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In ovo transplantation of enteric nervous system precursors from vagal to sacral neural crest results in extensive hindgut colonisation

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SUMMARY

The enteric nervous system (ENS) is derived from vagal and sacral neural crest cells (NCC). Within the embryonic avian gut, vagal NCC migrate in a rostrocaudal direction to form the majority of neurons and glia along the entire length of the gastrointestinal tract, whereas sacral NCC migrate in an opposing caudorostral direction, initially forming the nerve of Remak, and contribute a smaller number of ENS cells primarily to the distal hindgut. In this study, we have investigated the ability of vagal NCC, transplanted to the sacral region of the neuraxis, to colonise the chick hindgut and form the ENS in an experimentally generated hypoganglionic hindgut in ovo model. Results showed that when the vagal NC was transplanted into the sacral region of the neuraxis, vagal-derived ENS precursors immediately migrated away from the neural tube along characteristic pathways, with numerous cells colonising the gut mesenchyme by embryonic day (E) 4. By E7, the colorectum was extensively colonised by transplanted vagal NCC and the migration front had advanced caudorostrally to the level of the umbilicus. By E10, the stage at which sacral NCC begin to colonise the hindgut in large numbers, myenteric and submucosal plexuses in the hindgut almost entirely composed of transplanted vagal NCC, while the migration front had progressed into the pre-umbilical intestine, midway between the stomach and umbilicus. Immunohistochemical staining with the pan-neuronal marker, ANNA-1, revealed that the transplanted vagal NCC differentiated into enteric neurons, and whole-mount staining with NADPH-diaphorase showed that myenteric and submucosal ganglia formed interconnecting plexuses, similar to control animals. Furthermore, using an anti-RET antibody, widespread immunostaining was observed throughout the ENS, within a subpopulation of sacral NC-derived ENS precursors, and in the majority of transplanted vagal-to-sacral NCC. Our results demonstrate that: (1) a cell autonomous difference exists between the migration/signalling mechanisms used by sacral and vagal NCC, as transplanted vagal cells migrated along pathways normally followed by sacral cells, but did so in much larger numbers, earlier in development; (2) vagal NCC transplanted into the sacral neuraxis extensively colonised the hindgut, migrated in a caudorostral direction, differentiated into neuronal phenotypes, and formed enteric plexuses; (3) RET immunostaining occurred in vagal crest-derived ENS cells, the nerve of Remak and a subpopulation of sacral NCC within hindgut enteric ganglia.

Key words: Enteric nervous system, Quail, Chick, Neural crest cells, Gut, Neurons, Glia

INTRODUCTION

Neural crest cells (NCC) are embryonic multipotent stem cells that migrate along defined pathways to give rise to a wide variety of phenotypes including neuronal, mesenchymal, endocrine and pigment cells (Kalcheim and Le Douarin, 1999). Owing to their developmental properties, and the relative accessibility of the neural crest (NC) for experimental manipulation within embryos, NCC serve as a useful model for investigating the mechanisms involved in cell specification, migration, proliferation and differentiation, i.e. fundamental processes in the development of complex multicellular organisms.

In common with the remainder of the autonomic nervous system, the neurons and glia that constitute the enteric nervous system (ENS) are entirely derived from the NC (Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). The rhombencephalic (vagal) region of the NC, adjacent to somites 1-7, contributes the vast majority of ENS precursors along the entire length of the gut (Epstein et al., 1994; Le Douarin and Teillet, 1973). Upon leaving the NC, these vagal-derived precursors enter the foregut then migrate in a rostrocaudal direction, reaching the terminal hindgut of the chick at embryonic day (E) 8-8.5 (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973) and of the mouse at E14 (Kapur et al., 1992; Young et al., 1998). A second source of enteric precursors, the sacral NC, which is situated caudal to somite 28 in the chick and somite 24 in the mouse, contributes cells to the postumbilical gut only (Burns et al., 2000; Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973; Pomeranz and

Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991). Although initial studies (Pomeranz and Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991) suggested that sacral-derived precursors entered the hindgut early in development, before the arrival of vagal NCC, our studies in the chick embryo have demonstrated that sacral NC-derived precursors only begin to enter the gut after the colorectum has been colonised by vagal cells (Burns and Le Douarin, 1998). This finding suggested that in order to colonise the hindgut, sacral NCC may require an interaction with vagal NCC, or with factors or signalling molecules produced by them. However, we have subsequently reported that sacral NCC colonise the hindgut independently of vagal NCC-derived enteric precursors (Burns et al., 2000), a finding that has been confirmed in the chick using hindgut/NC co-culture experiments (Hearn and Newgreen, 2000), and in the mouse using *Wnt1-lacZ* transgene expression as an early marker of murine NCC (Kapur, 2000). Together, these data demonstrate that the hindgut ENS is formed by NCC that follow complex pathways to and within the gut. A first wave of vagal NCC migrate rostrocaudally (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973; Young et al., 1998), and later arriving sacral NCC invade the hindgut migrating in an opposing caudorostral direction (Burns et al., 2000; Hearn and Newgreen, 2000; Kapur, 2000).

In addition to the information concerning the spatiotemporal pathways that NCC follow, the recent understanding of the signalling mechanisms involved in the guidance of enteric precursors to and within the gut has rapidly progressed. Major defects in the ENS have been documented following the inactivation or overexpression of murine genes not previously known to affect the gut. Such genes include those encoding the components of the RET/GFRα1/GDNF, ECE-1/endothelin-3/endothelin-B and RET/GFRα2/neurturin signalling pathways, the homeobox gene *Hoxa4* and homeobox-related gene *Hox11l1*, the transcription factors *Sox10* and *Phox2b* (*Pmx2b* – Mouse Genome Informatics) and the proneural gene *Mash1* (*Ascl1* – Mouse Genome Informatics) (Cacalano et al.,, 1998; Enomoto et al., 1998; Gershon, 1998; Gershon, 1999; Manie et al., 2001; Pattyn et al., 1999; Taraviras and Pachnis, 1999; Tennyson et al., 1998). In mice, where some of these genes have been knocked out, a wide spectrum of enteric phenotypes can arise depending on the specific genes involved. Such phenotypes range from a complete absence of ENS cells along the entire gastrointestinal tract (*Sox10*$^{-/-}$), to varying degrees of regional deficiencies where sections of the gut are aganglionic (*Mash1*$^{-/-}$ and knockout of genes within the RET/GFRα1/GDNF and ECE-1/EDN3/EDNRB signalling pathways). Less obvious phenotypes include those where only specific neuronal subpopulations are affected (neurturin-null and *Gfra2*$^{-/-}$), and others where the numbers of neurons is actually increased within enteric ganglia (*Hox11l1*$^{-/-}$) (reviewed by Young and Newgreen, 2001a).

Many of the genes shown to affect ENS development in mice have also been implicated in humans with ENS disorders. For example, aganglionic megacolon or Hirschsprung's disease (HSCR), is characterised by the regional absence of enteric ganglion cells within the rectum and in a variable length of the colon (Kapur, 1999; Robertson et al., 1997; Wartiovaara et al., 1998). Susceptibility genes, including *RET*, *GDNF*, *EDN3* and *EDNRB* have been documented in individuals with HSCR, but the condition is genetically complex, with multiple modes of inheritance, incomplete penetrance and variable expressivity known to exist (Kapur, 1999; Wartiovaara et al., 1998). As a result, the length of aganglionic bowel can vary, with short or long segment, or even total gut aganglionosis having been described (Nemeth et al., 2001; Shimotake et al., 2001).

We have recently studied an in ovo hypoganglionic hindgut model in the chick embryo by ablating part of the vagal NC so that insufficient NCC are available to colonise the entire length of the gut. After such ablations, the post-umbilical bowel is free from enteric plexuses although small, isolated ganglia derived from the sacral crest do occur (Burns et al., 2000). We decided to use this developmental model to investigate the ability of vagal NCC to colonise the hindgut when grafted into the sacral region of the neuraxis. The rationale for this study is based on previous findings that demonstrate the strongly invasive nature of vagal NCC for the gut. Thus, heterotopic vagal NC grafting and backtransplantation experiments have shown that vagal-derived ENS precursors were capable of colonising the gut either when transplanted to another region of the neuraxis (Le Douarin and Teillet, 1974), or when segments of gut already colonised by vagal NCC were backtransplanted into younger embryos (Rothman et al., 1993). By contrast, Erickson and Goins (Erickson and Goins, 2000) recently proposed that sacral NCC have no special migratory properties that allow them to reach the gut, and it is the environment at the sacral level that is sufficient to allow NCC from any other axial levels to enter the gut mesenchyme. In this study we show that vagal NCC, transplanted into the sacral region, have the potential to invade the hindgut in sufficient numbers, and differentiate appropriately to form enteric plexuses and rescue an experimentally generated hypoganglionic hindgut phenotype in the chick embryo.

MATERIALS AND METHODS

Animals

Fertilised quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus domesticus*) eggs obtained from commercial sources were stored and incubated as previously described (Burns and Le Douarin, 2001). The developmental stages of embryos were determined either by counting the number of pairs of somites formed or by reference to developmental tables (Hamburger and Hamilton, 1951).

Microsurgical procedures: vagal neural crest ablation and heterotopic neural crest grafts

Access to the embryo was gained by cutting a window in the shell, then in order to facilitate visualisation of embryonic tissues, Indian ink, diluted 1:1 in phosphate-buffered saline (PBS), was injected into the sub-blastodermic cavity. The vitelline membrane was cut back to expose the embryo then the neural tube, including the NC, situated between somites 3-6 in 8- to 12-somite stage (ss) chick embryos, was excised using a fine microscalpel, as previously described (Burns et al., 2000) and as shown in Fig. 1.

In the same vagal NC-ablated chick embryos, approximately 24 hours later at 25 ss, the sacral region of the NC was removed as previously described (Burns and Le Douarin, 1998; Catala et al., 1995), and replaced with the vagal NC obtained from 10 ss quail embryos (Fig. 1). The grafted region consisted of the neural tube between somites 1-7, and during transplantation care was taken to ensure that the donor tissue was positioned in the same

rostrocaudal/dorsovental orientation. Additional experiments were performed using progressively smaller segments of donor vagal neural tube, down to ~1 somite in length, in order to test whether reduced numbers of vagal NCC could adequately colonise the hindgut to form enteric ganglia.

In a second series of grafts, following vagal NC ablation, the sacral crest was transplanted from 25 ss quail embryos into the vacant vagal region of the chick host embryos. In order to assess the ability of transplanted vagal NCC to migrate in a caudorostral direction, while normal vagal NCC were migrating in the opposing rostrocaudal direction, vagal-to-sacral transplantations were also performed without first ablating the vagal NC in the recipient embryo.

Immunohistochemistry

Intact embryos or dissected gastrointestinal tracts were fixed in Carnoy's fluid for 10-30 minutes, embedded in paraffin wax and sectioned at a thickness of 7.5 μm. After rehydration and rinsing in PBS, sections were placed in 10% serum in PBS for 45 minutes, then incubated overnight at 4°C with primary antibody. In this study, antibodies to QCPN (Developmental Studies Hybridoma Bank; mouse IgG_1, culture supernatant), ANNA-1 (Altermatt et al., 1991) (human IgG, diluted 1:1000 in PBS), GFAP (Dako; rabbit IgG, diluted 1:200) and RET (IBL, Japan; rabbit IgG, diluted 1:50) were used for immunostaining. Tissues were extensively rinsed in PBS prior to appropriate secondary antibody labelling (all Southern Biotechnology), which was performed for 1 hour at room temperature. Double-immunostaining with QCPN and ANNA-1 was performed sequentially on wax sections as previously described (Burns and Le Douarin, 1998). Double-labelling with RET and QCPN was sequentially performed on paraformaldehyde-fixed cryostat sections, prepared and treated as described (Burns and Le Douarin, 1998), using alkaline phosphatase (Vector) and DAB (Sigma) to identify RET- and QCPN-labelled cells respectively.

NADPH-diaphorase whole-mount staining

At E16, dissected segments of gut were cut open adjacent to the mesentery, pinned flat and fixed in 4% paraformaldehyde in PBS for 12 hours. After rinsing in PBS, staining for NADPH-diaphorase activity was performed by incubation in PBS (pH 7.4) containing 1 mg/ml β-NADPH, 0.5 mg/ml nitroblue tetrazolium and 0.05% Triton-X-100 (all Sigma) for 30-60 minutes at 37°C. The NADPH-diaphorase reaction product appeared as a dark blue granular deposit and the reaction was terminated by immersing the tissues in cold PBS.

Labelled tissues and sections were photographed using Fuji Provia 100 ASA or 400 ASA colour slide film with a Nikon microphot photomicroscope. The photographic slides were then scanned using a Nikon Coolscan III 35 mm digital scanner and figures prepared using Adobe Photoshop 6 software.

RESULTS

Microsurgical procedures

We have previously shown that a hypoganglionic hindgut phenotype can be generated in chick embryos by ablating the vagal NC in the region of somites 3-6 (Burns et al., 2000). When such ablations were performed in embryos aged between the 8 and 12 ss of development, the colorectum was always found to be devoid of enteric plexuses [10/10 experiments by Burns et al. (Burns et al., 2000)], with only cells of sacral NC origin present in the hindgut. However, this was not the case if ablations were performed at the 13 ss or later, as vagal crest-derived plexuses subsequently developed in these animals. In this current study, in order to replicate these previous findings and ensure that a hypoganglionic hindgut phenotype was generated, the vagal NC adjacent to somites 3-6 was ablated in chick embryos between 8 and 12 ss, with the majority of ablations occurring at 10 ss. In these operated embryos, ~1 day later at 25 ss, the sacral region of the NC was removed and replaced with the vagal neural tube from quail embryos (Fig. 1). In this study, from a total of 152 embryos operated upon, 59 survived the ablation/grafting procedure (38.8%) and were fixed at various stages of development.

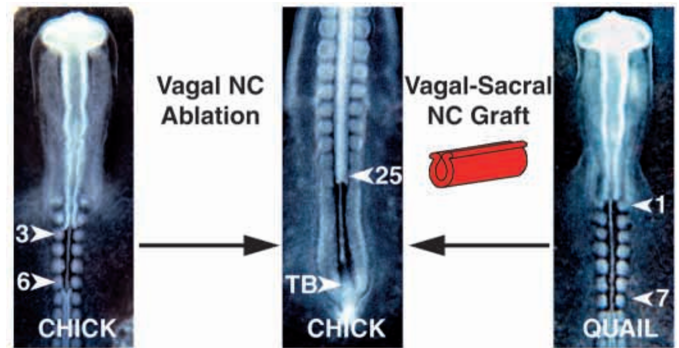


Fig. 1. Microsurgical grafting procedures. The neural tube, including the neural crest, situated between somites 3-6 was excised from chick embryos at 10 ss. Approximately 1 day later, at 25 ss, in the same NC-ablated chick embryos, the sacral region of the neuraxis was excised. The region involved corresponded to the neural tube caudal to the 25th pair of somites (25, arrowhead) and included the chordoneural hinge and rostral region of the tail bud (TB, arrowhead). After excision of the sacral NC, the vagal NC was removed from donor quail embryos at the 10 ss and grafted into the vacant sacral region of the neuraxis, ensuring that the appropriate rostrocaudal/dorsovental orientation was maintained.

Hindgut colonisation by vagal NCC transplanted to the sacral level

Embryos were first examined at E3, ~12 hours after transplantation of the vagal neural tube into the sacral region of the neuraxis. At this stage, NCC were present lateral to the neural tube underneath and within the ectoderm (Fig. 2A). Dorsoventral migration also occurred between the ectoderm and paraxial mesoderm, with transplanted cells migrating to the primitive gut wall where association with splanchnopleural mesenchyme occurred (Fig. 2A,B). The presence of transplanted vagal NCC within the gut at this early stage of development suggested that these cells commenced migration away from the neural tube immediately, or very soon after grafting to the new level of the neuraxis.

At E4, many transplanted vagal NCC were present within a dorsoventral pathway leading to the gut (Fig. 3). These cells appeared in streams leading away from the neural tube and were adjacent to the dorsal aorta. Occasional immunopositive cells were also observed within the mesonephros (Fig. 3A). Vagal-derived cells appeared to enter the gut within the outer layers of the mesenchyme, and eventually encircled the gut wall in a dorsoventral direction (Fig. 3A). In addition to the colorectum, at E4, vagal-derived cells had colonised the cecal region in large numbers and were present within the cecal buds and intestinal wall (Fig. 3B). At this stage of development, vagal cells accumulated in the dorsal mesentery, where they appeared to form the rudiment of the nerve of Remak (Fig. 3C).

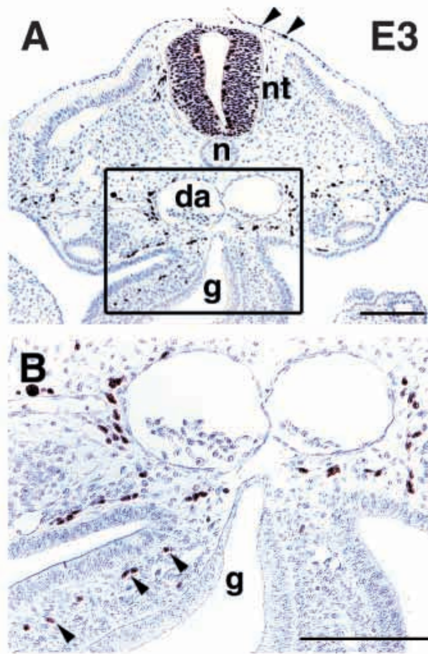


Fig. 2. Vagal-to-sacral heterotopic graft, E3. (A) Vagal NCC migrated away from the neural tube (nt), and entered a lateral pathway, underneath and within the ectoderm (arrowheads), and a dorsoventral pathway, lateral to the dorsal aorta (da) leading to the gut (g). (B) Boxed area in A; occasional QCPN+ cells (arrowheads) were evident within the splanchnopleural mesenchyme of the developing gut (g). Scale bars: 100 μm.

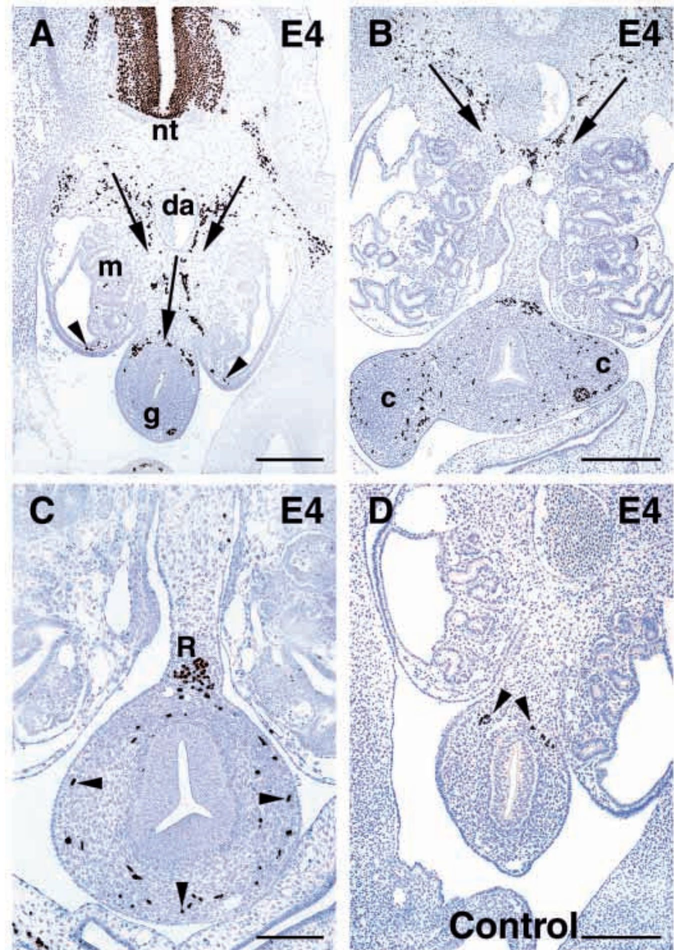


Fig. 3. Vagal-to-sacral heterotopic graft, E4. (A) Many QCPN+ cells entered the dorsoventral pathway. This extended from the dorsal neural tube (nt), lateral to the notochord and dorsal aorta (da), and into the dorsal aspect of the gut (g) (arrows, A and B). Occasional immunopositive cells (arrowheads) were present within the mesonephros (m). (B) At the level of the cecal gut, QCPN+ cells encircled the wall of the intestine, and were present within the outer mesenchymal layers of the cecal buds (c). (C) In the hindgut, transplanted vagal NCC accumulated to form the nerve of Remak (R), adjacent to the gut wall, and were present within the undifferentiated layers of the gut mesenchyme (arrowheads). (D) In control embryos, where isotopic and isochronic sacral NC grafts were performed, although occasional cells accumulated to form the rudiment of the nerve of Remak (arrowheads), no QCPN+ cells were observed within the gut wall. Scale bars: 200 μm in A,B,D; 100 μm in C.

In control animals (here the term ‘control’ refers to embryos where quail sacral NC was grafted to the equivalent region in chick embryos – this applies to Figs 3, 4, 5) at the same stage of development, the gut mesenchyme was free from sacral NC-derived cells and only occasional nerve of Remak precursors were adjacent to the outer layers of the gut wall (Fig. 3D).

At E7, heterotopically grafted vagal cells had colonised the colorectum, cecal region and post-umbilical intestine of vagal NC-ablated embryos. In the colorectum (Fig. 4A), numerous vagal graft-derived cells were present on either side of the circular muscle layers in the regions corresponding to the presumptive myenteric and submucosal plexuses, with the majority occurring in the dorsal myenteric plexus region of the gut wall, where cells were grouped into ganglia. In the cecal region, immunopositive cells were present on either side of the circular muscle layers of the intestine, while in the cecal buds, vagal-derived cells were situated adjacent to the outermost layers of the developing circular muscle layers (Fig. 4B). In the post-umbilical intestine, occasional vagal-derived cells encircled the outermost layers of the gut mesenchyme (Fig. 4C). In all regions of the intestine and hindgut examined at this stage, the nerve of Remak was poorly developed, and in transverse sections only very small groups of cells were apparent adjacent to the gut wall (Fig. 4A-C). In control animals at E7, sacral cells were absent or very sparse within the gut muscle layers, whereas the nerve of Remak was well developed and large ganglia were apparent in transverse hindgut sections (Burns and Le Douarin, 1998) (Fig. 4A′-C′).

At E10, the stage at which sacral NCC normally begin to colonise the hindgut in large numbers, transplanted vagal-derived cells were grouped on either side of the circular muscle layers in the colorectum (Fig. 5A,B) and appeared to comprise the vast majority of cells within the myenteric and submucosal plexuses (Fig. 5B). Transplanted vagal cells were also evident within the post-umbilical intestine, where the majority occurred external to the circular muscle layers within myenteric ganglia (Fig. 5C). Occasional vagal cells were also present within the pre-umbilical intestine (not shown). In control animals at E10, few sacral NC-derived cells were evident within the myenteric and submucosal plexus regions of the colorectum (Fig. 5A′,B′), and none were observed within the gut wall of the post-umbilical intestine (Fig. 5C′). Again,

at this stage of development, in transverse sections the nerve of Remak appeared to be much finer in diameter in vagal NC transplanted animals than in non-operated or sacral NC grafted embryos.

Neuronal fate of transplanted vagal NCC

The anatomical locations of vagal-derived cells within the hindgut, i.e. on either side of the circular muscle layer in plexus-like groupings (Fig. 6A), strongly suggested a neuronal fate for these NCC. Using the pan-neuronal marker ANNA-1 (Altermatt et al., 1991), enteric neurons were identified within the hindgut of vagal NC transplanted embryos as immunopositive groups of cells situated on either side of the circular muscle layer (Fig. 6B). When QCPN/ANNA-1 double antibody-labelled ganglia were examined in the same animals, all neurons were found to carry the QCPN-positive quail nucleus (Fig. 6C-C″) indicating that neurons within the colorectum were derived from vagal NCC transplanted into the sacral neuraxis.

Transplanted vagal NCC form enteric plexuses

Whole-mount NADPH-diaphorase staining was used to assess the ability of transplanted vagal NCC to form enteric plexuses within the colorectum of E16 chick embryos. In control animals (i.e. where no microsurgery was performed), nerve cell bodies of the myenteric (Fig. 7A), and submucosal plexuses were stained, as were interconnecting nerve fibres and nerve fibres within the muscle layers. After ablation of the vagal NC in the region of somites 3-6, although many nerve fibres were stained, the myenteric and submucosal plexus regions had few ganglia, although occasional NADPH-stained cell bodies were evident (Fig. 7B). In NC ablated/ vagal NCC transplanted animals, enteric plexuses appeared similar to those in controls. Many NADPH-stained neurons were evident within the submucosal and myenteric (Fig. 7C) ganglia, and interconnecting nerve fibres were evident within the plexuses and muscle layers.

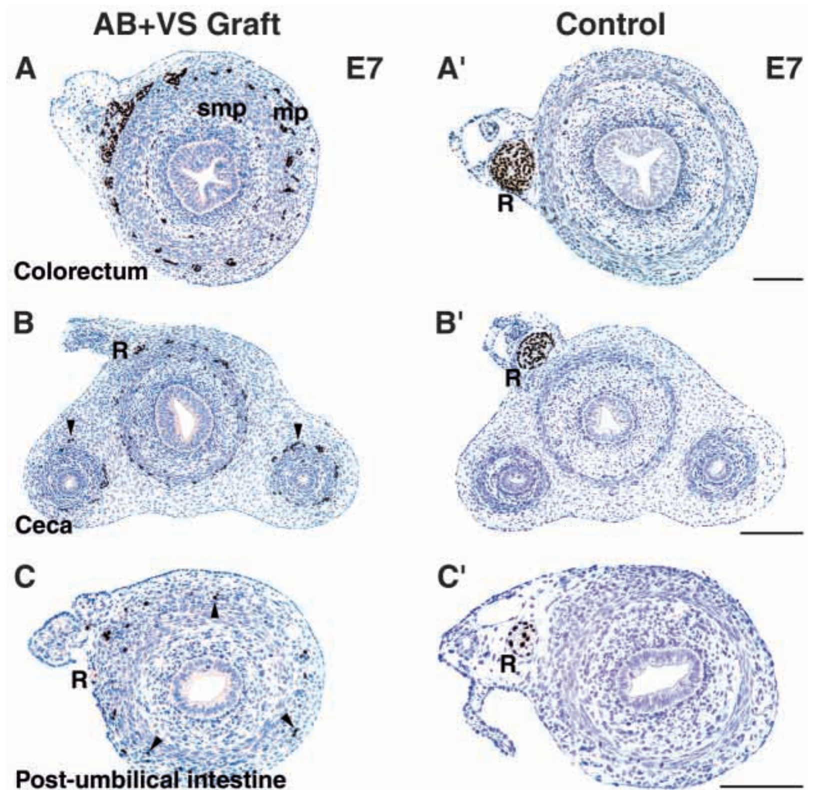


Fig. 4. Vagal-to-sacral heterotopic graft, E7. (A) In the colorectum, QCPN+ cells encircled the gut, within the region external to the circular muscle layer, corresponding to the myenteric plexus (mp) being heavily colonised. Internal to the circular muscle layer, in the region corresponding to the submucosal plexus (smp), QCPN+ cells were more sparsely distributed. (B) In the cecal region, the intestine wall and cecal buds were colonised by transplanted vagal NCC. In the intestine, QCPN+ cells were present on either side of the circular muscle wall, while in the cecal buds, immunopositive cells were only present external to the circular muscle layers (arrowheads). (C) In the post-umbilical intestine, QCPN+ cells were present external to the circular muscle layer (arrowheads). Note that in all regions, the nerve of Remak (R) was poorly developed compared with controls. (A′,B′,C′) In control embryos at E7 no sacral NCC were present within any regions of the gut wall. However, the nerve of Remak was well developed and large ganglia (R) were apparent in transverse sections. Scale bars: 100 μm.

RET immunostaining in vagal, sacral and transplanted vagal-sacral NCC

In order to determine whether sacral NCC possess any of the molecular markers known to be expressed by vagal NC-derived ENS precursors, we analysed, by immunolabelling, the presence of RET in the population of sacral NCC that colonise the gut, and in vagal NCC transplanted to the sacral region of the neuraxis. In controls (non-operated animals), widespread RET immunostaining was found within the nerve of Remak, in cells of the myenteric and submucosal plexuses, and in nerve fibres interconnecting the myenteric and submucosal ganglia (Fig. 8A). In experiments where the sacral NC from quail embryos was grafted into chick embryos, RET/QCPN double-labelled cells were observed within myenteric and submucosal ganglia (Fig. 8B,B′), thus confirming that a subpopulation of sacral NC-derived ENS precursors express RET. Vagal NCC, that were transplanted to the sacral region of the neuraxis, were also found to be RET-positive and were located within the nerve of Remak and within myenteric and submucosal ganglia (Fig. 8C,C′).

Transplanted and normal vagal NCC migrate in opposing directions within the intestine

Our results clearly demonstrate that transplanted vagal NCC migrated in a caudorostral direction and thus colonised the hindgut and contributed cells to more rostral regions, including the pre-umbilical intestine. As the normal population of vagal NCC migrates in a rostrocaudal direction, we decided to investigate further the spatiotemporal interactions of two vagal NCC populations that commenced migration at different regions of the neuraxis. To address this issue, vagal-to-sacral NC transplants were performed in host embryos where the vagal neural tube was not excised, so that normal rostrocaudal migration of vagal NCC could occur. At E4, vagal NCC had

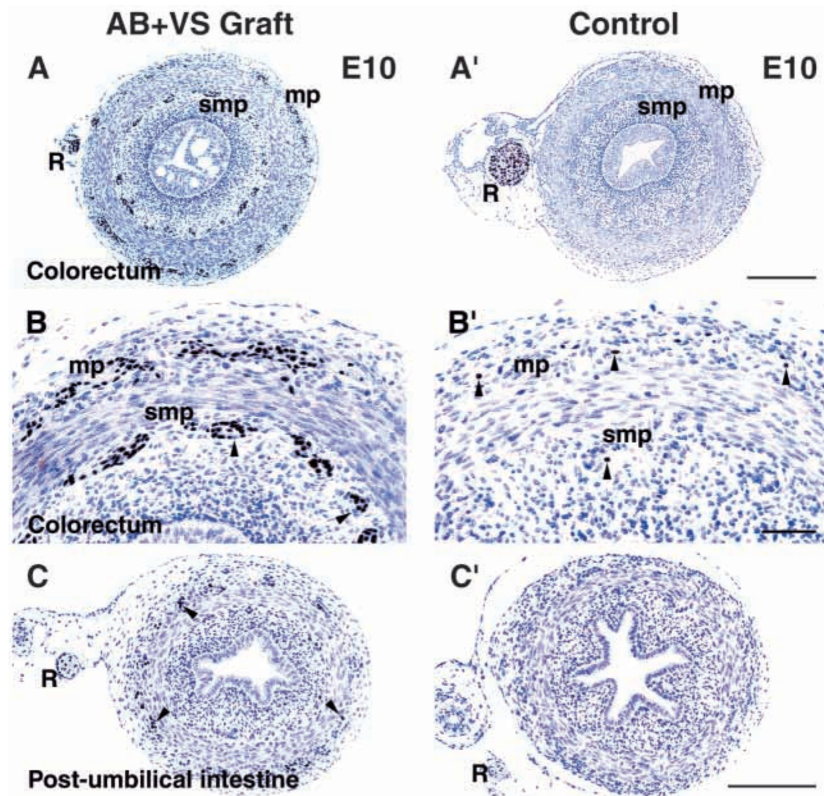


Fig. 5. Vagal-to-sacral heterotopic graft, E10. (A) In the colorectum, the myenteric (mp) and submucosal (smp) plexuses contained numerous QCPN+ cells. The nerve of Remak (R) appeared to be poorly developed in transverse sections. (B) Higher magnification revealed that the vast majority of plexus cells were QCPN+, with only a few exceptions in the submucosal plexus (arrowheads).(C) In the post-umbilical intestine, QCPN+ cells were mainly situated external to the circular muscle layers (arrowheads). (A′,B′,C′) In control gut at E10, only very occasional sacral-derived cells were present with the myenteric and submucosal plexuses of the colorectum (B, arrowheads). No immunopositive cells were present in the post-umbilical intestine at this stage of development. Scale bars: 200 µm in A,A′; 50 µm in B,B′; 100 µm in C,C′.

colonised most of the pre-umbilical intestine, while transplanted vagal-to-sacral NCC were present within the colorectum, cecal region, and the caudal part of the post-umbilical intestine. The region of bowel adjacent to the umbilicus was therefore free from NCC at this stage. At E5.5, vagal NCC were present at the level of the umbilicus, while the migration front of heterotopic vagal NCC had reached a similar position (see Fig. 9). During the subsequent 3 days of development, transplanted vagal NCC continued to migrate in a rostral direction, with the migration front of cells finally reaching midway within the pre-umbilical intestine. Over the same period of time, normal vagal NCC continued to migrate rostrocaudally. However, as the colorectum and cecal region were already extensively colonised by transplanted vagal NCC, normal vagal NCC were rarely observed in the colorectum (see Fig. 5 – the vast majority of enteric ganglion cells are derived from transplanted vagal NCC) and very few were observed in the cecal region. Mixed ganglia were, however, observed in the pre- and post-umbilical intestine (see Figs 5, 9).

Malformation of the nerve of Remak following vagal-to-sacral NC grafting

The nerve of Remak is a ganglionated nerve belonging to the autonomic nervous system that runs parallel to the intestine and extends from the distal cloaca, where it joins the pelvic plexus, to the duodeno-jejunal junction, where it is connected with the coeliac plexus (Teillet, 1978) (see Fig. 10). Whole-mount gut preparations demonstrated that the nerve was largest in diameter in the colorectum region (Fig. 10A), where distinct ganglia are evident (Fig. 10B), and

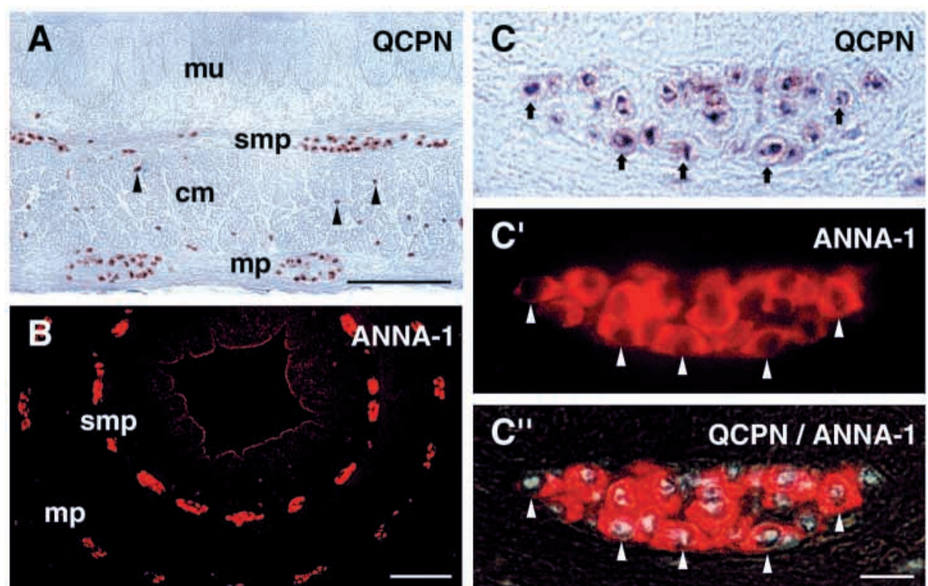
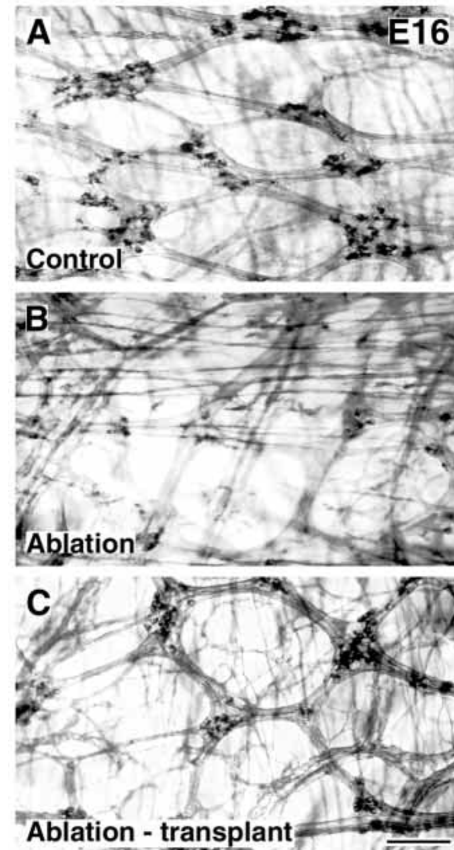


Fig. 6. Vagal-to-sacral heterotopic graft, E16. (A) In the colorectum, transplanted vagal NCC colonised the myenteric plexus (mp), the submucosal plexus (smp) and were also present (arrowheads) within the circular muscle layer (cm). No immunopositive cells were observed within the mucosa. (B) Using the pan-neuronal marker ANNA-1, neurons were labelled within the submucosal plexus (smp) and myenteric plexus (mp). (C,C′,C″) Double labelling with QCPN and ANNA-1 revealed that all ANNA-1-positive neurons carried the QCPN+ quail nucleus, indicating that these neurons were derived from transplanted vagal NCC. Scale bars: 100 µm in A,B; 10 µm in C″.

Fig. 7. NADPH-diaphorase staining, myenteric plexus, chick colorectum, E16. (A) The myenteric plexus in control (non-operated) animals consisted of interconnected ganglia containing NADPH+ neurons and nerve fibres. (B) In vagal NC-ablated animals, few ganglia were observed in either the myenteric or submucosal plexus. However, numerous nerve fibres and occasional NADPH+ neurons were evident. (C) Following vagal NC ablation/vagal-to-sacral transplantation, numerous NADPH+ neurons were evident within interconnected ganglia in both the myenteric and submucosal plexuses. Scale bar: 100 μm.



finer adjacent to the remainder of the gut (Fig. 10A). In embryos where the vagal NC was transplanted into the sacral region of the neuraxis, the nerve of Remak occurred in the same anatomical location, extending from the distal colorectum to the entrance of the bile and pancreatic ducts in the intestine (Fig. 10A′), but was much finer in diameter along its entire length (compare whole-mount preparations in Fig. 10A,B and cross-sections in Figs 4, 5). In the colorectum region, where the nerve was situated between the mesentery and gut wall, although much finer in calibre than in control animals, small discrete ganglia could still be distinguished (Fig. 10B′).

Sacral NCC transplanted into the vagal region of the neuraxis colonise the gut

In the next series of experiments (*n*=5), the sacral NC from 25 ss quail embryos was grafted to the vagal neuraxis of chick embryos at 10 ss. Chimeric embryos were examined at E8, the stage at which normal vagal NCC have colonised the entire length of the gut. In the stomach, numerous sacral NCC were present in the region external to the circular muscle layers, corresponding to the myenteric plexus (Fig. 11A). In the pre-umbilical intestine, transplanted sacral NCC were numerous within the myenteric plexus region, but few cells were evident within the submucosal region (Fig. 11B). In the colorectum, which is normally colonised by vagal NCC at this stage of development, no transplanted sacral NCC were observed (Fig. 11C).

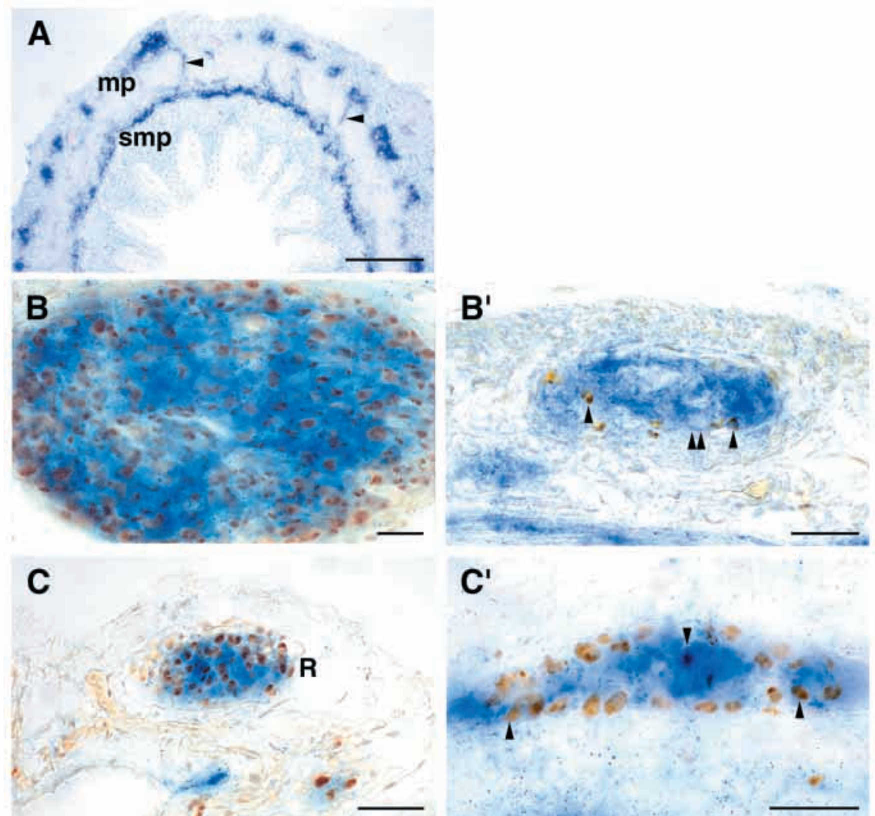


Fig. 8. Ret antibody labelling, chick hindgut, E16. (A) Widespread Ret staining (blue) was present within the myenteric plexus (mp), submucosal plexus (smp) and interconnecting nerve fibres spanning the circular muscular layer (arrowheads). (B,B′) Following orthotopic grafts of the sacral NC, double stained Ret (blue) and QCPN (brown) cells were present within the nerve of Remak (B), myenteric ganglia (B′) and submucosal ganglia (not shown). Double stained cells contained a brown nucleus and blue cytoplasm (arrowheads). Cells immunopositive for Ret and negative for QCPN were also observed (double arrowhead). (C,C′) Vagal-to-sacral heterotopic graft. Ret/QCPN double stained cells were present within the nerve of Remak (C), and within submucosal (arrowheads) and myenteric (not shown) ganglia. Scale bars: 50 μm in A; 25 μm in B,C; 10 μm in B′,C′.

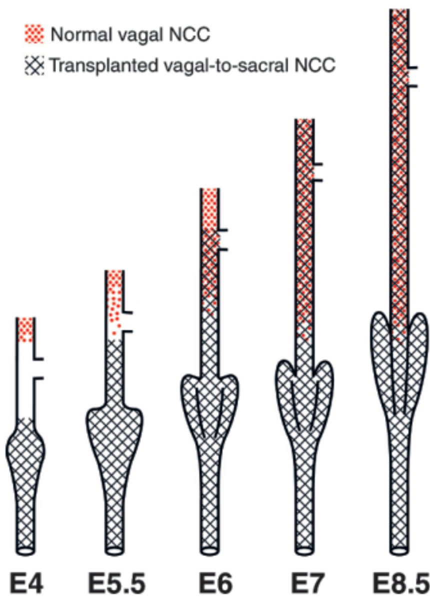


Fig. 9. The spatiotemporal progression of vagal NCC migration fronts within the developing chick gut. Normal vagal NCC (red dots) migrated rostrocaudally and transplanted vagal-to-sacral NCC (blue check) migrated caudorostrally. Intermingling of these two NCC populations occurred after E5.5.

DISCUSSION

In this study, we have demonstrated that when the vagal NC was transplanted into the sacral region of the neuraxis, vagal NCC entered the developing hindgut in large numbers, migrated in a caudorostral direction to the pre-umbilical intestine and formed enteric plexuses. These findings suggest that there are cell autonomous differences in the invasive capacity of vagal and sacral NCC, as vagal-derived NCC colonised the gut via migration pathways normally followed by sacral NCC, but did so in larger numbers, earlier in development. This more invasive nature of vagal NCC may be due to differences in gene expression that result following up- or downregulation of receptors or cell-signalling molecules involved in cell migration/proliferation. Such changes may be a consequence of the difference in age of the crest cells that colonise the gut. Vagal NCC enter migration pathways towards the gut at E1.5, whereas sacral cells begin to migrate towards the gut at E2.5. During this time, changes in cell signalling molecules, either already known or unknown, may occur that cause sacral NCC to be less invasive. In this study we examined the expression of RET, i.e. one of the principal cell-signalling receptors known to be necessary for ENS development (Schuchardt et al., 1994). We found RET immunostaining in vagal NCC, in agreement with the findings of Schiltz et al. (Schiltz et al., 1999), and in a subpopulation of sacral NCC that colonise the hindgut ENS, therefore suggesting that the difference in invasive capacity of sacral and vagal NCC is not due to a lack of RET expression by sacral cells. To our knowledge, this is the first report of the expression of a molecular marker, known to be involved in ENS development, within sacral NCC. However, further studies need to be performed to address the question of why one population of NCCs is more invasive of the gut than another.

Transplanted vagal NCC colonise the hindgut more rapidly than sacral NCC

Following heterotopic grafting of vagal NC into the sacral region of the neuraxis, we found that transplanted vagal NCC immediately began to migrate away from the neural tube along characteristic pathways. Transplanted cells were evident in a superficial lateral pathway, underneath and within the ectoderm, and within a dorsoventral pathway leading to the developing gut. These pathways are similar to those previously reported for migrating vagal NCC in the more rostral region of the embryo (Burns and Le Douarin, 1998). Although the majority of vagal-derived cells appeared within characteristic pathways, occasional transplanted cells were also observed in

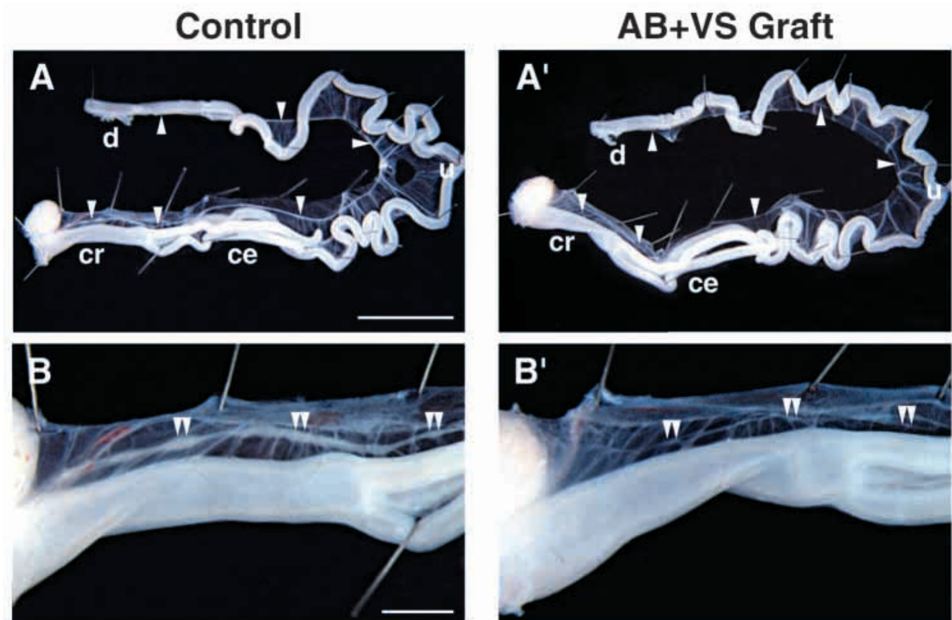


Fig. 10. Vagal-to-sacral heterotopic graft, E16 gut wholemount. (A) The nerve of Remak (arrowheads) extended from the cloaca, along the gut wall to the duodeno-jejunal junction, where the bile and pancreatic ducts (d) entered the gut. (B) Adjacent to the colorectum, the nerve was large in diameter and distinct ganglia were evident (B, double arrowheads). (A′) After vagal-to-sacral grafting, the nerve of Remak extended from the cloaca to the duodeno-jejunal junction, as in controls. However, the nerve was much finer in diameter. (B′) Adjacent to the colorectum, although extremely fine in diameter, the nerve of Remak contained small ganglia (double arrowheads). Scale bars: 10 mm in A; 2 mm in B.

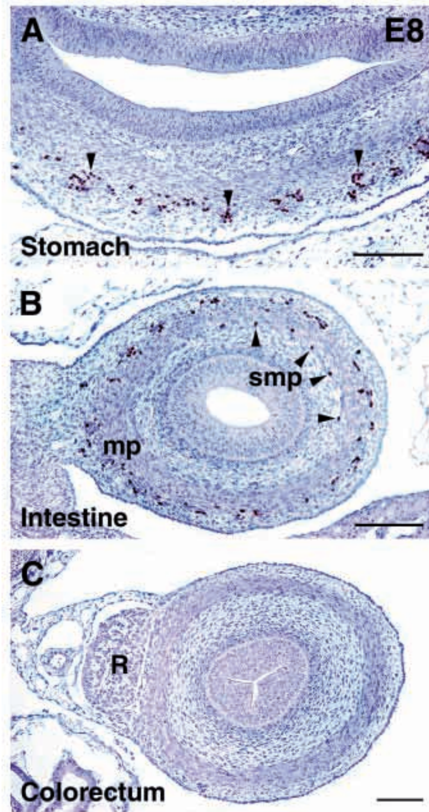


Fig. 11. Sacral-to-vagal heterotopic graft, E8. (A) Numerous transplanted sacral cells (arrowheads) were present external to the circular muscle layers, in the region corresponding to the myenteric plexus. (B) In the intestine, sacral cells were evident within the myenteric plexus (mp) external to the circular muscle. Occasional QCPN+ cells were present in the submucosal plexus region (smp) (arrowheads). (C) No transplanted sacral NC-derived cells were observed in the colorectum at this stage of development. Scale bars: 100 μm.

ectopic locations, such as the mesonephros. Although the fate of these cells was not determined in this investigation, in experiments where quail vagal NC was grafted to the 'adrenomedullary' region of the chick embryo (Le Douarin and Teillet, 1974), these authors subsequently documented connective cells and cartilage within the mesonephritic tissue as heterotopic vagal NC derivatives. A similar fate is likely for the transplanted vagal NCC observed within the mesonephros in our current study.

In contrast to the highly invasive transplanted vagal NCC that enter the developing gut mesenchyme in large numbers almost immediately, sacral NCC do not initially enter the gut, but accumulate in the region adjacent to the gut wall where they form the nerve of Remak from E4. We have previously shown that sacral NCC do not begin to enter the gut wall until at least 4 days later at E8, when the hindgut is already colonised by vagal NCC (Burns and Le Douarin, 1998). The mode of entry of sacral cells into the gut is via axons derived from the nerve of Remak that project to the outer layers of the gut wall, in the region corresponding to the presumptive myenteric plexus (Burns and Le Douarin, 1998). The study of Shepherd and Raper (Shepherd and Raper, 1999) demonstrated that prior to this stage, at E6, the secreted glycoprotein collapsin-1, which belongs to the semaphorin family of molecules (Luo et al., 1993) and acts as an axon repellent (Behar et al., 1996; Messersmith et al., 1995; Puschel et al., 1995), is expressed throughout the rectal wall. However, at E8, collapsin-1 expression retreats from the outer muscle layers to the inner submucosal and mucosal regions allowing axons to project from the nerve of Remak into the gut, which in turn facilitates entry of sacral NCC into the hindgut. Although it is not clear whether collapsin-1 affects the migration of sacral NCC directly, as it affects the axons along which they migrate to gain entry to the gut, collapsin-1 has been reported to be a repulsive signal for NCC migrating in both the hindbrain and trunk regions (Eickholt et al., 1999). Furthermore, these authors also reported that a collapsin-1 receptor, neuropilin-1, is expressed by migrating NCC in these regions, further supporting the functional role of this molecule for NCC patterning. Although this evidence is convincing for these cell populations, it appears that collapsin-1 does not play a role in patterning vagal NCC within the gut. Our previous study has shown that the rectum is colonised by vagal NCC during E7.5-8.5, and that the primary migration pathway for these cells is within the submucosal region of the hindgut (Burns and Le Douarin, 1998). According to the findings of Shepherd and Raper (Shepherd and Raper, 1999), this is the precise period of development when collapsin-1 expression becomes restricted to the sub-mucosal region of the hindgut. Therefore it seems highly unlikely that collapsin-1, in its function role as a repulsive signal for NCC (Eickholt et al., 1999), affects the migration of vagal NCC in this region of the embryo, as these cells are undergoing extensive migration in the specific gut region where collapsin-1 is highly expressed.

In terms of ENS development in the chick, it therefore appears that sacral NCC initially form the nerve of Remak beginning at E4, and projections from this nerve extend into the gut when collapsin-1 expression retreats from the outer muscle layers at E8. Sacral NCC then migrate along these penetrating nerve fibres into the gut and subsequently contribute to the enteric neuronal and glial populations. By contrast, vagal NCC appear to be inherently much more invasive. When transplanted to the sacral region of the neuraxis, these cells, which are unaffected by collapsin-1, immediately enter the developing gut mesenchyme in large numbers, and while an instructive cue to form the nerve of Remak appears to exist, fewer cells are available to contribute to this nerve with the result that the Remak is much reduced in size.

Prespecification of vagal NCC as ENS precursors may permit transplanted vagal NCC to invade the gut from different locations

Vagal NCC appear to be inherently more invasive of the gut than sacral NCC, as these cells were capable of migrating to the gut when (1) transplanted to the sacral level of the neuraxis (results of this study), (2) transplanted to the thoracic level of the neuraxis (Le Douarin and Teillet, 1974) and (3) sections of embryonic gut containing vagal NCC were backtransplanted into younger embryos (Rothman et al., 1993). It was initially suggested by Le Douarin and Teillet (Le Douarin and Teillet, 1974) that some vagal NCC may be pre-specified as ENS precursors, thus enabling them to follow a chemotactic guidance cue to the gut. This theory of pre-specification for

vagal NCC is supported by more recent findings which showed that premigratory vagal NCC express RET (Robertson and Mason, 1995) and *CCK-lacZ* (Lay et al., 1999), respectively. As both RET and CCK are subsequently expressed in enteric ganglia (in addition to other ganglia), it is possible that the NCC positive for RET and CCK prior to migration are those pre-specified to restricted lineages. The experiments carried out by Natarajan et al. (Natarajan et al., 1999) have confirmed that RET-positive cells isolated from mouse bowel are multipotential ENS progenitors. When small numbers, or even individual RET-positive cells were microinjected into the stomach of aganglionic gastrointestinal tracts that were grown in organ culture for 7 days, enteric neurons and glia were subsequently found in the oesophagus, and the small and large intestine, thus highlighting the extensive migratory and proliferative capacities of RET-positive vagal NCC. In this current investigation we have demonstrated that a subpopulation of sacral NCC within the myenteric and submucosal ganglia of the chick hindgut is RET positive. As these cells are less invasive of the gut than are vagal NCC, as discussed above, it appears that other factors, not including RET, account for the difference in invasive capacity of vagal and sacral NCC. To date, it is unclear whether sacral NCC express *CCK-lacZ*.

In contrast to the evidence concerning ENS prespecification for vagal NCC, Erickson and Goins (Erickson and Goins, 2000) have demonstrated that sacral NCC have no special prespecification or migratory properties that allow them to migrate to the gut, as these cells failed to colonise the gut when heterotopically grafted to the thoracic level of the neuraxis in the chick. However, these authors suggested that the environment at the sacral level is sufficient to allow NCC from other axial levels to enter the mesentery and gut mesenchyme, since thoracic NCC transplanted to the sacral level do colonise the gut. We also performed experiments to test the ability of sacral NC to invade the gut when grafted to the vagal region of the neuraxis. After such grafts, sacral NCC colonised the gut and were evident within the presumptive myenteric and to a lesser extent, submucosal regions. However, colonisation was less extensive than for normal vagal NCC at similar stages of development, because at E8, a stage at which the entire length of the gut is colonised by vagal ENS precursors, transplanted sacral cells were not found within the hindgut. It is possible that transplanted sacral NCC may be able to colonise the gut as they are exposed to stronger chemotactic signals when grafted to the vagal level of the neuraxis, or that preferential pathways leading to the gut from the vagal region are more permissive for migration, as originally suggested by Le Douarin and Teillet (Le Douarin and Teillet, 1974) following grafting of truncal crest to the vagal level. Therefore, although the migration pathways to the gut from the sacral level of the neuraxis appear to be sufficient to allow sacral (Burns and Le Douarin, 1998), and even transplanted thoracic NCC to reach the gut (Erickson and Goins, 2000), they do not appear to be as permissive as those at the vagal level where sacral NCC can colonise the gut in significant numbers.

Vagal NCC simultaneously migrate in opposing directions within the gut

We have demonstrated that vagal NCC transplanted to the sacral region of the neuraxis colonise the gut in a caudorostral direction, with the migration front of cells reaching the post-umbilical intestine by E4, the level of the umbilicus by E5.5 and the pre-umbilical intestine by E7. The most rostral region of gut populated by transplanted vagal NCC was found to be within the pre-umbilical intestine, midway between the umbilicus and stomach. This level was reached by E8.5, and even though later stages were examined, transplanted NCC were not observed in more rostral regions. This colonisation pattern is intriguing because in the course of their migration, transplanted vagal NCC encountered (in experiments where the vagal NC was not ablated) normal vagal NCC migrating in the opposing rostrocaudal direction, with the migration fronts of the two NCC populations crossing over and intermingling from E5.5 to E8. During this time period transplanted vagal NCC moved from the post-umbilical intestine to pre-umbilical intestine, while the converse was true of normal vagal NCC. However, as the cecal region and all of the colorectum was extensively colonised by transplanted vagal NCC as early as E4, migration of normal vagal NCC into these hindgut regions did not occur and careful examination of the enteric plexus revealed that virtually all cells were of graft origin (i.e. derived from transplanted vagal NCC).

The mechanisms that guide two populations of vagal NCC simultaneously in opposing directions in the gut is unclear, although a possible explanation may involve ‘cell population pressure’ as a source of migratory drive, as proposed by Hearn et al. (Hearn et al., 1998) for vagal NCC grown in vitro. This theory suggests that population pressure would cause NCC to move away from their point of entry to the gut. Therefore normal vagal NCC that enter the foregut migrate rostrocaudally to the unpopulated regions, and transplanted vagal cells that enter the hindgut in large numbers are ‘forced’ to move to less populated rostral regions. Some intermingling of these cells was found to occur in the intestine, but normal vagal NCC did not migrate to the hindgut, which was already colonised by transplanted NCC, while the rostral migration of transplanted vagal NCC was impeded when these cells encountered the normal vagal NCC that had extensively colonised the proximal intestine. However, the study by Natarajan et al. (Natarajan et al., 1999) outlined above seemingly provides evidence countering the ‘population pressure’ theory for gut colonisation. When these authors microinjected single RET-positive vagal-derived NCC into aneural mouse gut that was then grown in culture, individual cells were found to migrate relatively long distances from the point of entry. In these studies, cells were injected into the stomach region and the resulting migration was either rostrocaudally within the gut or caudorostrally into the oesophagus. It would be interesting, however, to inject isolated cells into the distal hindgut and map their subsequent migration from this alternative point of entry. In this current study, to test whether reducing the numbers of vagal NCC transplanted to the sacral region of the neuraxis affected colonisation of the gut, progressively smaller segments of vagal neural tube were used as donor grafts (not shown). Interestingly, even when the length of the grafted vagal neural tube was reduced by approximately 75%, similar spatiotemporal patterns of vagal NCC were observed within the hindgut, cecal region, and pre- and post-umbilical intestine. As the source of donor NCC was greatly reduced in these experiments, yet an apparently similar number of vagal cells were observed in the gut, at time points mirroring cells derived

from full-length grafts, it appears that vagal NCC are not only highly invasive of the gut, but are capable of altering proliferation in order to form enteric ganglia. This is in contrast to sacral NCC, which in the absence of vagal NCC, colonise the hindgut but do not compensate for lack of vagal-derived ENS precursors (Burns et al., 2000).

In addition to the 'population pressure' theory for gut colonisation, there are also undoubtedly cell-cell signalling interactions that affect the migration of vagal NCC to and within the gut. Interactions may be between NCC and extracellular matrix components within the migration pathways, and/or diffusable chemoattractive molecules that originate in the gut and attract vagal (or sacral) NCC. This latter idea is supported by studies such as those of Rothman et al. (Rothman et al., 1993) mentioned above. In these investigations, when sections of gut already colonised by vagal NCC were backtransplanted into younger embryos, the vagal-derived ENS precursors left the bowel and entered into new host migration pathways. Similarly, in the study of Fontaine-Perus et al. (Fontaine-Perus et al., 1988) when sensory ganglia containing cells of NC origin were either grafted into younger embryos, or cultured adjacent to segments of aneural gut, NC-derived cells migrated into the gut and became positioned within enteric ganglia. As the cells within the sensory ganglia had already reached their final migration sites prior to manipulation, the fact that these cells migrated out of the ganglia and colonised the gut provides strong evidence for the influence of chemotactic signals acting on vagal NCC. Although the precise nature of these signals is still unclear, recent work using organ-cultured gut and explants grown on collagen gel has demonstrated that GDNF, the ligand for the RET/GFRα1 receptor complex, is a chemoattractant for enteric neural cells that promotes directed axon outgrowth and migration of vagal NCC throughout the gut (Young et al., 2001b). Although the mechanism by which GDNF could promote the rostrocaudal migration of vagal NCC is not clear, Young et al. (Young et al., 2001b) surmised that GDNF protein levels could be higher in areas unoccupied by NCC which would act as GDNF 'sinks'. NCC migration could then be influenced by chemoattraction towards these areas with higher levels of GDNF. Although weak GDNF expression has been described in the chick hindgut early in development (Homma et al., 2000), it is unclear if such GDNF 'sinks' exist in embryonic chick gut that could account for the caudorostral migration of transplanted vagal NCC described in this study. In addition to its novel role as a chemoattractant for ENS precursors, GDNF has previously been shown to be necessary for the proliferation and survival of ENS precursors (Hearn et al., 1998; Heuckeroth et al., 1998; Chalazonitis et al., 1998), while EDN3 appears to modulate the effect of GDNF by inhibiting the differentiation of migrating ENS precursors (Hearn et al., 1998; Wu et al., 1999), thus ensuring that sufficient cells are available to colonise the entire length of the gut. Interestingly, in mutations affecting EDN3, either spontaneous (Bolande, 1975; Jacobs-Cohen et al., 1987; Kapur et al., 1993) or targeted (Baynash et al., 1994), only the distal hindgut is aganglionic, suggesting that ENS precursors differentiate, and thus stop migration, before they colonise the terminal bowel. Humans with Hirschsprung's disease also have been identified with *EDN3* mutations and have a similar phenotype of aganglionic hindgut (Bidaud et al., 1997b; Kusafuka and Puri, 1997; Oue and Puri, 1999; Kenny et al., 2000).

In conclusion, we have shown that vagal and sacral NCC possess different invasive capacities of the gut. Vagal NCC, when transplanted to the sacral region of the neuraxis, immediately colonised the gut mesenchyme in large numbers, migrated in a caudorostral direction, differentiated into neuronal phenotypes, and formed enteric plexuses. We have also shown that RET, which is essential for ENS development, is expressed in vagal crest-derived ENS cells, sacral crest-derived cells that comprise the nerve of Remak and within a subpopulation of sacral NCC within hindgut enteric ganglia.

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