Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 305 (2007) 287–299

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Effects of different regions of the developing gut on the migration of enteric neural crest-derived cells: A role for Sema3A, but not Sema3F

R.B. Anderson [a,*], A.J. Bergner [a], M. Taniguchi [b], H. Fujisawa [c], A. Forrai [d,1], L. Robb [d], H.M. Young [a,*]

[a] *Department of Anatomy and Cell Biology, University of Melbourne, 3010, VIC, Australia*

[b] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, Japan*

[c] *The 21st Century COE Program, Division of Biological Science, Nagoya University Graduate School of Science, Chikusa-ku, Nagoya 464-8602, Japan*

[d] *The Walter and Eliza Hall Institute of Medical Research, Parkville, 3050, VIC, Australia*

Received for publication 24 January 2007; revised 13 February 2007; accepted 13 February 2007
Available online 21 February 2007

Abstract

The enteric nervous system arises from vagal (caudal hindbrain) and sacral level neural crest-derived cells that migrate into and along the developing gut. Data from previous studies have suggested that (i) there may be gradients along the gut that induce the caudally directed migration of vagal enteric neural precursors (ENPs), (ii) exposure to the caecum might alter the migratory ability of vagal ENPs and (iii) Sema3A might regulate the entry into the hindgut of ENPs derived from sacral neural crest. Using co-cultures we show that there is no detectable gradient of chemoattractive molecules along the pre-caecal gut that specifically promotes the caudally directed migration of vagal ENPs, although vagal ENPs migrate faster caudally than rostrally along explants of hindgut. Exposure to the caecum did not alter the rate at which ENPs colonized explants of hindgut, but it did alter the ability of ENPs to colonize the midgut. The co-cultures also revealed that there is localized expression of a repulsive cue in the distal hindgut, which might delay the entry of sacral ENPs. We show that Sema3A is expressed by the hindgut mesenchyme and its receptor, neuropilin-1, is expressed by migrating ENPs. Furthermore, there is premature entry of sacral ENPs and extrinsic axons into the distal hindgut of fetal mice lacking Sema3A. These data show that Sema3A expressed by the distal hindgut regulates the entry of sacral ENPs and extrinsic axons into the hindgut. ENPs did not express neuropilin-2 and there was no detectable change in the timetable by which ENPs colonize the gut in mice lacking neuropilin-2.

Keywords: Neural crest; Migration; Gastrointestinal tract; Semaphorin 3A; Neuropilin-1; Semaphorin 3F; Neuropilin-2

Introduction

The enteric nervous system (ENS) is an extensive system of neurons and glial cells within the gut wall. In most gut regions, the ENS is capable of independently modulating or controlling many gut functions including motility and secretion, although the CNS can also influence these reflexes (Gershon, 2005; Furness, 2006). The vast majority of enteric neurons and glial cells arise from neural crest cells in the caudal hindbrain termed "vagal" neural crest cells, although sacral neural crest cells also contribute some neurons and glia, principally in the distal hindgut (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Kapur et al., 1992; Durbec et al., 1996; Burns and Le Douarin, 1998; Kapur, 2000; Burns, 2005; Anderson et al., 2006;). After emigrating from the hindbrain, vagal enteric neural precursors (ENPs) migrate into the foregut, and then along the gut, within the mesenchyme, to colonize the entire gastrointestinal tract (Baetge and Gershon, 1989; Kapur et al., 1992; Newgreen et al., 1996; Burns and Le Douarin, 1998; Young et al., 1998; Natarajan et al., 1999; Conner et al., 2003). In mice it takes over 4 days, or approximately 25% of the gestation period, for ENPs to colonize the entire gastrointestinal tract (Kapur, 1999; Young and Newgreen, 2001), and in humans the process takes approximately 3 weeks (Fu et al., 2003; Wallace and Burns, 2005). As the gut is elongating during the colonization process, ENPs are thought to migrate further than

* Corresponding authors. Fax: +613 9347 5219.
E-mail addresses: rba@unimelb.edu.au (R.B. Anderson), h.young@unimelb.edu.au (H.M. Young).
[1] Current address: Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2XY, UK.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.02.020

any other population of neural crest-derived cells (Newgreen et al., 1996; Newgreen and Young, 2002). During the developmental stages that ENP migration occurs, gut morphogenesis has commenced, and some parts of the gut have begun to adopt distinct regional identities.

Migration of ENPs through the gut is regulated by multiple factors, including the number of ENPs (Burns et al., 2000; Simpson et al., 2007), growth of the gut (Landman et al., 2003) and molecules expressed by the gut mesenchyme including glial cell line-derived neurotrophic factor (GDNF), endothelin-3 and netrins (Jiang et al., 2003). GDNF is expressed by the gut mesenchyme, and studies in vitro have shown that GDNF is chemoattractive to ENPs (Young et al., 2001; Natarajan et al., 2002; Iwashita et al., 2003). At the developmental stages at which ENPs are colonizing the small intestine and caecum of fetal mice, the expression of GDNF is maximal in the pre-caecal midgut and caecum (Natarajan et al., 2002). This has led to the suggestion that GDNF might promote the colonization of the small intestine by ENPs (Natarajan et al., 2002). The expression of GDNF in the hindgut is lower than that of the caecum (Natarajan et al., 2002), but it is unknown how this impacts on the migration of ENPs.

Endothelin-3 is expressed in the developing gut, and its receptor, endothelin receptor B (EDNRB), is expressed by migrating ENPs (Leibl et al., 1999; Lee et al., 2003). The expression of endothelin-3 in the developing gut is highest in the caecum than other gut regions (Leibl et al., 1999; Barlow et al., 2003). As endothelin-3 reduces the chemoattractive effects of GDNF in vitro, it has been proposed that exposure to the caecum might alter the migratory properties of ENPs (Barlow et al., 2003; Kruger et al., 2003). Furthermore, endothelin-3 also promotes the proliferation and inhibits the differentiation of ENPs, but does not appear to affect ENP survival (Hearn et al., 1998; Wu et al., 1999; Bondurand et al., 2006). As signaling via EDNRB receptors is only required for normal ENS development between E11.5 and E12.5, which is when ENPs are colonizing the caecum (Shin et al., 1999; Woodward et al., 2000), it has also been proposed that migration through the caecum is important for colonization of the hindgut by ENPs. However, it is currently unclear whether ENPs that have not been exposed to the caecum can colonize the hindgut as well as ENPs that have been exposed to the caecum.

ENPs derived from sacral neural crest cells migrate to the vicinity of the distal hindgut and then undergo a waiting period for several days and do not enter the distal hindgut until shortly before or shortly after vagal ENPs colonize this region (Burns and Le Douarin, 1998; Kapur, 2000; Druckenbrod and Epstein, 2005; Anderson et al., 2006; Nagy et al., 2006). Sacral ENPs are thought to migrate into the hindgut of chick embryos along the axons of extrinsic neurons (Burns and Le Douarin, 1998; Burns, 2005). In a number of regions of the developing nervous system where migrating cells or axons halt for a period of time before navigating to their correct target, it has been demonstrated that the waiting periods are mediated by the expression of repulsive cues (Watanabe et al., 2006; Renzi et al., 2000). In chick embryos, Sema3A is expressed by the mesenchyme of the distal large intestine, and neuropilin-1 is expressed by axons of neurons in the nerve of Remak, which is located parallel to, but outside, the gut in birds (Shepherd and Raper, 1999). The axons of neurons in Remak's nerve undergo a waiting period and do not penetrate the outer layers of the gut wall until the expression of Sema3A recedes to more internal layers, and *in vitro*, Sema3A is repulsive to the neurites of neurons in the nerve of Remak (Shepherd and Raper, 1999). It has therefore been proposed that Sema3A regulates the entry of intrinsic axons into the hindgut (Shepherd and Raper, 1999), although to date there are no *in vivo* data. As sacral ENPs accompany extrinsic axons into the hindgut (Burns and Le Douarin, 1998), it has also been suggested that Sema3A directly or indirectly, via its repulsive effect on extrinsic axons, regulates the entry of sacral ENPs into the hindgut (Shepherd and Raper, 1999; Young and Newgreen, 2001; Newgreen and Young, 2002; Burns, 2005; Burns and Thapar, 2006). Sema3A-neuropilin-1 signaling has not yet been shown to influence the migration of ENPs, although Sema3A has been shown to be repulsive to trunk and cranial neural crest cell populations in vitro (Eickholt et al., 1999) and to be essential for the correct migration of sympathetic neuron precursors (Kawasaki et al., 2002). Recent studies in vivo have shown that neuropilin-2 and its ligand, Sema3F, influence the migration pathways of some cranial and trunk neural crest cells (Osborne et al., 2005; Yu and Moens, 2005; Gammill et al., 2006a,b). Although Sema3F is expressed at high levels in the distal hindgut of rat embryos (Giger et al., 1998), the involvement of Sema3F-neuropilin-2 signaling in ENP migration has not previously been investigated.

In this study we tested a number of theories about the guidance of ENPs along the developing gut that have been proposed based on data from previous studies. The rate of migration of ENPs along explants of pre-caecal and post-caecal gut was compared to determine whether the migratory speed of ENPs differs in different regions of the gastrointestinal tract, and the abilities of ENPs derived from the pre-caecal gut or the caecum to migrate along explants of hindgut was also compared. To determine if there are gradients along the pre- and post-caecal gut that influence the migration of ENPs, we used co-cultures to compare the effect of direction of migration on the rate of migration of ENPs. Finally, we examined the expression of Sema3A and Sema3F and their receptors, neuropilin-1 and neuropilin-2, in the gut of fetal mice, and the entry of sacral ENPs into the distal hindgut in mice lacking members of the Sema3A or Sema3F signaling pathways.

Materials and methods

Animals

All mice were on a C57/BL6 background. Timed pregnant mice were killed by cervical dislocation and the embryos removed. The morning on which a copulatory plug was observed was designated E0.5. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Ret-TGM mice: *Ret*-TGM mice have had cDNA encoding tau-EGFP-myc (TGM) inserted into the first coding exon of the *Ret* gene (Enomoto et al., 2001) and all enteric neural crest-derived cells in the gut of $Ret^{TGM/+}$ embryos express GFP (Young et al., 2004). The genotype of adult Ret^{TGM} mice was determined by PCR using the primers and conditions reported previously (Enomoto et al.,

2001). Male mice heterozygous for the *Ret*-TGM allele ($Ret^{TGM/+}$ mice) were mated to wild-type (C57/BL6) or $Ret^{TGM/+}$ females. $Ret^{TGM/TGM}$ and $Ret^{TGM/+}$ embryos were identified by screening the stomachs for GFP^+ cells as described previously (Yan et al., 2004); $Ret^{TGM/TGM}$ mice have a small number of GFP^+ cells in the stomach, $Ret^{TGM/+}$ mice have abundant GFP^+ cells and $Ret^{+/+}$ mice have no GFP^+ cells.

DβH-nlacZ mice: ENPs in *DβH-nlacZ* mice express lacZ (Kapur et al., 1992). *DβH-nlacZ* mice were bred and genotyped as described previously (Stewart et al., 2003).

Sema3A null mutant mice: Sema3A mutant mice, generated by targeted gene disruption (Taniguchi et al., 1997), were examined.

Nrp2 null mutant mice: $Nrp2^{gt/+}$ mice were derived from a W9.5 ES cell line identified during a gene trap screen (Robb et al., 2001; Tarrant et al., 2002). Molecular cloning of the gene trap fusion transcript and mapping of the *Nrp2* genomic locus by Southern analysis localized the gene trap insertion site to intron 11. The insertion interrupted the *Nrp2* coding sequence at cDNA position 1786 (amino acid −Asp). Southern analysis was used to genotype offspring of heterozygous matings using genomic DNA digested with *Eco*RI and probed with a radiolabeled *Nrp2* cDNA fragment. Mice were crossed more than 6 generations onto the C57BL/6 background. Decreased overall body size and increased perinatal lethality was observed in $Nrp2^{gt/gt}$ mice. Staining for β-galactosidase and whole-mount immunohistochemistry with antineurofilament (2H3) antibody demonstrated that $Nrp2^{gt/gt}$ embryos phenocopied published targeted and gene trap mutations of the *Nrp2* locus (Chen et al., 2000; Giger et al., 2000; Takashima et al., 2002; Walz et al., 2002).

Co-cultures

Explants of gut were suspended across a "V" cut in a piece of filter paper (Hearn et al., 1999), and small segments of gut or connective tissue containing β-gal^+ or GFP^+ ENPs were placed in close contact with one or both ends of the suspended explant and cultured as described previously (Young et al., 2002).

Immunohistochemistry and histochemistry

Whole-mount or frozen sections were processed for immunohistochemistry as described previously (Young et al., 2002). The primary antisera used were 1:400 goat anti-GFP (Rockland, USA), 1:1000 rabbit anti-β-gal (Cappel-ICN, USA), 1:200 rabbit anti-neuropilin-1 (Kitsukawa et al., 1997), 1:250 rabbit anti-p75 (1:100, Promega) and 1:2000 human anti-Hu (Fairman et al., 1995). The secondary antisera were anti-sheep FITC (1:100, Jackson Immunoresearch, PA, USA), anti-human Texas red (1:100, Jackson Immunoresearch) or biotinylated donkey anti-rabbit, 1:100 (Jackson), followed by streptavidin-Cy-5, 1:100 (Amersham). X-gal histochemistry was performed as described previously (Stewart et al., 2003).

In situ hybridization

Whole-mount in situ hybridization of E11.5–E14.5 dissected gut tissue was performed as described previously (Young et al., 2001). Templates for Sema3A and Sema3F riboprobes were kindly provided by Dr. Andreas Puschel (Westfalische Wilhelms-Universitat, Germany). Some whole-mount in situ hybridization preparations were sectioned. After cryoprotection in 20% sucrose/phosphate buffer saline at 4 °C, gut tissue was embedded in OCT compound, and 16 μm sections were prepared using a cryostat.

Results

Effects of different gut regions and migration direction on ENP migration

To examine the ability of enteric neural precursors (ENPs) from different sources to colonize different gut regions and to migrate in different directions, co-cultures were established between segments of gut containing ENPs grown with suspended explants of aneural pre-caecal midgut or post-caecal gut (Young et al., 2002) (Fig. 1). In E11.5 mice, the migratory wave front of vagal ENPs is in the caecum (Kapur et al., 1992). Two regions of gut containing ENPs were used, the pre-caecal midgut and the caecum (Fig. 1), from $Ret^{TGM/+}$ or *DβH-nlacZ* mice; in these mice, ENPs express GFP or lacZ (Enomoto et al., 2001; Kapur et al., 1992). We also used sacral neural crest-derived cells for some experiments.

Comparison of ability of vagal ENPs derived from the caecum or pre-caecal midgut to colonize the pre- and post-caecal gut

It is unknown whether ENPs migrate at the same rate through different regions of the gastrointestinal tract. Additionally, it has been proposed that exposure to the caecum might be important for the colonization of the hindgut by ENPs because the expression of some molecules is higher in the caecum than elsewhere along the gastrointestinal tract (Kruger et al., 2003; Leibl et al., 1999). Although the caecum of E11.5 mice contains vagal ENPs at the migratory wave front and therefore potentially the cells with the best migratory ability, it has been previously shown that ENPs behind the wave front are also capable of colonizing aneural explants of gut (Sidebotham et al., 2002; Simpson et al., 2007). We therefore compared the ability of ENPs from the caecum and pre-caecal midgut to colonize explants of aneural pre-caecal midgut and post-caecal gut (Fig. 1). The pre-caecal midgut of $Ret^{TGM/TGM}$ mice was used as recipient gut because it lacks ENPs (Schuchardt et al., 1994), and it is currently not technically feasible to obtain aneural midgut explants from wild-type mice. Aneural post-caecal gut was obtained from $Ret^{TGM/TGM}$ mice or wild-type mice.

Ability of ENPs from the pre-caecal midgut to migrate caudally through explants of aneural pre-caecal midgut or hindgut. When explants of pre-caecal midgut were placed at the rostral end of explants of aneural pre-caecal midgut or aneural post-caecal gut (from either $Ret^{TGM/TGM}$ or wild-type mice) and cultured for 3 days, GFP^+ or β-gal^+ ENPs had migrated up to 600–700 μm and colonized each type of recipient gut equally well (Figs. 1B, C, F).

Ability of ENPs from the caecum to migrate caudally through explants of aneural pre-caecal midgut or hindgut. When explants of caecum were placed adjacent to explants of aneural midgut, caecal-derived GFP^+ or β-gal^+ ENPs usually colonized only a short length (average of ~200 μm) of the recipient midgut explants (Fig. 1D). In contrast, caecal-derived ENPs migrated around 600–700 μm along explants of aneural hindgut, which is similar to ENPs derived from pre-caecal gut (Figs. 1E, F). These results show that exposure to the caecum does not affect the ability of ENPs to colonize aneural segments of post-caecal gut, as pre-caecal and caecal-derived ENPs colonized explants of aneural hindgut equally well. However, as caecal-derived ENPs did not migrate far along explants of midgut, exposure to the caecum appears to change the characteristics of ENPs so that the pre-caecal midgut is no longer favorable for migration. Although Ret is expressed exclusively by ENPs in the gut, a recent study has shown

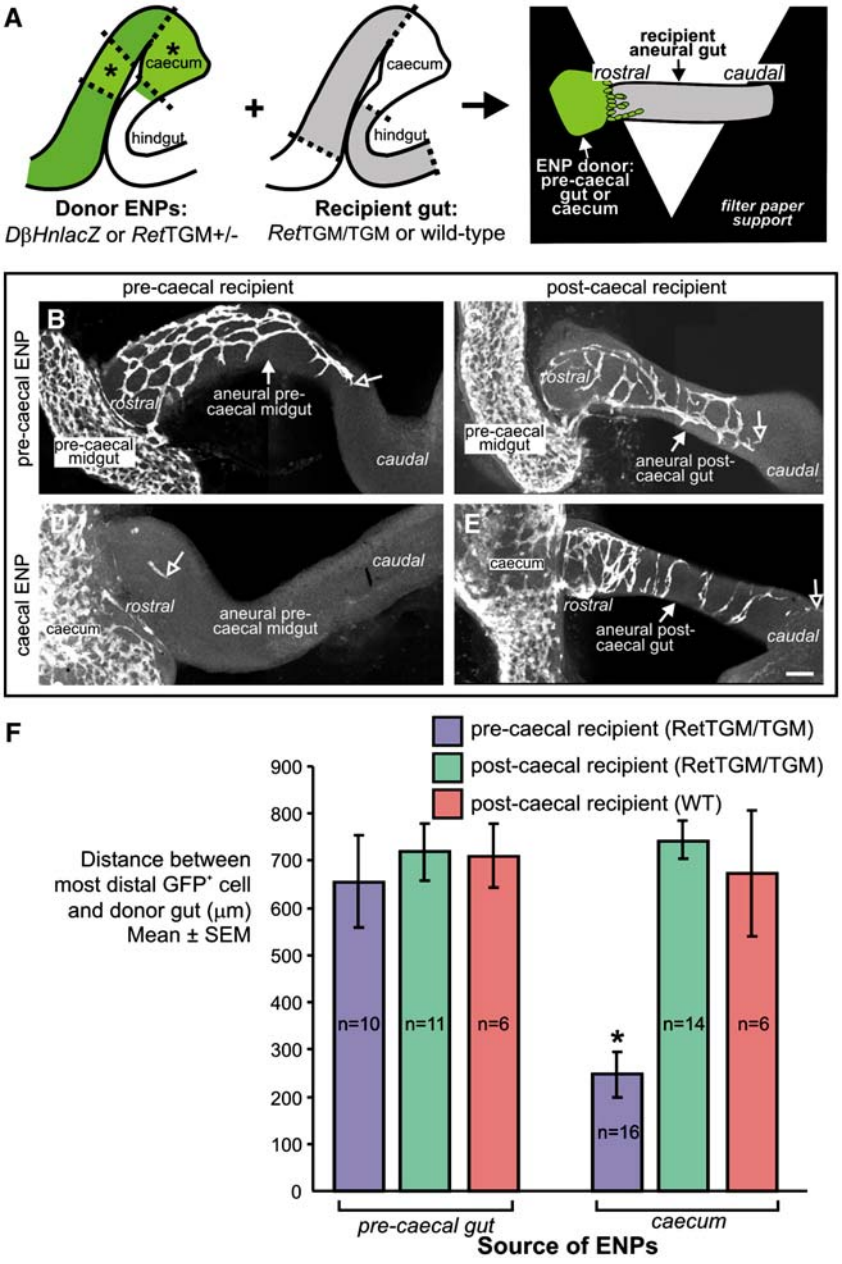

Fig. 1. Comparison of distance that pre-caecal and caecal ENCs migrate in recipient pre-caecal and post-caecal gut. (A) Diagram showing regions of gut used in co-cultures. Segments of pre-caecal or caecum (*asterisks*) from mice in which ENCs express GFP or lacZ were placed in contact with the rostral end of segments of aneural pre-caecal or post-caecal gut recipient gut (shown in grey). The recipient gut was suspended across a "V" cut in a piece of filter paper as described previously (Hearn et al., 1999). Recipient aneural pre-caecal gut came from *Ret*$^{TGM/TGM}$ mice and recipient aneural post-caecal gut came from *Ret* $^{TGM/TGM}$ or wild-type mice. (B–E) Recipient gut explants after 3 days in culture; the most distal cell is indicated with an open arrow. Pre-caecal ENPs colonized both pre-caecal (B) and post-caecal (C) recipient gut equally well. Caecal ENPs colonized pre-caecal recipient explants poorly (D) but migrated a similar distance along post-caecal recipients (E) to pre-caecal ENPs (C). Scale bar: 100 μm. (F) Quantification (mean ± SEM) of distance from edge of donor explant to most distal GFP$^+$ or lacZ$^+$ cell in different co-cultures. Caecal ENCs migrate significantly shorter distances in explants of aneural midgut than other combinations (ANOVA followed by Tukey's test).

abnormal gene expression in other cell types in the gut of fetal Ret null mice (Vohra et al., 2006). Nonetheless, ENPs colonized explants of hindgut from wild-type and *Ret*$^{TGM/TGM}$ mice equally well (Fig. 1F).

Are there gradients along the midgut and hindgut?

Is there a gradient along the pre-caecal midgut to which midgut-derived ENPs respond? Vagal ENPs normally migrate in a rostral-to-caudal direction along the developing gut. At E11, GDNF expression is maximum in the pre-caecal small intestine and caecum (Natarajan et al., 2002), and it is possible that a gradient of GDNF could promote the directional migration of vagal ENPs towards the caecum. To determine whether vagal ENPs are equally capable of migrating rostrally as they are of migrating caudally along explants of midgut, explants of recipient midgut up to the caecum from *Ret*$^{TGM/TGM}$ mice were co-cultured with

segments of pre-caecal midgut from E11.5 *Ret*$^{TGM/+}$ mice placed at both the rostral and caudal ends (Fig. 2A). The recipient gut included the most distal small intestine, which like the caecum, shows high GDNF mRNA expression (see Fig. 2F of Natarajan et al., 2002). There was no significant difference in the distance that ENPs migrated from both ends after 3 days in culture (Figs. 2A, B). These data show that there is no detectable gradient along the pre-caecal midgut to which midgut-derived ENPs respond.

Is there a gradient along the post-caecal gut to which caecal-derived ENPs respond? Wild-type aneural post-caecal gut was used as recipient gut for the following experiments. When segments of E11.5 caecum were placed adjacent to the rostral end of the post-caecal gut from wild-type mice and cultured for 4 days, the recipient hindgut was well colonized by GFP$^+$ or β-gal$^+$ ENPs (n=25; Figs. 3A–C). In the vast majority (28/32) of explants in which E11.5 caecum was placed adjacent to distal end of segments of aneural hindgut, no GFP$^+$ or β-gal$^+$ cells were present in the recipient explants after 4 days in culture (Figs. 3D–F). In 4/32 explants, a small number (<20) of GFP$^+$ or β-gal$^+$ cells were present in the recipient gut, but they had migrated only a short distance. To determine whether only the most distal end of E11.5 hindgut is inhibitory to the entry of vagal ENPs, E11.5 caecum containing donor ENPs was placed adjacent to the caudal end of the middle region of E11.5 post-caecal gut (Fig. 3G). The recipient post-caecal gut explants were usually completely colonized by GFP$^+$ or β-gal$^+$ cells after 4 days in culture (Fig. 3H). To examine whether vagal neural crest cells migrate rostrally at the same speed as they migrate caudally, co-cultures were established in which caeca from E11.5 *Ret*$^{TGM/+}$ or *DβH-nlacZ* mice were placed at both ends of explants of the middle part of E11.5 hindgut (Fig. 3I) and cultured for 24 h only. Measurement of the distance from each donor caecum to the furthermost GFP$^+$ or β-gal$^+$ cell in each explant revealed that cells migrated significantly further from rostral-to-caudal than from caudal-to-rostral (Figs. 3I, J; P<0.05; two-tailed t test). Thus, although vagal ENPs are capable of migrating rostrally along the middle regions of E11.5 hindgut, they migrate faster caudally. These data show that the distal end of the hindgut is inhibitory to migration of vagal crest-derived cells, and that there may be a gradient of a repulsive cue(s) along the hindgut.

Co-cultures of sacral neural crest-derived cells with aneural hindgut. Sacral neural crest-derived cells, which give rise to both pelvic ganglia and to some neurons in the hindgut, coalesce into clusters adjacent to the distal hindgut from E11.5 (Kapur, 2000). Co-cultures were established in which the connective tissue containing GFP$^+$ sacral crest cells from E11.5 *Ret*$^{TGM/+}$ mice was placed at the anal end of explants of the entire length of post-caecal gut from E11.5 wild-type mice (Fig. 4A). In 4/5 explants of recipient gut, no GFP$^+$ cells were observed after 3 days in culture (Fig. 4B); in one explant we counted 15 GFP$^+$ cells, which had migrated a maximum of 50 μm into the explant. Sacral neural crest cells were also placed adjacent to the caudal end of the middle region of E11.5 hindgut (Fig. 4C). In 5/6 co-cultures, GFP$^+$ cells were present within the recipient gut, but typically only colonized 100–300 μm of the gut (Fig. 4D). These data show that at E11.5 there is either an absence of an attractive cue or the transient expression of a repulsive cue, which prevents crest-derived cells from entering the E11.5 distal hindgut.

Expression of Sema3A, Sema3F, neuropilin-1 and neuropilin-2 in the developing mouse gut

In both chick and mice, sacral neural crest cells directly adjacent to the distal hindgut undergo a "waiting period" and only colonize the hindgut after it has been colonized by vagal neural crest cells (Burns and Le Douarin, 1998; Kapur, 2000). The above *in vitro* studies suggest that there may be localized expression of a cue that is inhibitory to ENPs in the distal hindgut. Previous studies have shown that Sema3A is transiently expressed in the outer mesenchyme of the distal hindgut of embryonic chicks and is repulsive to the axons of extrinsic neurons that project into the hindgut (Shepherd and Raper, 1999). Sema3F is also expressed at high levels in the distal hindgut of fetal rats (Giger et al., 1998). Neuropilin-1 and neuropilin-2 form part of the receptor complex for

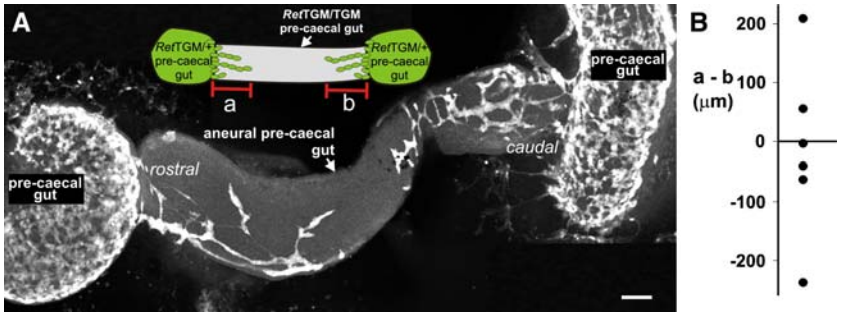

Fig. 2. (A) Co-culture with segments of pre-caecal gut from a *Ret*$^{TGM/+}$ embryo placed in contact with both the rostral and caudal ends of a segment of aneural midgut from an E11.5 *Ret*$^{TGM/TGM}$ embryo. The distances from the edge of the rostral donor explant to the most distal cell ("a") and the edge of the caudal donor explant to the most distal cell ("b") were measured. Scale bar: 100 μm. (B) Quantification of the differences between caudal migration ("a") and rostral migration ("b") in each explant. There was no significant difference in the distance that ENPs migrated from each end after 3 days in culture (two-tailed t test where the null hypothesis was that the mean difference between the distance the cells migrated caudally and rostrally in each preparation is not different from 0).

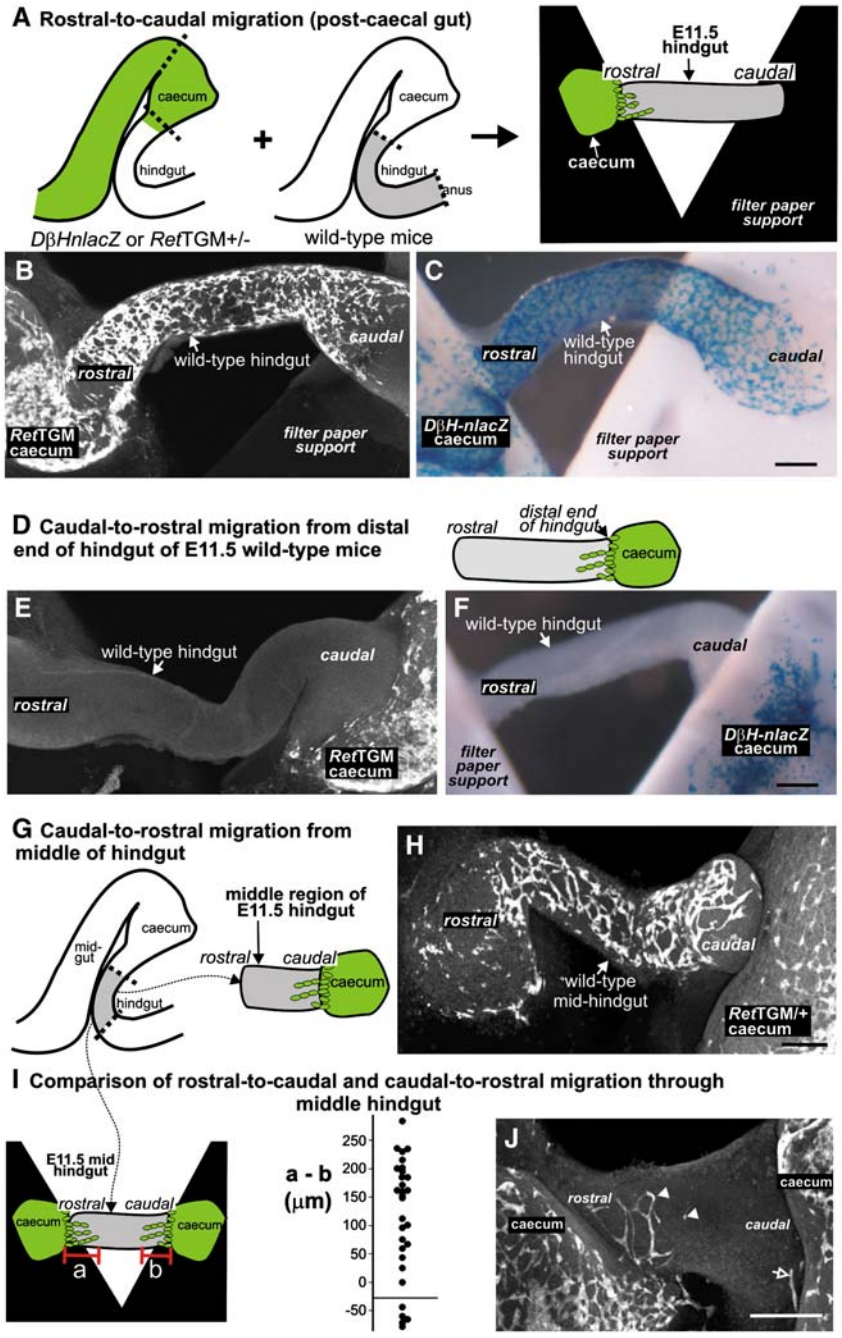

Fig. 3. Colonization of aneural explants of post-caecal gut by caecal ENPs. (A–C) Co-cultures to examine caudal migration. (A) Segments of caecum from mice in which ENCs express GFP or lacZ were placed in contact with the rostral end of segments of aneural post-caecal recipient gut. After 4 days in culture, GFP^+ (B) or β-gal^+ (C) cells were present along the entire length of recipient gut. (D–F) Co-cultures to examine rostral migration from the distal end of the hindgut. After 4 days in culture, few if any GFP^+ (E) or β-gal^+ (F) cells were present in the recipient gut. (G–H) Co-cultures to examine rostral migration from the middle region of the hindgut. GFP^+ cells can enter and migrate rostrally through the middle region of the post-caecal gut (H). (I–J) Comparison of caudal and rostral migration through the middle region of post-caecal gut. (I) Quantification of the differences between caudal migration ("a") and rostral migration ("b") in each explant. Cells migrated significantly further caudally than rostrally ($P<0.05$; two-tailed t test). (J) Co-culture showing more extensive caudally directed migration (*arrowheads*) than rostral migration (*open arrow*). Scale bars: 100 μm.

Sema3A and Sema3F, respectively (Fujisawa, 2004). We examined the expression of Sema3A and Sema3F in the gut of developing mice using in situ hybridization, the presence of neuropilin-1 protein using immunohistochemistry (Kitsukawa et al., 1997) and the expression of neuropilin-2 (Nrp2) using $Nrp2^{gt/+}$ mice.

Expression of Sema3A

The distribution of Sema3A mRNA was examined in whole mounts and sections of gut from E11.5, E12.5 and E14.5 mice. At all stages examined, Sema3A mRNA was expressed in the caecum and along most of the post-caecal gut, with the highest levels of expression in the caecum and

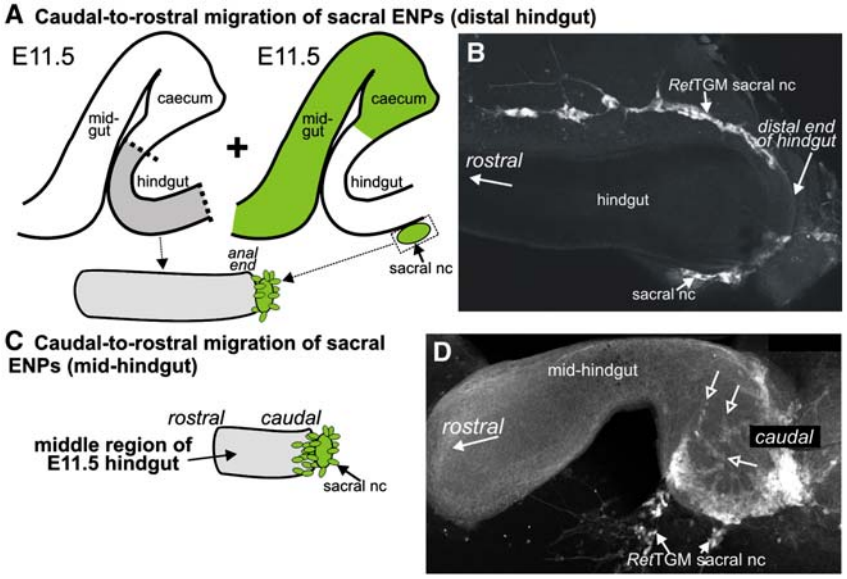

Fig. 4. Colonization of aneural segments of hindgut by sacral neural crest (*nc*)-derived cells. (A) Diagram showing that the mesentery adjacent to the distal hindgut containing GFP$^+$ sacral crest-derived cells from *Ret*$^{TGM/+}$ embryos was co-cultured with explants of aneural hindgut. (B) After 3 days in culture, there were no GFP$^+$ cells within the recipient gut. (C) Sacral crest-derived cells were placed at the caudal end of segments of the middle region of post-caecal gut. (D) After 3 days in culture, some GFP$^+$ cells were present in the recipient gut (*open arrows*), but they had not migrated far from the donor tissue.

the distal end of the hindgut (Figs. 5A, E). The middle regions of the small intestine also showed higher levels of expression than caudal small intestine. Transverse sections through the distal hindgut of E11.5 mice showed that Sem3A was expressed by the inner mesenchyme; the outer 2–3 layers of mesenchymal cells and the epithelial cells usually showed no Sema3A expression (Fig. 5B). Sacral crest-derived cells are thought to enter the distal hindgut around E14.5 (Anderson et al., 2006; Kapur, 2000). At E14.5, Sema3A was still expressed by the distal colon (Fig. 5E). Examination of sections of distal colon revealed that Sema3A was expressed by the inner mesenchyme (Fig. 5F), but the outer mesenchymal layer that lacked Sema3A expression was many more cell layers thicker than in E11.5 mice. The circular muscle layer was apparent using DIC optics at E14.5. Sema3A was expressed by the mesenchyme internal to the circular muscle layer, but not by the circular muscle.

Neuropilin-1 immunoreactivity

Virtually all vagal crest-derived (GFP$^+$) cells in the midgut and caecum of E11.5–E14.5 *Ret*$^{TGM/+}$ mice showed neuropilin-1 immunoreactivity (Figs. 5G–I). Neuropilin-1 immunoreactivity appeared to be restricted to crest-derived (GFP$^+$) cells, and mesenchymal cells did not show detectable staining. Many sacral crest-derived cells adjacent to the hindgut also showed neuropilin-1 immunostaining (Figs. 5J–L).

Expression of Sema3F

The pattern of expression of Sema3F was very similar to that of Sema3A, with highest expression in the caecum and distal hindgut (Fig. 5C). Like Sema3A, Sema3F was expressed by inner mesenchymal cells, but not by the gut mucosa (Fig. 5D).

Expression of neuropilin-2

In E11.5–E14.5 *Nrp2*$^{gt/+}$ mice, β-gal$^+$ cells were present in the outer part of the mesenchyme (Fig. 5N). The β-gal$^+$ cells showed a different distribution pattern from crest-derived cells, and double-labeling with an antibody to Hu (to label enteric neurons) at E11.5 and E14.5 revealed no overlap between β-gal and Hu immunostaining (Figs. 5M–O). In E11.5 mice, the β-gal$^+$ cells were roundish in shape, but in E14.5 mice the β-gal$^+$ cells in the small intestine were spindle-shaped and oriented around the circumference of the gut, with a shape and distribution similar to circular smooth muscle cells (data not shown). These data show that neuropilin-2 is expressed by gut mesenchymal cells, probably longitudinal and circular muscle cells and their precursors, but crest-derived cells do not express neuropilin-2.

There is premature entry of sacral neural crest-derived cells into the hindgut of mice lacking Sema3A in vivo, but no change in the timetable by which sacral crest-derived cells colonize the gut of mice lacking neuropilin-2

The entry of sacral neural crest-derived cells into the hindgut of *Sema3A*$^{+/+}$, *Sema3A*$^{+/-}$ and *Sema3A*$^{-/-}$ mice was examined at E12.5 and E13.5 using antibodies to p75, to label neural crest-derived cells (Chalazonitis et al., 1998), and Tuj-1 to label axons. No p75$^+$ cells or Tuj1$^+$ fibres were observed in whole-mount preparations or in sections (Figs. 6A–E) of the distal hindgut of E12.5 or E13.5 *Sema3A*$^{+/+}$ or *Sema3A*$^{+/-}$ embryos. However, p75$^+$ cells and Tuj1$^+$ fibres, which presumably arise from pelvic ganglia, were observed within the hindgut of E12.5 and E13.5 *Sema3A*$^{-/-}$ embryos (Figs. 6F–J). All p75$^+$ cells were closely associated with Tuj1$^+$ fibres, but not all Tuj1$^+$ fibres were accompanied by p75$^+$ cells. In transverse sections through the distal small intestine of E13.5 *Sema3A*$^{-/-}$ mice, p75$^+$ cells were

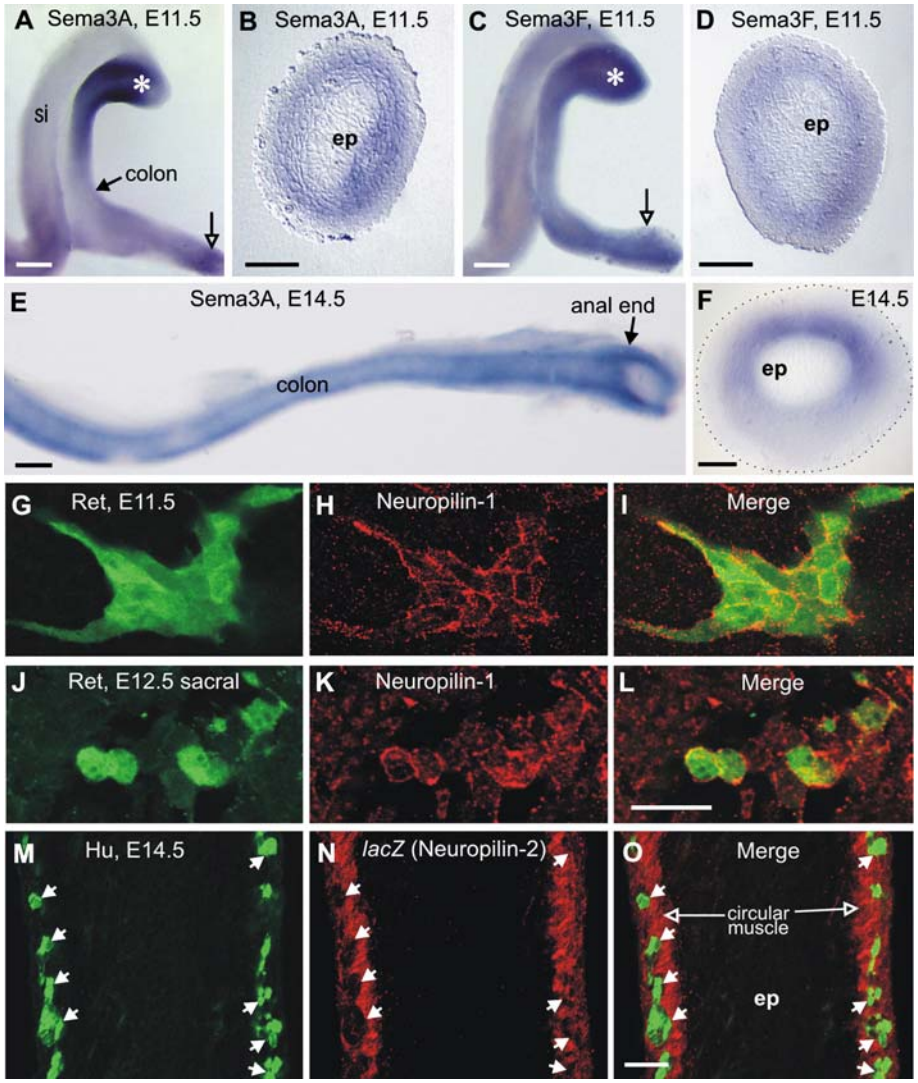

Fig. 5. Expression of Sema3A, Sema3F, neuropilin-1 and neuropilin-2 in the developing gut. (A, B) Sema3A expression in the gut of E11.5 mice revealed by *in situ* hybridization. (A) In whole mounts, Sema3A expression is highest in the caecum and distal hindgut (*open arrow*). Scale bar: 200 μm. (B) Transverse section through the distal hindgut. Sema3A is expressed by the inner mesenchyme. (C, D) Sema3F expression in the gut of E11.5 mice. (C) In whole mounts, Sema3F expression is highest in the caecum and distal hindgut (*open arrow*). Scale bar: 200 μm. (D) Transverse section through the distal hindgut. Sema3F is expressed by the inner mesenchyme. (E, F) Sema3A expression in the gut of E14.5 mice. (E) Whole-mount preparation of the colon. (F) Transverse section of distal colon. Sema3A is expressed in the inner mesenchyme. The outer mesenchymal layer lacking Sema3A expression is thicker than at E11.5 (see panel B). Scale bar: 50 μm. (G–I) Confocal microscope images of neuropilin-1 immunoreactivity in the colon of an E11.5 $Ret^{TGM/+}$ mouse. Neuropilin-1 immunostaining (H) is present on the cell surface of most GFP^+ cells. (G, I) These cells are derived from the vagal neural crest. (J–L) Sacral crest-derived cells that are found in the mesentery adjacent to the distal hindgut also show neuropilin-1 immunostaining. Scale bar: 25 μm. (M–O) Optical (longitudinal) section through the small intestine of an E14.5 $Nrp2^{gt/+}$ mouse. The circular muscle layer in the outer mesenchyme shows β-gal staining (N, lacZ expression), but the Hu^+ ENPs (*arrows*, M, O) do not show β-gal staining. Scale bar: 25 μm. *Ep*-epithelium.

quite sparse and we did not observe more than three $p75^+$ cells per section, but $Tuj1^+$ axons were commonly observed.

In the colon of fetal mice, vagal ENPs settle in the outer mesenchyme, on the serosal side of the circular muscle layer (McKeown et al., 2001). It is possible that the high levels of the Sema3A expressed by the inner mesenchyme influences the location within the mesenchyme that vagal ENPs migrate and settle. However, in transverse sections through the proximal and mid-colon, there was no detectable difference in the location of ENPs between E12.5 and E13.5 $Sema3A^{-/-}$, $Sema3A^{+/+}$ and $Sema3A^{+/-}$ embryos.

We also examined the entry of sacral crest-derived cells into the hindgut of E11.5–E13.5 mice lacking neuropilin-2 ($Nrp2^{gt/gt}$ mice) using an antibody to p75. No premature entry of $p75^+$ cells into the hindgut was observed (data not shown). These data are consistent with the above observations that neuropilin-2 is not expressed by enteric crest-derived cells.

Discussion

Are there gradients along the gut that promote the caudal migration of ENPs?

ENPs derived from the vagal neural crest enter the foregut and then migrate caudally to colonize the entire length of the gastrointestinal tract. A number of studies have shown that ENP

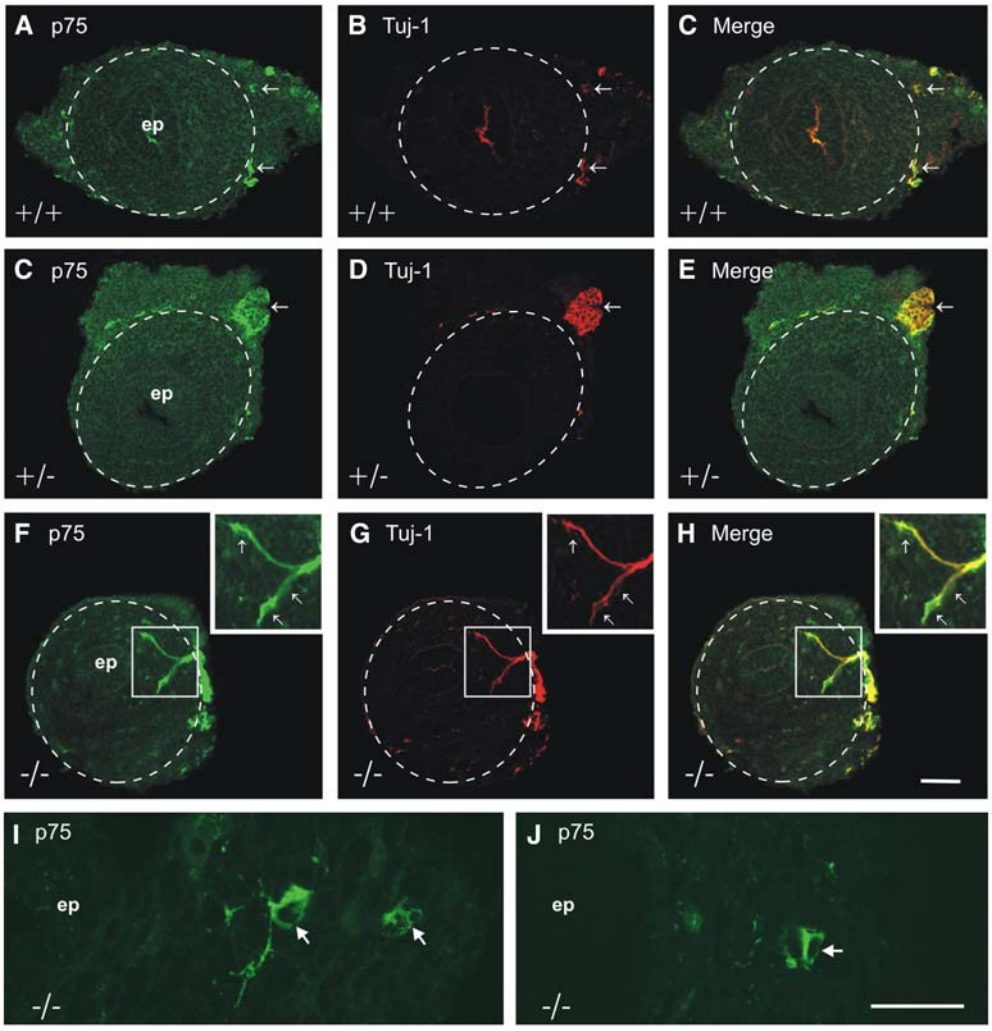

Fig. 6. Frozen transverse sections through the distal colon of E13.5 *Sema3A*$^{+/+}$ (A–C), *Sema3A*$^{+/-}$ (C–E) and *Sema*3A$^{-/-}$ (F–J) mice, showing p75 and Tuj-1 immunostaining. (A–E) Although p75+ and Tuj-1+ are closely opposed to the colon in *Sema3A*$^{+/+}$ and *Sema3A*$^{+/-}$ mice, there are none within the gut wall. (F–H) In *Sema*3A$^{-/-}$ mice, prominent Tuj-1^{+} nerve fibres and sparse p75^{+} cells are present (*arrows* in inset). (I, J) Higher magnification images of p75^{+} cell bodies within the hindgut of E13.5 *Sema3A*$^{-/-}$ embryos. Scale bars: 50 μm.

cell number is important in the migration of ENPs (Burns et al., 2000; Peters-van der Sanden et al., 1993; Simpson et al., 2007), but it is also possible that the graded expression of attractive or repulsive cues along the gut could promote the caudal migration of vagal ENPs. GDNF is expressed by the developing gut (Golden et al., 1999; Natarajan et al., 2002; Young et al., 2001), and several studies have shown that GDNF is attractive to enteric ENPs in vitro (Barlow et al., 2003; Fu et al., 2004; Iwashita et al., 2003; Kruger et al., 2003; Nagy and Goldstein, 2006; Natarajan et al., 2002; Young et al., 2001). It is very likely that the expression of GDNF by the gut mesenchyme induces the entry of ENPs derived from vagal neural crest into the foregut (Natarajan et al., 2002) and retains them within the gut (Young et al., 2001). However, the high levels of expression of GDNF in the pre-caecal small intestine and caecum could also promote the directional migration of vagal ENPs along the pre-caecal gut (Natarajan et al., 2002). Our results using co-cultures showed that vagal ENPs migrate rostrally at the same speed as they migrate caudally through explants of aneural midgut, and thus we found no evidence for a gradient of chemoattractive molecules along the pre-caecal gut that specifically promotes the caudally directed migration of vagal ENPs. Moreover, although the levels of GDNF are thought to be lower in the hindgut than the midgut (Natarajan et al., 2002), we found that ENPs migrate equally well along explants of post-caecal and pre-caecal gut. It therefore seems possible that GDNF expressed by the gut mesenchyme might promote ENP migration non-directionally and retain ENPs within the gut, but ENP proliferation at the migration wave front is likely to be the major factor driving migration into uncolonized regions of the gut (Simpson et al., 2007). Our data support the idea that vagal ENPs migrate caudally simply because they enter the rostral end of the developing gut.

Effect of the caecum on ENP migration

Data from a number of previous studies have lead to the suggestion that exposure to the caecum is important for the

colonization of the hindgut by ENPs (Kruger et al., 2003; Lee et al., 2003; Leibl et al., 1999; Shin et al., 1999). Furthermore, using time-lapse imaging, Druckenbrod and Epstein (2005) have shown that the migratory behavior of ENPs differs in the caecum from other parts of the gut. Previous studies have shown that ENPs that have never been exposed to the caecum are capable of colonizing aneural explants of hindgut (Sidebotham et al., 2002; Simpson et al., 2007; Young et al., 2002), but it is possible that exposure to the caecum changes the migration rate of ENPs. In the current study, we showed that ENPs derived from pre-caecal gut migrate through explants of aneural post-caecal gut at the same rate as ENPs derived from the caecum.

An unexpected result was that ENPs from the caecum colonized aneural explants of pre-caecal gut significantly slower than explants of post-caecal gut. This suggests that exposure to the caecum alters of the properties of vagal ENPs so that the pre-caecal gut is no longer an attractive environment in which to migrate. One candidate mediator of this effect is endothelin-3, which is highly expressed in the caecum, and it would be interesting to compare the migratory abilities of pre-caecal and caecal ENPs from endothelin-3 or Ednrb null mice in explants of aneural pre-caecal gut. Regardless of the gut region, migrating ENPs preferentially migrate into uncolonized regions of the gut (Druckenbrod and Epstein, 2005, 2007; Young et al., 2004), and so the functional significance of the observation is unclear.

Sacral ENPs do not give rise to a substantial ENS in cultured explants of hindgut

When the mesentery containing sacral crest-derived cells was co-cultured with explants of mid-hindgut, ENPs were found within the recipient gut, but they did not migrate far or form a very extensive ENS. This suggests that, as in chick and quail embryos (Burns et al., 2000; Hearn and Newgreen, 2000), sacral crest-derived cells in mice do not have the same capacity to form an ENS as vagal ENPs.

Sema3A regulates the entry of sacral ENPs and extrinsic axons into the distal hindgut

Studies in avian embryos showed that ENPs derived from the sacral neural crest undergo a waiting period outside the gut before entering the distal hindgut (Burns and Le Douarin, 1998). In mice, sacral ENPs and extrinsic axons are present adjacent to the distal hindgut from E11.5 (Young et al., 1998) but are not thought to enter the gut until around E14.5 (Anderson et al., 2006; Kapur, 2000). Our data are the first *in vivo* evidence that Sema3A, at least in part, is responsible for the delay, as there was premature entry of both sacral ENPs and extrinsic axons into the distal hindgut of Sema3A null mice. The results extend previous *in vitro* assays and expression studies in chick embryo that suggested that Sema3A regulates the entry of extrinsic axons into the chick hindgut (Shepherd and Raper, 1999).

Sema3A is a secreted glycoprotein (Luo et al., 1993). The pattern of Sema3A expression within the distal colon of developing mice was similar to that reported previously in chick embryos (Shepherd and Raper, 1999). Within the distal hindgut of fetal mice, Sema3A mRNA expression was highest in the inner mesenchyme, and there was little or no expression in the outer mesenchyme. The outer mesenchymal layers lacking Sema3A expression increased in thickness between E11.5 (when sacral crest cells are present adjacent to the distal hindgut) and E14.5 (when sacral ENPs enter the hindgut), and so the levels of Sema3A protein in the outermost layers are likely to be lower at E14.5 than at E11.5. Furthermore, at E14.5 the circular muscle layer has started to develop. The developing circular muscle layer does not express Sema3A and may provide a barrier to diffusion of Sema3A from the inner mesenchyme.

It has previously been suggested that Sema3A might only be inhibitory to extrinsic axons, and that any effect on sacral ENPs is indirect because they require extrinsic axons in order to enter the gut (Burns, 2005). We were unable to resolve whether Sema3A is directly repulsive to both extrinsic axons and sacral ENPs. Although extrinsic fibres were common, only a small number of sacral ENPs were observed to enter the hindgut prematurely in Sema3A null mice. This may indicate that there are multiple repulsive cues that regulate the entry of sacral ENPs into the hindgut or that sacral ENPs do not normally contribute many neurons to the ENS in mice.

Our study showed that Sema3A is also expressed at high levels by the caecum and rostral post-caecal gut. Although migrating vagal ENPs expressed neuropilin-1, the timetable by which vagal ENPs colonized the mid and hindgut was not noticeably different in Sema3A null mice. Thus, the functional significance of the expression of Sema3A by the caecum is unclear. Although expression is maximal in the caecum and distal hindgut, Sema3A is expressed by the inner mesenchyme along the entire colon. Vagal ENPs colonize that outer layers of the developing gut, and one possibility was that Sema3A contributes to the concentric layering of the developing gut by repelling vagal ENPs away from the inner mesenchyme. However, vagal ENPs were found in the outer mesenchyme of the colon of E14.5 mice lacking Sema3A, as they are in wild-type mice. Thus, although both vagal and sacral ENPs express neuropilin-1, we could only find a defect in sacral ENPs in *Sema3A*$^{-/-}$ mice. Previous studies have shown that the responsiveness of neurons to a single guidance cue can vary depending on the combination of co-receptors expressed (Hong et al., 1999), the levels of intracellular signaling molecules including cyclic nucleotides and Ca^{2+} (Henley et al., 2004; Nishiyama et al., 2003), the composition of the extracellular matrix (Hopker et al., 1999) or the presence of chemokines (Chalasani et al., 2003), neurotrophic growth factors including GDNF (Wanigasekara and Keast, 2006) or other soluble molecules (Castellani et al., 2002). Both vagal and sacral ENPs express neuropilin-1, and we do not currently know why they apparently show different responses to Sema3A. Future studies are required to reveal molecular differences between vagal and sacral ENPs.

Neuropilin-2 is expressed by many pre-migratory and early post-migratory neural crest cells, and Sema3F/neuropilin-2 signaling plays roles in the migration of some cranial and trunk

neural crest cells (Gammill et al., 2006a,b; Osborne et al., 2005; Yu and Moens, 2005). In the current study, we were unable to detect neuropilin-2 expression by vagal ENPs within the gut, but it may be expressed prior to their entry into the gut. We were unable to detect any ENP migration defects in mice lacking neuropilin-2, which is not surprising since it is not expressed by ENPs. Our data suggest that Sema3F/neuropilin-2 signaling does not play a role in ENP migration, although we cannot rule out the possibility that Sema3F could act through other receptors. As Sema3F was expressed by the inner mesenchyme and neuropilin-2 was expressed by the outer mesenchyme of the developing gut, this signaling pathway may play some role in gut mesenchyme development.

Conclusions

Our data suggest it is unlikely that there is a gradient of chemoattractive molecules along the pre-caecal gut that specifically promotes the caudally directed migration of vagal ENPs. Vagal ENPs probably migrate caudally because they enter the rostral end of the developing gut, and factors such as GDNF produced by the gut mesenchyme promote their survival and proliferation and retain them within the gut. The significance of high endothelin-3 and GDNF expression by the caecum on the migration of ENPs remains unclear as exposure to the caecum does not alter the rate at which ENPs colonize explants of hindgut. Sema3A expressed by the hindgut delays the entry of sacral ENPs and extrinsic axons. Unlike some other neural crest-derived populations, Sema3F/neuropilin-2 signaling does not appear to influence ENP migration as ENPs do not express neuropilin-2 and there is no detectable change in the timetable by which ENPs colonize the gut in mice lacking neuropilin-2.

Acknowledgments

This work was supported by NHMRC project grants 400017 and 350311. We thank Don Newgreen for his suggestion of using the middle sections of hindgut in the co-culture experiments, for interesting discussions and for valuable comments on the manuscript. We also thank Hideki Enomoto and Jeff Milbrandt for providing the *Ret*^{TGM} mice, Christiana Ruhrberg for advice with PCR protocols, Miles Epstein for the anti-Hu and Andreas Puschel for in situ probes.

References

Anderson, R.B., Stewart, A.L., Young, H.M., 2006. Phenotypes of neural-crest-derived cells in vagal and sacral pathways. Cell Tissue Res. 323, 11–25.

Baetge, G., 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.

Barlow, A., de Graaff, E., Pachnis, V., 2003. Enteric nervous system progenitors are coordinately controlled by the g protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron 40, 905–916.

Bondurand, N., Natarajan, D., Barlow, A., Thapar, N., Pachnis, V., 2006. Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling. Development 133, 2075–2086.

Burns, A.J., 2005. Migration of neural crest-derived enteric nervous system precursor cells to and within the gastrointestinal tract. Int. J. Dev. Biol. 49, 143–150.

Burns, A.J., Le Douarin, N.M., 1998. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. Development 125, 4335–4347.

Burns, A.J., Thapar, N., 2006. Advances in ontogeny of the enteric nervous system. Neurogastroenterol. Motil. 18, 876–887.

Burns, A.J., Champeval, D., Le Douarin, N.M., 2000. Sacral neural crest cells colonise aganglionic hindgut in vivo but fail to compensate for lack of enteric ganglia. Dev. Biol. 219, 30–43.

Castellani, V., De Angelis, E., Kenwrick, S., Rougon, G., 2002. Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A. EMBO J. 21, 6348–6357.

Chalasani, S.H., Sabelko, K.A., Sunshine, M.J., Littman, D.R., Raper, J.A., 2003. A chemokine, SDF-1, reduces the effectiveness of multiple axonal repellents and is required for normal axon pathfinding. J. Neurosci. 23, 1360–1371.

Chalazonitis, A., Rothman, T.P., Chen, J., Gershon, M.D., 1998. Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 in vitro and in vivo. Dev. Biol. 204, 385–406.

Chen, H., Bagri, A., Zupicich, J.A., Zou, Y., Stoeckli, E., Pleasure, S.J., Lowenstein, D.H., Skarnes, W.C., Chedotal, A., Tessier-Lavigne, M., 2000. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25, 43–56.

Conner, P.J., Focke, P.J., Noden, D.M., Epstein, M.L., 2003. Appearance of neurons and glia with respect to the wavefront during colonization of the avian gut by neural crest cells. Dev. Dyn. 226, 91–98.

Druckenbrod, N.R., Epstein, M.L., 2005. The pattern of neural crest advance in the cecum and colon. Dev. Biol. 287, 125–133.

Druckenbrod, N.R., Epstein, M.L., 2007. Behavior of enteric neural crest-derived cells varies with respect to the migratory wavefront. Dev. Dyn. 236, 84–92.

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

Eickholt, B.J., Mackenzie, S.L., Graham, A., Walsh, F.S., Doherty, P., 1999. Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. Development 126, 2181–2189.

Enomoto, H., Crawford, P.A., Gorodinsky, A., Heuckeroth, R.O., Johnson Jr., E.M., Milbrandt, J., 2001. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development 128, 3963–3974.

Fairman, C.L., Clagett-Dame, M., Lennon, V.A., Epstein, M.L., 1995. Appearance of neurons in the developing chick gut. Dev. Dyn. 204, 192–201.

Fu, M., Chi Hang Lui, V., Har Sham, M., Nga Yin Cheung, A., Kwong Hang Tam, P., 2003. HOXB5 expression is spatially and temporarily regulated in human embryonic gut during neural crest cell colonization and differentiation of enteric neuroblasts. Dev. Dyn. 228, 1–10.

Fu, M., Lui, V.C., Sham, M.H., Pachnis, V., Tam, P.K., 2004. Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. J. Cell Biol. 166, 673–684.

Fujisawa, H., 2004. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. J. Neurobiol. 59, 24–33.

Furness, J.B., 2006. The Enteric Nervous System. Blackwell Publishing, Massachusetts, USA.

Gammill, L.S., Gonzalez, C., Bronner-Fraser, M., 2006a. Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. J. Neurobiol.

Gammill, L.S., Gonzalez, C., Gu, C., Bronner-Fraser, M., 2006b. Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development 133, 99–106.

Gershon, M.D., 2005. Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. J. Clin. Gastroenterol. 39, S184–S193.

Giger, R.J., Urquhart, E.R., Gillespie, S.K., Levengood, D.V., Ginty, D.D., Kolodkin, A.L., 1998. Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 21, 1079–1092.

Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., Kolodkin, A.L., Ginty, D.D., Geppert, M., 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 25, 29–41.

Golden, J.P., DeMaro, J.A., Osborne, P.A., Milbrandt, J., Johnson Jr., E.M., 1999. Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp. Neurol. 158, 504–528.

Hearn, C., Newgreen, D., 2000. Lumbo-sacral neural crest contributes to the avian enteric nervous system independently of vagal neural crest. Dev. Dyn. 218, 525–530.

Hearn, C.J., Murphy, M., Newgreen, D., 1998. GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev. Biol. 197, 93–105.

Hearn, C.J., Young, H.M., Ciampoli, D., Lomax, A.E., Newgreen, D., 1999. Catenary cultures of embryonic gastrointestinal tract support organ morphogenesis, motility, neural crest cell migration, and cell differentiation. Dev. Dyn. 214, 239–247.

Henley, J.R., Huang, K.H., Wang, D., Poo, M.M., 2004. Calcium mediates bidirectional growth cone turning induced by myelin-associated glycoprotein. Neuron 44, 909–916.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M., Stein, E., 1999. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97, 927–941.

Hopker, V.H., Shewan, D., Tessier-Lavigne, M., Poo, M., Holt, C., 1999. Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. Nature 401, 69–73.

Iwashita, T., Kruger, G.M., Pardal, R., Kiel, M.J., Morrison, S.J., 2003. Hirschsprung disease is linked to defects in neural crest stem cell function. Science 301, 972–976.

Jiang, Y., Liu, M.T., Gershon, M.D., 2003. Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas. Dev. Biol. 258, 364–384.

Kapur, R.P., 1999. Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. Pediatr. Dev. Pathol. 2, 559–569.

Kapur, R.P., 2000. Colonization of the murine hindgut by sacral crest-derived neural precursors: experimental support for an evolutionarily conserved model. Dev. Biol. 227, 146–155.

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

Kawasaki, T., Bekku, Y., Suto, F., Kitsukawa, T., Taniguchi, M., Nagatsu, I., Nagatsu, T., Itoh, K., Yagi, T., Fujisawa, H., 2002. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. Development 129, 671–680.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., Fujisawa, H., 1997. Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron 19, 995–1005.

Kruger, G.M., Mosher, J.T., Tsai, Y.H., Yeager, K.J., Iwashita, T., Gariepy, C.E., Morrison, S.J., 2003. Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. Neuron 40, 917–929.

Landman, K.A., Pettet, G.J., Newgreen, D.F., 2003. Mathematical models of cell colonization of uniformly growing domains. Bull. Math Biol. 65, 235–262.

Le Douarin, N.M., 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.

Lee, H.O., Levorse, J.M., Shin, M.K., 2003. The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Dev. Biol. 259, 162–175.

Leibl, M.A., Ota, T., Woodward, M.N., Kenny, S.E., Lloyd, D.A., Vaillant, C.R., Edgar, D.H., 1999. Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. Gut 44, 246–252.

Luo, Y., Raible, D., Raper, J.A., 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217–227.

McKeown, S.J., Chow, C.W., Young, H.M., 2001. Development of the submucous plexus in the large intestine of the mouse. Cell Tissue Res. 303, 301–305.

Nagy, N., Goldstein, A.M., 2006. Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. Dev. Biol. 293, 203–217.

Nagy, N., Brewer, K.C., Mwizerwa, O., Goldstein, A.M., 2006. Pelvic plexus contributes ganglion cells to the hindgut enteric nervous system. Dev. Dyn.

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

Natarajan, D., Marcos-Gutierrez, C., Pachnis, V., de Graaff, E., 2002. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129, 5151–5160.

Newgreen, D., Young, H.M., 2002. Enteric nervous system: development and developmental disturbances-part 2. Pediatr. Dev. Pathol. 5, 329–349.

Newgreen, D.F., Southwell, B., Hartley, L., Allan, I.J., 1996. Migration of enteric neural crest cells in relation to growth of the gut in avian embryos. Acta Anat. 157, 105–115.

Nishiyama, M., Hoshino, A., Tsai, L., Henley, J.R., Goshima, Y., Tessier-Lavigne, M., Poo, M.M., Hong, K., 2003. Cyclic AMP/GMP-dependent modulation of Ca2+ channels sets the polarity of nerve growth-cone turning. Nature 423, 990–995.

Osborne, N.J., Begbie, J., Chilton, J.K., Schmidt, H., Eickholt, B.J., 2005. Semaphorin/neuropilin signaling influences the positioning of migratory neural crest cells within the hindbrain region of the chick. Dev. Dyn. 232, 939–949.

Peters-van der Sanden, M.J., Kirby, M.L., Gittenberger-de Groot, A., Tibboel, D., Mulder, M.P., Meijers, C., 1993. Ablation of various regions within the avian vagal neural crest has differential effects on ganglion formation in the fore-, mid- and hindgut. Dev. Dyn. 196, 183–194.

Renzi, M.J., Wexler, T.L., Raper, J.A., 2000. Olfactory sensory axons expressing a dominant-negative semaphorin receptor enter the CNS early and overshoot their target. Neuron 28, 437–447.

Robb, L., Tarrant, J., Groom, J., Ibrahim, M., Li, R., Borobakas, B., Wright, M.D., 2001. Molecular characterisation of mouse and human TSSC6: evidence that TSSC6 is a genuine member of the tetraspanin superfamily and is expressed specifically in haematopoietic organs. Biochim. Biophys. Acta 1522, 31–41.

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

Shepherd, I.T., Raper, J.A., 1999. Collapsin-1/semaphorin D is a repellent for chick ganglion of Remak axons. Dev. Biol. 212, 42–53.

Shin, M.K., Levorse, J.M., Ingram, R.S., Tilghman, S.M., 1999. The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature 402, 496–501.

Sidebotham, E.L., Kenny, S.E., Lloyd, D.A., Vaillant, C.R., Edgar, D.H., 2002. Location of stem cells for the enteric nervous system. Pediatr. Surg. Int. 18, 581–585.

Simpson, M.J., Landman, K.A., Hughes, B.D., and D.F., N., 2007. Cell proliferation drives neural crest cell invasion of the intestine. Dev. Biol. 302, 553–568.

Stewart, A.L., Anderson, R.B., Young, H.M., 2003. Characterization of lacZ-expressing cells in the gut of embryonic and adult DbetaH-nlacZ mice. J. Comp. Neurol. 464, 208–219.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y., Kitsukawa, T., Fujisawa, H., Klagsbrun, M., Hori, M., 2002. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc. Natl. Acad. Sci. U. S. A. 99, 3657–3662.

Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., Yagi, T., 1997. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron 19, 519–530.

Tarrant, J.M., Groom, J., Metcalf, D., Li, R., Borobokas, B., Wright, M.D., Tarlinton, D., Robb, L., 2002. The absence of Tssc6, a member of the tetraspanin superfamily, does not affect lymphoid development but enhances in vitro T-cell proliferative responses. Mol. Cell Biol. 22, 5006–5018.

Vohra, B.P., Tsuji, K., Nagashimada, M., Uesaka, T., Wind, D., Fu, M., Armon, J., Enomoto, H., Heuckeroth, R.O., 2006. Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. Dev. Biol. 298, 259–271.

Wallace, A.S., Burns, A.J., 2005. Development of the enteric nervous system, smooth muscle and interstitial cells of Cajal in the human gastrointestinal tract. Cell Tissue Res. 319, 367–382.

Walz, A., Rodriguez, I., Mombaerts, P., 2002. Aberrant sensory innervation of the olfactory bulb in neuropilin-2 mutant mice. J. Neurosci. 22, 4025–4035.

Wanigasekara, Y., Keast, J.R., 2006. Nerve growth factor, glial cell line-derived neurotrophic factor and neurturin prevent semaphorin 3A-mediated growth cone collapse in adult sensory neurons. Neuroscience 142, 369–379.

Watanabe, K., Tamamaki, N., Furuta, T., Ackerman, S.L., Ikenaka, K., Ono, K., 2006. Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord. Development 133, 1379–1387.

Woodward, M.N., Kenny, S.E., Vaillant, C., Lloyd, D.A., Edgar, D.H., 2000. Time-dependent effects of endothelin-3 on enteric nervous system development in an organ culture model of Hirschsprung's disease. J. Pediatr. Surg. 35, 25–29.

Wu, J.J., Chen, J.X., Rothman, T.P., Gershon, M.D., 1999. Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. Development 126, 1161–1173.

Yan, H., Bergner, A.J., Enomoto, H., Milbrandt, J., Newgreen, D.F., Young, H.M., 2004. Neural cells in the esophagus respond to glial cell line-derived neurotrophic factor and neurturin, and are RET-dependent. Dev. Biol. 272, 118–133.

Yntema, C.L., Hammond, W.S., 1954. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J. Comp. Neurol. 101, 515–541.

Young, H.M., Newgreen, D., 2001. Enteric neural crest-derived cells: origin, identification, migration, and differentiation. Anat. Rec. 262, 1–15.

Young, H.M., Hearn, C.J., Ciampoli, D., Southwell, B.R., Brunet, J.F., 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., Hearn, C.J., Farlie, P.G., Canty, A.J., Thomas, P.Q., Newgreen, D.F., 2001. GDNF is a chemoattractant for enteric neural cells. Dev. Biol. 229, 503–516.

Young, H.M., Jones, B.R., McKeown, S.J., 2002. The projections of early enteric neurons are influenced by the direction of neural crest cell migration. J. Neurosci. 22, 6005–6018.

Young, H.M., Bergner, A.J., Anderson, R.B., Enomoto, H., Milbrandt, J., Newgreen, D.F., Whitington, P.M., 2004. Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev. Biol. 270, 455–473.

Yu, H.H., Moens, C.B., 2005. Semaphorin signaling guides cranial neural crest cell migration in zebrafish. Dev. Biol. 280, 373–385.