagus and the stomach of these animals, suggesting that only a subset of the neural crest forming the ganglia of the foregut require ret function. Also, our lineage studies indicate that, in mouse embryos, the ENS of the foregut has a dual origin in the anterior vagal neural crest of the posterior hindbrain, as well as the neural crest of the anterior trunk at the level of somites 6 and 7. Although at this point we cannot exclude the possibility that, in embryos homozygous for *ret.k*$^-$, a subset of surviving foregut ganglia are derivatives of the cranial neural crest, our studies strongly suggest that the ENS ganglia affected by this mutation originate in the anterior vagal neural crest, while neurons and glial cells that develop normally in the foregut of *ret.k*$^-$ embryos originate from the trunk neural crest. The effects of the *ret.k*$^-$ mutation on the development of the ENS are similar to the effects of surgical ablation of the vagal neural crest in chick embryos, which results in elimination of the mid- and hindgut ENS but has minimal effects on the development of the foregut ENS (Peters van der Sanden et al., 1993a). However, in these studies, the entire vagal crest region was ablated, including the anterior trunk neural crest which, according to our studies on mammalian embryos, contributes to the foregut ENS. It is possible that species-specific differences or partial regeneration of the neuroepithelium subsequent to surgical ablation (Scherson et al., 1993) could account for this apparent discrepancy.

In addition to its effects on the development of the ENS, the *ret.k*$^-$ mutation specifically eliminates the SCG, the most anterior ganglion of the sympathetic chain, without affecting the other more posteriorly located sympathetic ganglia. We have used gene expression and lineage studies to show that, in mammalian embryos, at least a subpopulation of the progenitors of the SCG is derived from the anterior vagal neural crest. Therefore, it appears that, in the developing sympathetic nervous system, as in the developing ENS, the *ret.k*$^-$ mutation affects primarily derivatives of the anterior vagal crest, while structures originating in the trunk neural crest, such as the sympathetic ganglia posterior to the SCG and the adrenal medulla, are independent of the ret signal transduction pathway.

The sympathetic ganglia and the adrenal medulla of the vertebrate embryo are derived from the sympathoadrenal (SA) lineage of the PNS. A common bipotential progenitor of this lineage, derived from the neural crest of the trunk, is capable of generating sympathetic neurons or adrenal chromaffin cells depending on the specific microenvironment into which it migrates (Anderson, 1993). Our experiments suggest that the SA progenitor is also capable of generating a subset of enteric ganglia in the mammalian foregut, and thus establish a close lineal relationship between the foregut ENS, the sympathetic ganglia posterior to the SCG and the adrenal chromaffin cells. However, our genetic, gene expression and lineage studies indicate that the remainder of the ENS (with the exception of the hindgut; see below) and the SCG constitute a unique lineage of the PNS, which we term sympathoenteric (SE) lineage. Despite the phenotypic similarities between the SA and SE progenitors and the generation of overlapping cellular phenotypes, the two lineages differ in several respects: the SA lineage is derived from the trunk neural crest, contributes to the majority of the sympathetic ganglia and a subset of cells of the foregut ENS and is independent of the ret RTK. In contrast, the SE lineage is derived from the anterior vagal neural crest, contributes to the formation of the ENS and the SCG and is dependent on the ret signal transduction pathway. We suggest that, by analogy to the SA lineage, a bipotential progenitor of the SE lineage can either migrate into the embryonic bowel and give rise to the complex cellular phenotypes of the ENS, or

remain in close association with the dorsal aorta and develop into the sympathetic neurons of the SCG (Fig. 7).

Studies in avian embryos are also consistent with the hypothesis of distinct SA and SE lineages: for example, trunk neural crest cells heterotopically transplanted into the vagal region can migrate into the bowel, but give rise to inappropriate cell types (i.e. melanoblasts) in the gut wall of the recipient embryos (Peters van der Sanden et al., 1993b). Furthermore, vagal neural crest in culture have greater propensity to generate "enteric" neuronal phenotypes, compared to similar cultures of trunk neural crest (Peters van der Sanden et al., 1993b; Newgreen et al., 1980). These studies, as well as our current studies, suggest that the developmental repertoire of the vagal and the trunk neural crest are to some extent distinct and independent of the target tissues that they populate. One possibility is that the differential genetic behaviour (i.e. the differential sensitivity to the *ret.k*$^-$ mutation) of these topographically distinct groups of cells is a consequence of their distinct sites of origin along the anteroposterior axis of the neuroepithelium, and the absolute requirement of *c-ret* by the SE lineage reflects a unique genetic programme bestowed by the posterior hindbrain. However, at this point, we cannot exclude the possibility that the differential responses of the SE and SA lineages to the *ret.k*$^-$ mutation are determined by the distinct microenvironments encountered during migration from their sites of origin to the foregut. One approach to distinguish between these two possibilities would be to examine the autonomic nervous system of chimaeric chick embryos, which have received as orthotopic or heterotopic transplants, vagal or trunk neural crest from homozygous *ret.k*$^-$ mouse embryos.

The effects of the *ret.k*$^-$ mutation, combined with expression of *c-ret* in the neuroblasts of the SE lineage suggests that the gene functions in a cell autonomous fashion to promote the generation of enteric or sympathetic neurons. Our experiments indicate that the normal *c-ret* function is required during the early stages of neurogenesis of the ENS and the SCG. In the case of the ENS, the effects of the *ret.k*$^-$ mutation are already evident prior to the coalescence of the enteric neuroblasts to form enteric ganglia (E10.5). Similarly, the SCG has already been eliminated by a stage (E12.5) at which it is normally composed of dividing neuroblasts (Hendry, 1977). Despite our better understanding of the stage of neurogenesis during which the *c-ret* function is required, the cellular functions of the ret receptor are at present not clear. However, the phenotypic analysis of the *ret.k*$^-$ mutant embryos presented here suggests that ret controls the survival or proliferation of the SE neuroblasts. Such a role is evident in the case of the SCG, since its anlage forms normally in E10.5 *ret.k*$^-$ mouse embryos, but is subsequently eliminated and is consistent with our analysis of the ENS phenotype. We suggest that lack of signalling by the ret receptor leads to inappropriate cell death or failure of the cells to divide after a certain stage of neurogenesis. Consistent with this suggested role of the *c-ret* proto-oncogene in the survival or proliferation of the neuroblast derivatives of the SE lineage, we have recently established that ret-deficient vagal SE progenitors in mutant embryos are capable of migrating into the bowel and the SCG anlage and differentiating into neuroblasts (P. D. and V. P., unpublished observations). The apparent role of the ret receptor in the survival or proliferation of the SE neuroblasts is analogous to that of the trk subfamily of RTKs, which promote the survival of various cells types of the PNS, including sympathetic neurons, upon activation by the cognate neurotrophins (Snider, 1994; Birren et al., 1993; Davies, 1994). This suggests that the (as yet unknown) ret ligand is a neurotrophic factor that plays a critical role during the early stages of neurogenesis of the ENS and SCG. Although our findings suggest a major role of ret signalling in the survival or proliferation of the SE lineage progenitors, we cannot exclude the possibility that the *ret.k*$^-$ mutation also affects other aspects of the development of the autonomic nervous system, such as migration or differentiation of neural crest precursors.

In addition to its cell autonomous effect on the derivatives of the SE lineage, it is likely that the *ret.k*$^-$ mutation also affects indirectly other lineages that contribute to the formation of the ENS. For example, in situ hybridization experiments on normal mouse embryos at various stages of development have failed to detect expression of c-ret mRNA in the sacral component of the hindgut ENS. Despite this, the vast majority of the enteric ganglia is eliminated from the hindgut of *ret.k*$^-$ embryos, with only a small number of neurons surviving near the anus (P. D. and V. P., unpublished data). Similar results have been reported from experiments in which the entire vagal neural crest region has been ablated in chicken embryos (Yntema and Hammond, 1954; Peters van der Sanden et al., 1993a). These findings suggest that an interaction between vagal and sacral neural crest derivatives is required for proper development of the ENS in the hindgut of vertebrate embryos.

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