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The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system

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SUMMARY

The majority of the enteric nervous system is derived from vagal neural crest cells (NCC), which migrate to the developing gut, proliferate, form plexuses and differentiate into neurons and glia. However, for some time, controversy has existed as to whether cells from the sacral region of the neural crest also contribute to the enteric nervous system. The aim of this study was to investigate the spatiotemporal migration of vagal and sacral NCC within the developing gut and to determine whether the sacral neural crest contributes neurons and glia to the ENS. We utilised quail-chick chimeric grafting in conjunction with antibody labelling to identify graft-derived cells, neurons and glia. We found that vagal NCC migrated ventrally within the embryo and accumulated in the caudal branchial arches before entering the pharyngeal region and colonising the entire length of the gut in a proximodistal direction. During migration, vagal crest cells followed different pathways depending on the region of the gut being colonised. In the pre-umbilical intestine, NCC were evenly distributed throughout the splanchnopleural mesenchyme while, in the post-umbilical intestine, they occurred adjacent to the serosal epithelium. Behind this migration front, NCC became organised into the presumptive Auerbach's and Meissner's plexuses situated on either side of the developing circular muscle layer. The colorectum was found to be colonised in a complex manner. Vagal NCC initially migrated within the submucosa, internal to the circular muscle layer, before migrating outwards, adjacent to blood vessels, towards the myenteric plexus region. In contrast, sacral NCC, which also formed the entire nerve of Remak, were primarily located in the presumptive myenteric plexus region and subsequently migrated inwards towards the submucosal ganglia. Although present throughout the post-umbilical gut, sacral NCC were most numerous in the distal colorectum where they constituted up to 17% of enteric neurons, as identified by double antibody labelling using the quail-cell-specific marker, QCPN and the neuron-specific marker, ANNA-1. Sacral NCC were also immunopositive for the glial-specific antibody, GFAP, thus demonstrating that this region of the neural crest contributes neurons and glia to the enteric nervous system.

Key words: Enteric, Neuron, Glia, Neural crest, Avian, Gut

INTRODUCTION

The embryonic origin of the neurons and glia that form the enteric nervous system (ENS), the intrinsic innervation of the gastrointestinal tract, has long been a subject of investigation. Although Yntema and Hammond (1954, 1955) originally suggested that enteric neuronal precursors are derived from the neural crest, it was in a land-mark study by Le Douarin and Teillet (1973) that the precise location of the neural crest émigrés was determined. These authors, utilising quail-chick grafts, demonstrated that vagal NCC, originating between somites 1 and 7, migrate in a proximal-to-distal direction forming the majority of the ENS along the entire length of the gut. The sacral neural crest, caudal to the 28th pair of somites, was also shown to contribute to the ENS, although only distal to the level of the umbilicus. In the last 25 years, numerous studies have supported the findings of Le Douarin and Teillet concerning the contribution of the vagal neural crest to the ENS (e.g. Cochard and Le Douarin, 1982; Epstein et al., 1994; Tucker et al., 1986), but the contribution of the sacral neural crest to the ENS has remained controversial, with conflicting findings being reported. For example, in studies where the normal proximodistal migration of vagal NCC was prevented, either by excision and culturing of aneural hindgut segments in vitro (Allan and Newgreen, 1980), or by severing the bowel in vivo (Meijers et al., 1989), an ascending contribution to the distal portion of the gut from sacral NCC was not observed. In contrast, when the sacral neural crest was labelled using the fluorescent carbocyanine dye, DiI, (Serbedzija et al., 1991), and/or a replication retrovirus (Pomeranz et al., 1991),

positively marked cells were identified within the post-umbilical bowel. However, although both of these techniques have been useful for demonstrating that a contribution from the sacral neural crest to the hindgut does exist, due to technical limitations of the experimental procedures, the time course and precise migration pathways of migrating sacral NCC were not resolved. Furthermore, these studies were unable to ascertain the cellular derivatives of sacral NCC within the gut, an important issue that has remained unresolved prior to this current study.

The recent development of the monoclonal antibody (mAb) QCPN (available from the Developmental Studies Hybridoma Bank), which recognises a species-specific antigen carried by all quail cells, but not chick cells, has recently provided an invaluable tool for studying the migration of quail cells in quail-chick interspecies chimeric grafts. Although mAbs such as HNK-1 and NC-1 (Vincent et al., 1983; Vincent and Thiery, 1984) have previously been used in immunohistochemical studies to investigate the migration of neural crest cells within the developing gut (Tucker et al., 1986; Pomeranz and Gershon, 1990; Epstein et al., 1991; Luider et al., 1992), the evidence obtained from these studies has not always been conclusive as, in addition to NCC, HNK-1/NC-1 antibodies also recognise a carbohydrate epitope that is expressed by other cell types (Vincent and Thiery, 1984; Newgreen et al., 1990; Meijers et al., 1989). Therefore, due to the limitations of previous studies, we feel it is necessary to address the issue of NCC migration within the gut, using a method that allows individual NCC to be clearly identified and which also permits double labelling of graft-derived cells with other antibodies to enable confirmation of their phenotype.

Here we report the use of quail-chick chimeric grafting in conjunction with antibody labelling to determine the precise time course and migration routes of vagal- and sacral-derived NCC within the developing gut, paying particular attention to the hindgut, which we show to be colonised in a complex manner. This region of the bowel is particularly important developmentally, as failure of NCC to fully colonise the hindgut results in aganglionic megacolon, or Hirschsprung's disease in humans. This condition is characterised by the absence of enteric neurons and glia within the rectum and in a variable length of the distal colon. Aganglionosis may result from a defect in the migration, proliferation or survival of NCC, and/or may result from a defect in the local gut environment. In this study, we demonstrate that the sacral neural crest contributes significantly to the neuronal and glial populations of the ENS within the distal hindgut, raising the question of why cells from this neuraxis fail to compensate for the proximodistal deficiency of vagal-derived NCC in the aganglionic megacolon of mammals.

MATERIALS AND METHODS

Construction of quail-chick chimeras

Fertilised quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus domesticus*) eggs were obtained from commercial sources and maintained as previously described (Catala et al., 1995). The stages of developing embryos were determined either by the number of pairs of somites formed or by reference to the developmental tables of Hamburger and Hamilton (HH) (1951). Isotopic and isochronic grafts consisting of the neural tube from the vagal and sacral regions of the neural crest were performed. The vagal neural tube corresponding to the region between somites 1 and 7 in 10- to 12-somite-stage chick host embryos was surgically removed (Fig. 1A) and replaced by an equivalent stage-matched neural tube from a quail donor, as previously described by Le Douarin and Teillet (1973). Grafts involving the sacral neural crest were performed at the 25-somite stage, as demonstrated by Catala et al. (1995). This grafted region consisted of the neural tube caudal to the 25th pair of somites, and included the posterior neuropore to the level of the chordoneural hinge and the rostral region of the tail bud (Fig. 6A).

Immunohistochemistry

Whole 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 5-7.5 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 QCPN mAb (undiluted supernatant). Following extensive rinsing in PBS, bound antibody was labelled for 1 hour with goat anti-mouse IgG_1 horseradish peroxidase (Southern Biotechnology), diluted 1:50-100 in PBS, and then visualised with diaminobenzidine (DAB, Sigma). Sections were counterstained with Gill's haematoxylin, dehydrated, cleared in toluene and mounted. To obtain frozen sections, tissues were fixed for 6 hours to overnight in freshly made 4% paraformaldehyde in PBS at pH 7.4, rinsed for several hours in PBS containing 15% sucrose, and finally immersed in PBS containing sucrose (15%) and gelatin (7.5%), warmed to 37°C. Specimens were then rapidly frozen in liquid nitrogen-cooled isopentane at −65°C, sectioned at a thickness of 10-20 μm using a cryostat microtome and collected on gelatin-coated microscope slides.

In this study, antibodies to QCPN (Developmental studies hybridoma bank, culture supernatant), 200 kDa neurofilament (Sigma, rabbit IgG, diluted 1:200 in PBS), HNK-1(Tucker et al., 1984, mouse IgM, culture supernatant), 13F4 (Rong et al., 1987, mouse IgM, culture supernatant), QH-1 (Pardanaud et al., 1987, mouse IgM, diluted 1:500 in PBS), ANNA-1 (Altermatt et al., 1991; human IgG, diluted 1:1000, see below) and GFAP (Dako, rabbit IgG, diluted 1:200 in PBS) were used for immunostaining. Where double immunostaining with QCPN and ANNA-1 was performed, QCPN labelling was carried out first as described above but, after secondary labelling and DAB staining, sections were rinsed extensively in PBS, then incubated overnight at 4°C with ANNA-1, diluted 1:1000 in PBS containing 20% goat serum, 0.3% Triton-X-100, and 0.05% sodium azide. Bound ANNA-1 was then labelled with biotinylated anti-human IgG (1:100 in PBS), and visualised with alkaline phosphatase in Tris buffer (Vector AP kit III). Similar sequential incubations were also performed when double labelling with QCPN and GFAP or neurofilament antibodies was required.

NADPH-diaphorase staining

Paraformaldehyde-fixed frozen sections (obtained as described above) were stained for NADPH-diaphorase activity by incubation in PBS (pH 7.4) containing 1 mg/ml β-NADPH and 0.5 mg/ml Nitroblue tetrazolium (both Sigma) for 10-30 minutes at 37°C. The NADPH-diaphorase reaction product appeared as a dark-blue granular deposit and the reaction was terminated by immersing the sections in cold PBS. For double labelling with QCPN, sections were incubated with the primary and secondary antibodies as described above, then the NADPH-d reaction was performed prior to the DAB revelation, which was carried out last. Sections were finally rinsed in PBS and mounted in an aqueous mounting medium.

Quantitative analysis

In order to quantify the number of immunopositive cells in various regions of the gut, dissected gastrointestinal tracts were serially sectioned and collected on microscope slides. A fraction of the total number of slides was selected e.g. every second or fourth slide as

appropriate, depending on the total number of slides. Then, on each selected slide, five non-adjacent sections, chosen at random, were examined and the numbers of immunopositive cells recorded. For statistical analysis, a minimum of three animals was examined at each stage of development.

RESULTS

Vagal NCC colonise the entire bowel via proximodistal migration

Approximately 1 day after grafts were performed, at HH16-17 (E2-2.5), QCPN-positive cells were observed adjacent to the grafted donor neural tube in the regions of the dorsolateral pathway, underneath and within the ectoderm (Fig. 1B), and within the ventral pathway, adjacent to the myotome, leading towards the developing pharyngeal gut (Fig. 1C). By HH20, large numbers of vagal-derived cells had accumulated within the caudal branchial arches (Fig. 1D), from where they entered the gut mesenchyme, and migrated distally. By HH25, immunopositive cells were evenly distributed within the gizzard (Fig. 1E) and the duodenum (Fig. 1F), while the migration front was evident as occasional cells located within the dorsal aspect of the gut wall, proximal to the umbilicus (Fig. 1G). By HH26 (E5), graft-derived cells were present within the entire pre-umbilical gut, including the esophagus (Fig. 2A), proventriculus (Fig. 2B) and gizzard (Fig. 2C). At this stage, the caudal migration front was at the level of the umbilicus, where NCC were randomly scattered throughout the gut mesenchyme (Fig. 2D). In the post-umbilical intestine at HH28 (E5.5-6), the migration front consisted of a few cells within the outermost layers of the splanchnopleural mesenchyme, adjacent to the serosal epithelium (Fig. 2E). At HH29 (E6-6.5), the migration front of graft-derived cells had reached the cecal region. Here immunopositive cells were evenly distributed within the intestinal mesenchyme and occasional cells were also present within the developing cecal buds. By HH30 (E7), the entire cecal region including the cecal buds was colonised and the migration front of cells was within the area of the cecal-colorectal junction. Graft-derived cells were grouped on either side of the developing circular muscle layer, in the regions corresponding to the presumptive external myenteric (Auerbach's) plexus and the internal submucosal (Meissner's) plexus (Fig. 2G). The circular smooth muscle layer was evident in transverse histological sections as a dense band of cells encircling the gut between the submucosa and the serosa (Fig. 2G), which was immunopositive for the muscle-specific antibody, 13F4 (Fig. 2H).

At HH31 (E7.5), graft-derived cells were first observed within the colorectum. The migration front appeared as infrequent, isolated cells within the submucosal region adjacent to the inner aspect of the circular muscle layer (Fig. 3Aiv), while rostrally, QCPN-positive cells entirely encircled

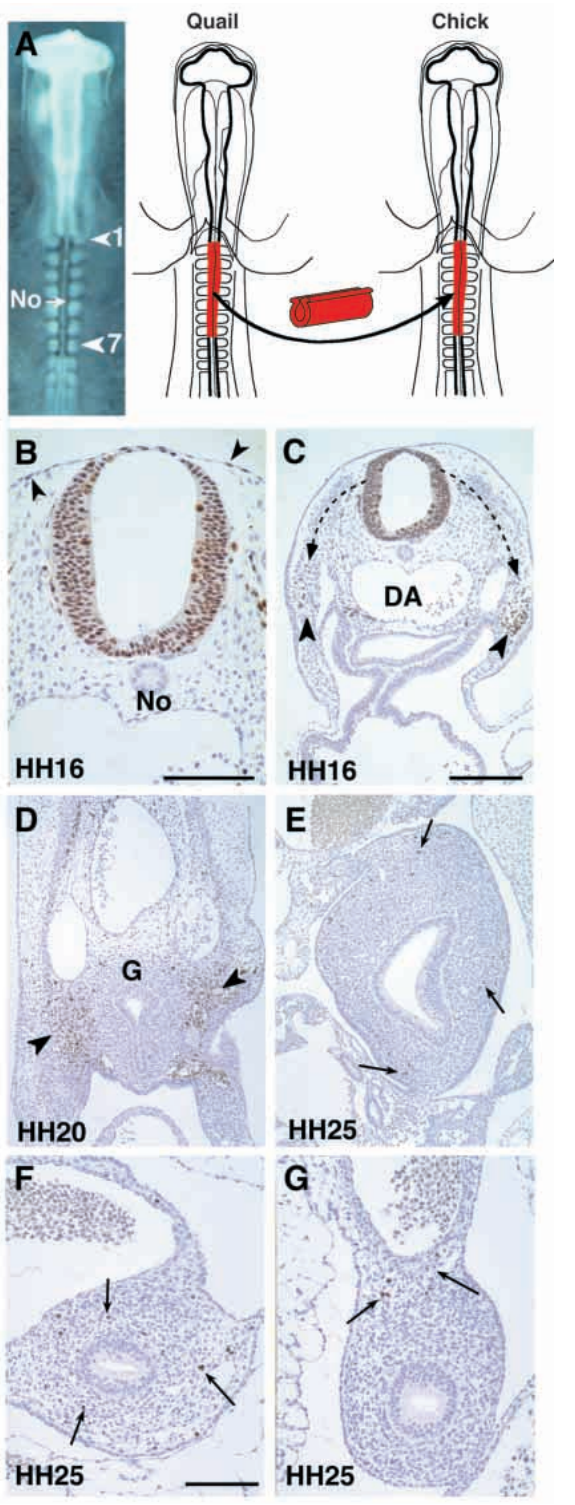

Fig. 1. Quail-chick grafting of the vagal neural tube and early migration of NCC. (A) The 'vagal' neural tube between somites 1 and 7 (arrowheads) was surgically removed from 10-somite-stage chick embryos leaving the underlying notochord (No, arrow) intact, and replaced with the vagal neural tube from quail embryos at the equivalent stage of development. (B,C) Vagal region, HH16. NCC were adjacent to the immunopositive quail neural tube underneath and within the ectoderm (arrowheads), and within the ventral pathway (dashed arrows) lateral to the dorsal aorta (DA) where they accumulated in the caudal branchial arches (arrowheads). (D) Pharyngeal gut, HH20. Large numbers of NCC were present within the caudal branchial arches (arrowheads), adjacent to the splanchnopleural mesenchyme of the developing pharyngeal gut (G). (E,F) Gizzard, duodenum, HH25. NCC (arrows) were evenly scattered within the splanchnopleural mesenchyme. (G) Pre-umbilical small intestine, HH25. The migration front of NCC (arrows) was within the dorsal aspect of the developing gut, adjacent to the dorsal aorta. Bars: B,F,G, 100 μm; C-E, 200 μm.

the submucosal region of the gut (Fig. 3Aii). By HH33-34 (E8.5), the migration front had reached the terminal colorectum (Fig. 3Bv); therefore, at this stage, the entire length of the gut was colonised by vagal NCC. In the rostral colorectum, graft-derived cells were most numerous within the submucosal region where they were clustered together to form primitive ganglia (Fig. 3Bi). Immunopositive cells were also observed external to the circular muscle layer, at the level of the presumptive myenteric plexus (Fig. 3Bi,ii). These cells reached this outer plexus via migration through the circular muscle from the already colonised submucosa (Fig. 3Biii) and appeared to traverse the circular muscle layer adjacent to blood vessels which interconnected the myentric and submucosal plexuses (Fig. 4). By HH35-36 (E9.5-10), the submucosal and myenteric ganglion regions of the entire colorectum were colonised by vagal NCC which were grouped into distinct ganglia (Fig. 3C).

Anti-neurofilament expression primarily occurs in the myenteric plexus region

When vagal grafted tissues were double stained with QCPN and anti-neurofilament mAbs at HH33, neurofilament-positive fibre tracts derived from the nerve of Remak were found to penetrate the gut wall external to the circular muscle layer, at the level of the myenteric plexus (Fig. 5A). At this stage, anti-neurofilament labelling was restricted to the myenteric plexus region, and was not present within the submucosal ganglia of either the intestine or hindgut (Fig. 5B,C). Although we have demonstrated that, in the colorectum, vagal NCC colonise the submucosal region at least 1 day before the myenteric plexus region exists, it appears that NCC in the later forming myenteric plexus develop a neuronal phenotype first.

Sacral NCC colonise the hindgut via distal-proximal migration

Early development of the nerve of Remak

Approximately 1 day after sacral neural crest grafts (Fig. 6A) were performed, NCC had migrated from the neural tube and formed dorsal root ganglia (Fig. 6B). These cells also migrated ventrally, in close proximity to the dorsal aorta and accumulated within the dorsal wall of the developing mesorectum by HH24 (E4.5) (Fig. 6C,D), forming the rudiment of the nerve of Remak (Fig. 6E,F). Graft-derived cells comprising the nerve of Remak migrated along the axis of the gut in a rostral direction reaching their most anterior level, the opening of the bile and pancreatic ducts, by HH29 (E6). In the hindgut, the nerve of Remak was large in calibre and was situated adjacent to the dorsal aspect of the gut wall (Fig. 7A), whereas rostrally, at the levels of the ileum and jejunum, the nerve was fine in calibre and consisted of small ganglia distributed along its length.

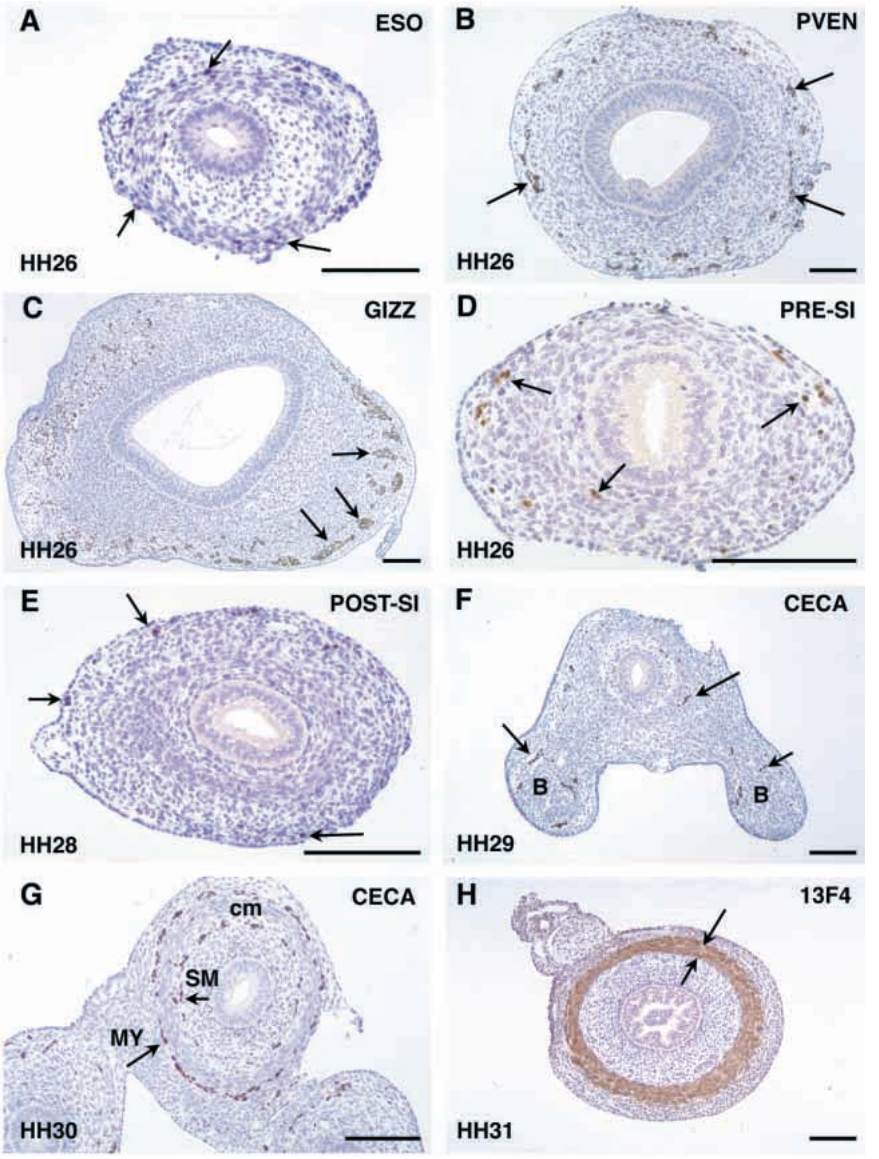

Fig. 2. Proximodistal migration of vagal neural-crest-derived cells. (A-C) Esophagus, proventriculus and gizzard, HH26. NCC (arrows) were distributed within the outer layers of the splanchnopleural mesenchyme. Within the gizzard (panel C), crest cells were grouped together to form presumptive ganglia (arrows). (D) Pre-umbilical intestine, HH26. QCPN-immunopositive cells (arrows) were scattered throughout the splanchnopleural mesenchyme. (E) Post-umbilical small intestine, HH28. The migration front of NCC (arrows) was within the outermost layers of the splanchnopleural mesenchyme, adjacent to the serosal epithelium. (F) Cecal region, HH29. NCC (arrows) were within the mesenchyme of the intestine and the cecal buds (B). (G) Cecal region, HH30. NCC were grouped into presumptive ganglionic plexuses, external (Auerbach's or myenteric plexus, MY arrow) and internal (Meissner's or submucosal plexus, SM arrow) to the developing circular muscle layer (CM). (H) Colorectum, 13F4 antibody staining, HH31. A dense circular band (arrows) of smooth muscle encircling the gut was 13F4-immunopositive. Bars, 100 μm.

Table 1. Number (mean±s.e.m.) of sacral neural crest derived (QCPN-positive) cells per histological section within different regions of the developing gut

Embryonic day		Colorectum			Ceca		Post-umbilical small intestine
		Distal	Mid	Rostral	Distal	Rostral	
8		6.5 (0.6)	2.4 (0.3)	2.1 (0.3)	2.9 (0.4)	2.7 (0.5)	1.3 (0.3)
10	myenteric plexus	20.3 (2.4)	5.6 (0.8)	1.4 (0.3)	2.9 (0.5)	2.2 (0.6)	0.9 (0.6)
	submucosal plexus	0.6 (0.2)	0.2 (0.1)	0	0	0	0
12	myenteric plexus	173.5 (8.5)	57.0 (4.7)	26.7 (3.0)	16.7 (2.9)	10.4 (2.2)	2.9 (0.6)
	submucosal plexus	17.3 (3.4)	5.5 (0.8)	5.0 (1.2)	4.1 (1.2)	2.5 (0.6)	0
16	myenteric plexus	244.8 (19.8)	103.0 (13.0)	22.0 (2.5)	22.8 (2.8)	5.8 (1.1)	3.1 (0.5)
	submucosal plexus	10.7 (1.5)	10.1 (1.9)	0.8 (0.3)	3.6 (0.7)	0.6 (0.5)	0

Contribution of sacral NCC to the ENS

Sacral NCC were observed in the hindgut from HH31 (E7.5). These cells occurred in close association with nerve fibres derived from Remak's nerve, which penetrated the gut wall in a dorsoventral direction primarily at the level of the presumptive myenteric plexus (Fig. 7A,B). In the next few days of development (HH31-36, E7.5-10), the length of nerve fibres and numbers of associated cells penetrating the gut wall increased (Fig. 7C). At this time, the majority of sacral NCC within the gut were associated with nerve fibre tracts and few sacral cells were observed within enteric ganglia. From E10, the number of sacral NCC within the gut increased dramatically, with the maximum increase in numbers occurring between days 10 and 12 (Table 1; Fig. 8), when sacral-derived cells became incorporated within myenteric and submucosal ganglia (Fig. 7D-F). The vast majority of graft-derived cells were present within the colorectum and were much less numerous in the ceca and the post-umbilical intestine, appearing as only very occasional cells on histological sections (see Table 2). Sacral graft-derived cells were never observed in any region of the gut proximal to the umbilicus. Within the hindgut, sacral NCC were present along a distal-proximal gradient, being most numerous in the distal colorectum (244.8±19.8, mean ± s.e.m., cells per section at E16) and less numerous towards the rectal-cecal junction (22±2.5 cells per section at E16). These numbers fell to 5.8±1.1 and 3.1±0.5 cells per section in the rostral ceca and post-umbilical intestine respectively (Table 1). The distribution of sacral-crest-derived cells also followed a dorsoventral pattern within the hindgut. Although they entirely encircled the most distal hindgut, proximally, they were more dorsally located and occurred close to Remak's nerve. In addition, sacral NCC were more numerous in the myenteric plexus region, compared to the submucosal plexus region (see Fig. 7D). For example, at E16, 244.8±19.8 cells per section was present in the myenteric plexus region of the distal colorectum compared with 10.7±1.5 within the submucosa of the same sample. This finding was mirrored in all regions of the gut examined (see Table 1).

To investigate whether the surgical grafting procedure between quail and chick embryos affected the migration of NCC, sections of control chick gut were examined using the neural crest cell antibody, HNK-1 (Tucker et al., 1984). Although this antibody does not distinguish between vagal and

Table 2. Percentage of sacral neural crest derived neurons within the colorectum at embryonic day 16

	No. Neurons		No. QCPN		No. Neurons/ QCPN		% Neurons/ QCPN	
	MY	SM	MY	SM	MY	SM	MY	SM
Rostral-colorectum	163 (17.2)	225.6 (16.1)	18.6 (4.4)	0.9 (0.35)	0.3 (0.3)	0.1 (0.1)	0.3 (0.3)	0.1 (0.1)
Mid-colorectum	143.8 (11.6)	187.4 (9.0)	160.4 (30.4)	12.3 (2.9)	7.7 (2.9)	2.8 (1.0)	5.2 (1.7)	2.0 (0.9)
Distal-colorectum	166.1 (8.5)	158.4 (21.3)	237.8 (20.6)	12.3 (3.5)	28.7 (5.9)	2.2 (1.2)	17.4 (3.4)	1.3 (0.6)

Number (mean±s.e.m.) of neurons, was determined by ANNA-1 immunoreactivity and sacral neural crest derived cells by QCPN immunoreactivity. MY, myenteric plexus region; SM, submucosal plexus region.

sacral NCC, since it indiscriminately labels all NCC, spatiotemporal staining patterns were similar to the findings for grafted tissues described above. At HH32 (E8), HNK-1-positive cells were present at the level of the submucosa (corresponding to the vagal-derived cells in Fig. 3), and Remak-derived nerve fibres also penetrated the gut wall, exterior to the circular muscle layer (Fig. 5D) corresponding to Figs 5A and 7B,C. Immunopositive fibres interconnecting the myenteric and submucosal regions were also observed, although it was not possible to determine from which plexus these fibres originated.

Phenotypic derivatives of sacral NCC within the ENS

Neuronal labelling with ANNA-1 immunohistochemistry

The neuronal-specific marker ANNA-1 labelled all enteric neurons within the myenteric and submucosal ganglia (Fig. 9A), and neurons within the nerve of Remak. ANNA-1-immunopositive cells contained large, round, unstained nuclei, with densely stained cell bodies (Fig. 9B). Since QCPN specifically labels the perinuclear cytoplasm of quail cells, it was therefore possible to identify ANNA-1-positive neurons, QCPN-positive cells and double-labelled (i.e. sacral neural-crest-derived) neurons within individual ganglia (Fig. 9B). In order to quantify the number of sacral-crest-derived neurons within the colorectum, the number of neurons, QCPN-positive cells and double-labelled cells were counted and the data presented in Table 2. The colorectum was arbitrarily divided into three regions of equal length – rostral (nearest the cecal-colorectal junction), mid and distal. In the distal region, sacral NCC constituted 17.4%±3.4 (mean ± s.e.m.) of neurons within the myenteric plexus and 1.3%±0.6 within the submucosal plexus. This compared to 5.2%±1.7 and 2%±0.9 in the mid hindgut and 0.3%±0.3 and 0.1%±0.1 in the myenteric and submucosal plexuses of the rostral hindgut. The maximum number of sacral-crest-derived neurons recorded in any specimen was 31%, within the myenteric plexus of the distal colorectum at E16.

Neuronal labelling with NADPH-diaphorase histochemistry

NADPH-diaphorase is co-localised in neurons containing nitric oxide synthase (Ward et al., 1992), the enzyme involved in the production of the inhibitory neurotransmitter, nitric oxide. NADPH-d histochemistry was performed to investigate whether sacral NCC contribute specifically to this neuronal subpopulation within the colorectum. NADPH-d labelled neurons within the myenteric and submucosal ganglia of the hindgut and the nerve of Remak (Fig. 9C,D). When double labelling with QCPN was performed, double-stained cells were observed within the nerve of Remak (Fig. 9C), although such cells were scarce within enteric ganglia (Fig. 9D) and did not constitute a significant contribution from the sacral neural crest (data not shown).

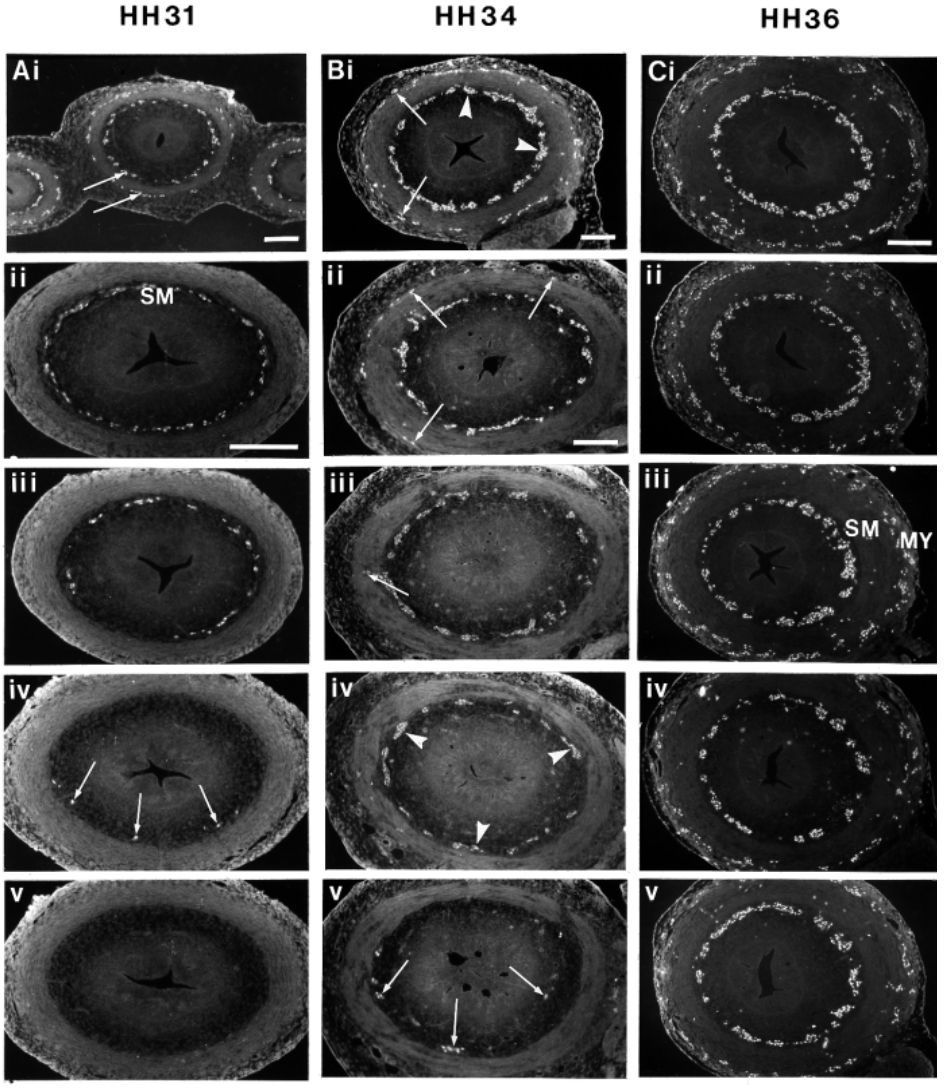

Fig. 3. Colonisation of the colorectum by vagal neural-crest-derived cells. (A) Colorectum, HH31. In the cecal region (panel i), presumptive ganglia (arrows) were present on either side of the circular muscle layer, in both the intestine and the cecal buds. The dorsal colorectum (panel v) did not contain NCC, while in the mid colorectum (panel iv), the migration front of cells (arrows) was within the submucosa. Rostrally (panel ii), NCC fully encircled the gut within the submucosa (SM). (B) Colorectum, HH34. The migration front was within the submucosa of the most distal region (panel v, arrows). Presumptive submucosal ganglia were present rostrally (panels i, iv, arrowheads). Some QCPN-immunopositive cells were observed external to the circular muscle layer (panel i,ii, arrows) and occasional NCC appeared to penetrate into the muscle layer from the submucosal region (panel iii, arrow). (C) Colorectum, HH35. NCC were grouped into myenteric (MY) and submucosal (SM) ganglia along the entire length of the colorectum. Bars, 100 μm.

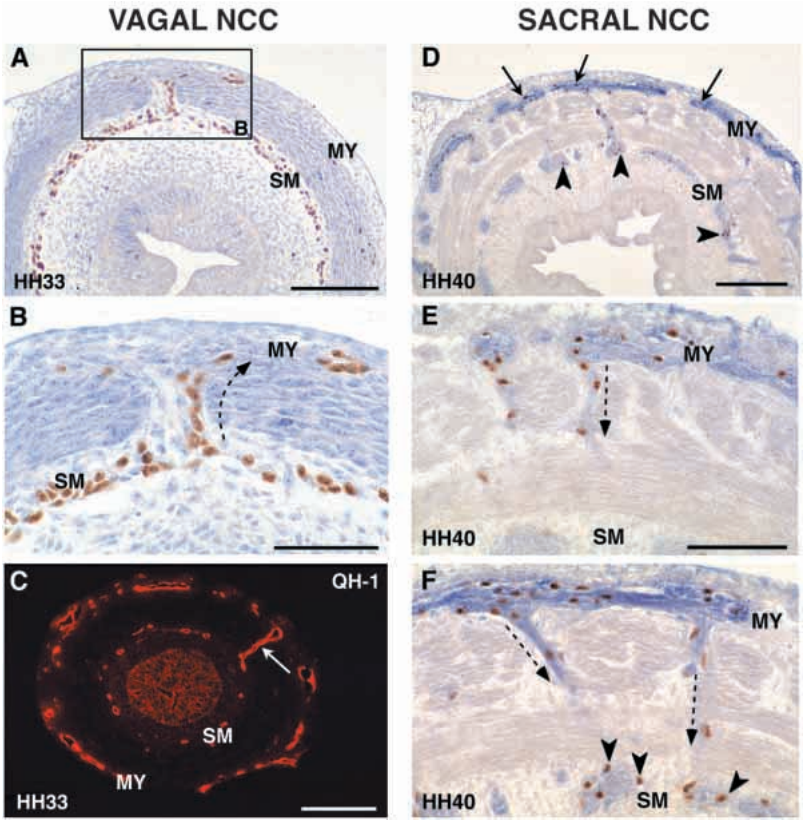

Fig. 4. Vagal and sacral NCC colonisation of the myenteric and submucosal plexus regions of the colorectum. (A,B) Colorectum HH33. Many NCC were present within the submucosal (SM) region. Some crest-derived cells within the circular muscle layer interconnected (dashed arrow) the submucosa and the presumptive myenteric (MY) plexus, external to the circular muscle layer. (C) Colorectum HH33, anti-QH-1 antibody staining. Blood vessels were present within the submucosa (SM) and the presumptive myenteric plexus (MY), and interconnected (arrow) these two regions. (D) Colorectum HH40. Many sacral NCC were present within the anti-neurofilament-positive (blue stained) myenteric plexus region (arrows), although occasional cells were also evident within the submucosal region (arrowheads). (E,F) Colorectum HH40. Sacral NCC were found within nerve fibre tracts penetrating the muscle layers (dashed arrows). These nerve fibres extended from the myenteric plexus (MY) to the submucosal region (SM), where NCC became incorporated within ganglia (arrowheads). Bars: A,C,D, 100 μm; B,E,F, 50 μm.

Glial labelling with GFAP immunohistochemistry

As the majority of sacral NCC within the hindgut were found to be non-neuronal, their phenotype was investigated using antibodies directed against glial fibrillary acidic protein (GFAP), which occurs in high levels in cytoplasmic intermediate filaments of all glial cells. In double-labelling experiments with QCPN, GFAP-immunopositive cells were present within the nerve of Remak (Fig. 9E), where large neurons were often surrounded by smaller GFAP/QCPN immunopositive cells. Numerous double-labelled cells were also evident within myenteric and submucosal ganglia (Fig. 9F).

DISCUSSION

We have utilised quail-chick chimeric grafting and antibody labelling to determine the precise migration routes of vagal and sacral NCC within the developing gut of the chick embryo. In addition, we have determined the extent of the contribution from the sacral neural crest to the ENS and demonstrated that, in addition to glia, cells from this region of the neural crest contribute significantly to the neuronal population, particularly in the distal colorectum.

Colonisation of the bowel by vagal NCC proceeds according to different migration routes in the pre- and post-umbilical gut

On leaving the neural crest, vagal NCC migrated along two pathways, the dorsolateral pathway underneath the ectoderm, and the ventral pathway within the anterior moiety of the sclerotome of each somite, towards the developing pharyngeal gut. Cells in this latter pathway accumulated within the caudal branchial arches, as previously shown by Noden (1975), Ciment and Weston (1983), Payette et al. (1984) and Tucker et al. (1986). On entering the gut on E3 of development, NCC migrated in a caudal direction, and reached the umbilicus by E5, the ceca by E6.5 and the cecal-colorectal junction by E7. The colorectum was colonised between E7.5 and 10. The general proximodistal progression of NCC was similar to the temporal sequence observed by Tucker et al. (1986) and Epstein et al. (1991) using the neural-crest-specific antibodies NC-1 and HNK-1, respectively, and Fairman et al. (1995) using the neuron-specific antibody ANNA-1. This once more confirms that the developmental processes observed in quail-chick chimeras accurately reflects those occurring in non-grafted embryos.

As vagal NCC migrated within the bowel, a developmental change in their distribution was observed. In the pre-umbilical intestine, the migration front occurred close to the dorsal aspect of the gut wall while, in the post-umbilical intestine, QCPN-immunopositive cells were located within the outermost layers of the developing splanchnopleural mesenchyme, adjacent to the serosal epithelium. Behind this migration front, NCC were scattered within the splanchnopleural mesenchyme, but as the circular muscle layer developed, they became organised into presumptive ganglia internal and external to the muscle layer, as previously shown by Tucker et al. (1986), using the NCC marker NC-1. We also observed an as yet undescribed phenomenon: in the colorectum, vagal NCC primarily colonised the submucosal region, internal to the circular muscle from E7-8.5 from where they subsequently migrated

through the muscle layer, adjacent to blood vessels, to colonise the myenteric plexus region 1-2 days later.

It was previously believed that during colonisation of the hindgut, the presumptive myenteric plexus region was the first area to be colonised by NCC. This theory was based on information obtained from studies employing markers for the early identification of enteric neurons in the chick (Epstein et al., 1980; Gershon et al., 1980; Payette et al., 1984), the mouse (Pham et al., 1991) and the rat (Matini et al., 1997). In all these studies, neuronal markers were first observed in the myenteric plexus and, consequently, it was postulated that the submucosal plexus arises from the secondary migration of neural precursors from the myenteric plexus. However, this does not appear to be the case in the chick, as we have clearly shown that the submucosal region of the colorectum is primarily colonised by NCC, approximately 24 hours before the myenteric plexus forms. When double-labelling experiments were performed on grafted tissues, using QCPN and anti-neurofilament antibodies, the myenteric plexus of the colorectum and the cecal region was found to express neurofilament first, in agreement with the study of Payette et al. (1984). It therefore appears that, in the chick, although NCC primarily colonise the submucosal plexus of the colorectum, the ganglion cells within the myenteric plexus are the first to develop a neuronal phenotype. It is not yet clear what cellular interactions trigger neuronal differentiation in the cells occupying these sites and further investigation is needed in this area.

Colonisation of the bowel by sacral NCC proceeds according to distal-proximal migration and results in the formation of enteric neurons and glia

The contribution of the posterior neural crest to the hindgut has been shown by Le Douarin and Teillet (1973) to correspond to the segment of neuraxis located caudal to the 28th pair of somites. More recently, the data presented by Catala et al. (1995), concerning the development of the tail bud, were invaluable in determining the precise stage and territories of the embryo required for sacral NCC to colonise the bowel.

Sacral NCC were found to migrate in a distal-to-proximal direction, forming the entire nerve of Remak adjacent to the gut wall from E4. However, few sacral crest cells were observed within the wall of the hindgut prior to E7, when NCC associated with fibre tracts derived from the nerve of Remak penetrated the wall of the colorectum, primarily at the level of the myenteric plexus. The numbers of sacral NCC within the hindgut increased dramatically between E10 and E12, although they were always most numerous within the distal colorectum, where they fully encircled the gut and constituted up to 17% of neurons within the myenteric plexus. Sacral-derived cells were also found to be immunopositive for the glial-specific marker GFAP, thus confirming that this region of the neural crest contributes neurons and glia to the ENS. In the mid and rostral colorectum, NCC were evident along a dorsoventral gradient, as they did not fully encircle the gut but occurred closer to the nerve of Remak, as previously described by Serbedzija et al. (1991). In the cecal region and post-umbilical intestine, sacral NCC were evident in much smaller numbers, constituting less than 1% of enteric neurons and were only present close to the nerve of Remak. Sacral graft-derived cells were not observed within the gut proximal to the level of the umbilicus even at the latest time of observation (E17).

In previous studies, where the contribution of the sacral neural crest to the gut was investigated using either antibody staining (Pomeranz and Gershon, 1990) or by cell marking with microinjection of fluorescent dye or replication-deficient retrovirus (Pomeranz et al., 1991; Serbedzija et al., 1991), labelled cells were observed within the gut much earlier than was found in this study and in previous quail-chick grafting experiments (Le Douarin and Teillet, 1973; Teillet, 1978; Catala et al., 1995). Using the neural crest cell antibody NC-1, Pomeranz and Gershon (1990) observed two immunopositive streams of cells, described as rostral and caudal, extending from the sacral crest to the hindgut early in development at E3.5. The rostral group, which co-expressed neural markers, became embedded within the mesenchyme of the dorsal bowel by E4, and gave rise to the ganglion of Remak.

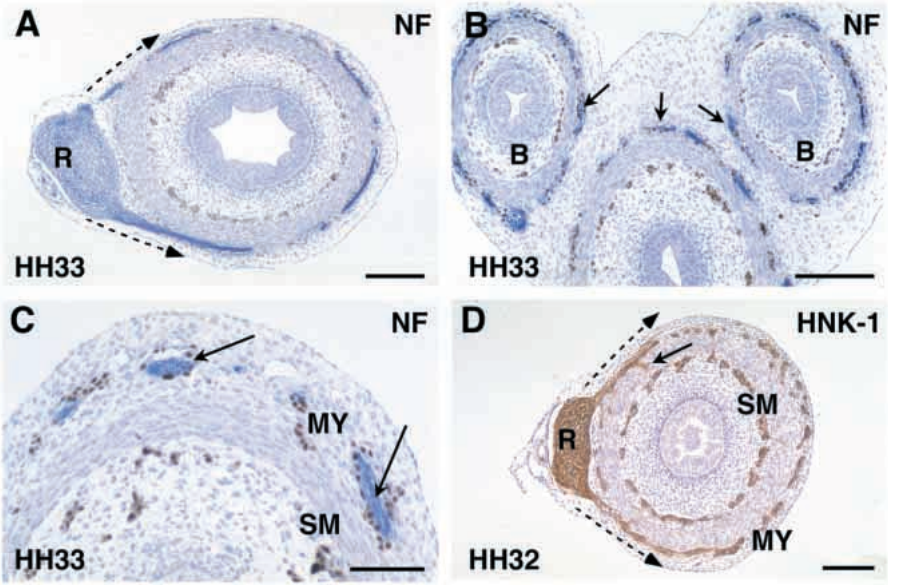

Fig. 5. Anti-neurofilament antibody and QCPN double labelling within the colorectal and cecal regions. (A) Colorectum, HH33. Anti-neurofilament antibody (blue) stained the nerve of Remak (R) and nerve fibre tracts which penetrated the gut external to the circular muscle at the level of the myenteric plexus (dashed arrows). (B) Ceca, HH33. Anti-neurofilament immunostaining occurred only in the myenteric plexus region (arrows) of the intestine and cecal buds (B). (C) Colorectum, HH33. High magnification confirmed that anti-neurofilament staining (arrows) was restricted to the myenteric (MY) ganglia and was absent within the submucosal region (SM). (D) Colorectum, HNK-1 antibody labelling, HH32. Immunostaining was present within the nerve of Remak (R), and Remak-derived nerve fibres which penetrated into the gut (dashed arrows). The myenteric (MY) and submucosal (SM) plexus regions, and fibres interconnecting the plexuses (arrow) were also immunopositive. Bars: A,B,D, 100 μm; C, 50 μm.

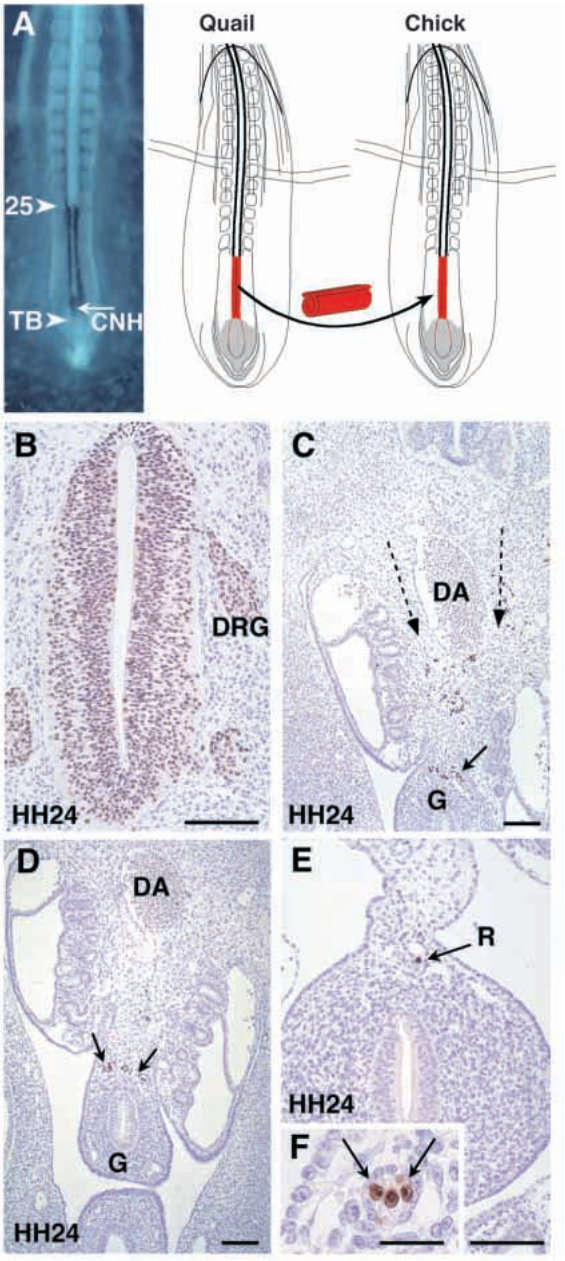

Fig. 6. Quail-chick sacral neural crest grafting and early migration of NCC. (A) The sacral neural tube was surgically removed from chick embryos and replaced with the equivalent tissue from quail embryos at the same stage of development. Grafts consisted of the neural tube caudal to the 25th pair of somites (25, arrowhead) and included the chordoneural hinge (CNH, arrow) and rostral region of the tail bud (TB, arrowhead). (B) Sacral region, HH24. NCC formed dorsal root ganglia (DRG) adjacent to the neural tube. (C,D) Sacral region, HH24. NCC migrated ventrally (dashed arrows) from the neural tube, in close proximity to the dorsal aorta (DA), and accumulated (arrows) within the dorsal wall of the hindgut (G). (E,F) Hindgut, HH24. The primitive nerve of Remak consisted of a few cells (arrows) aggregated within the dorsal wall of the colorectum. Bars: B-E, 100 μm; F, 25 μm.

This observation is in general agreement with the findings of this current study, as sacral NCC were shown to aggregate within the dorsal wall of the mesorectum forming the rudiment of Remak's nerve from E4. Similarly, using the neuron-specific marker ANNA-1, Fairman et al. (1995) observed immunopositive cells within the developing nerve of Remak from E3.5. Although the caudal stream of NC-1-positive cells described by Pomeranz and Gershon (1990) encircling the gut at E5 did not express neuronal markers, when sections of gut containing only this population of cells were cultured, neurons and glia developed in the explants, suggesting the presence of neurogenic cells within the gut at this stage. As we have not observed sacral NCC within the gut wall prior to E7, and not in significant numbers before E10, a discrepancy clearly exists between these studies. As NC-1 is not entirely neural crest-specific (Vincent and Thiery, 1984; Newgreen et al., 1990), it is possible that the immunopositive cells described as encircling the gut at E5 were of mesenchymal origin, a theory supported by the fact that they did not co-express neuronal markers. However, why the explants cultured at E4-5 developed neurons and glia is not clear as, in other similar culture experiments carried out using hindgut explants at similar of later stages (E6-7), neurons and glia did not develop (Allen and Newgreen, 1980; Smith et al., 1977). A possible explanation is that the explants were contaminated with sacral NCC derived from the nerve of Remak, although the developing nerve was apparently removed prior to culture.

In other experiments where the sacral neural crest was labelled using either the vital fluorescent dye, DiI or the replication-deficient retrovirus LZ10, positively stained cells were also observed within the post-umbilical gut at E4-5. When Pomeranz et al. (1991) injected DiI or LZ10 into the sacral neural crest, positive cells were observed within Remaks's nerve and within the post-umbilical bowel. In apparent agreement with the above findings, Serbedzija et al. (1991), injected DiI into the sacral crest of both chick and mouse embryos, and found that at E4 labelled cells were observed within the mesenchyme surrounding the gut and also within the gut epithelium. These observations, like the finding of Pomeranz et al. (1990) with NC-1, would suggest that sacral NCC appear within the hindgut at E4-5, i.e. at least 2 days before we observed the first sacral crest cells within the hindgut and 5-7 days before significant numbers of graft-derived cells were observed within enteric ganglia. It is likely, however, that, in the studies of Pomeranz et al. (1991) and Serbedzija et al. (1991), as well as sacral NCC, other germ layers were also labelled, as in addition to presumptive ganglion cells, cells within the gut mesenchyme and the epithelium were also positively marked. Therefore in the studies described above, it is possible that among the labelled cells (NC-1, DiI or LZ10), not only were sacral NCC contributing to the ENS present, but also cells of mesenchymal or other origin. As double labelling with neuronal or glial markers was not performed in the studies of Pomeranz et al. (1991) and Serbedzija et al. (1991), the phenotype of labelled cells was not determined while, in the study of Pomeranz et al. (1990), the early NC-1-positive cells encircling the gut were found to be negative for neuronal markers. In contrast, in this current study, we have double-labelled sacral NCC in the gut with neuronal and glia markers, thus confirming their phenotype. Our findings regarding the timing of appearance of NCC within the hindgut are supported by the studies of Payette et al. (1984) and Fairman et al. (1995) using neurofilament and ANNA-1 antibodies, respectively, as markers for the early development of neurons. Although

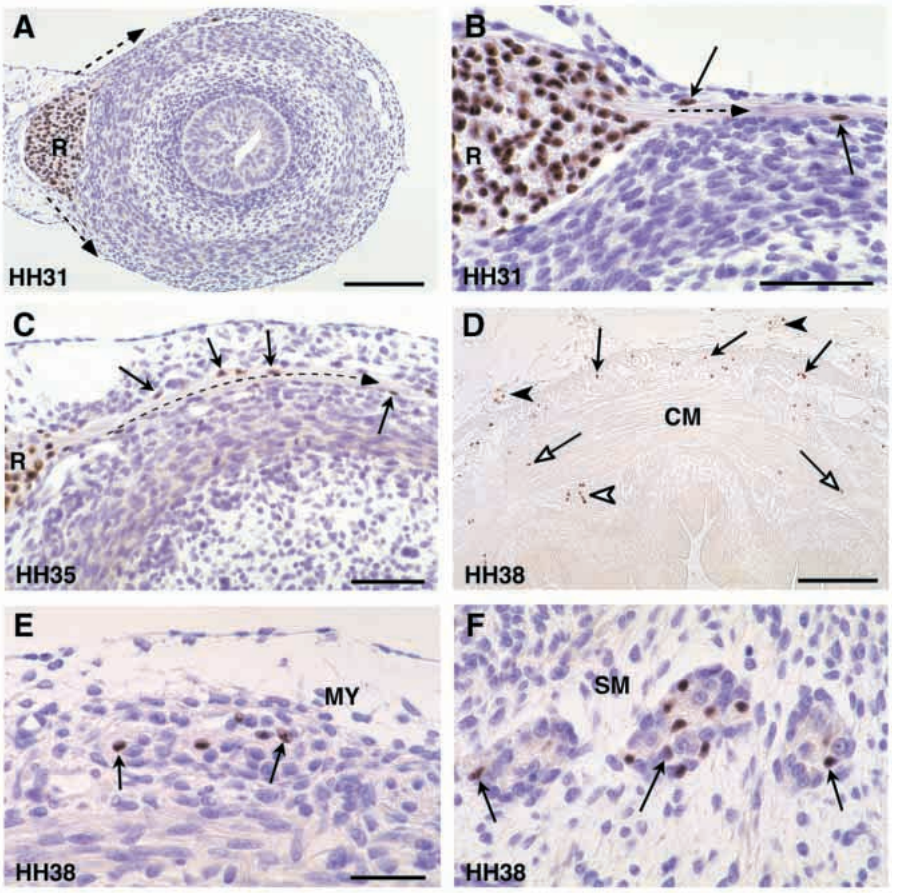

Fig. 7. Penetration of the gut wall by nerve fibres derived from the nerve of Remak. (A,B) Colorectum, HH31. Fibre tracts derived from the nerve of Remak (R) penetrated the gut wall (dashed arrows) external to the circular muscle layer, at the level of the presumptive myenteric plexus. Sacral NCC (arrows) were adjacent to nerve fibre tracts. (C) Colorectum, HH35. Many sacral NCC (arrows) were associated along the nerve fibres which penetrated the gut wall (dashed arrow). (D) HH38. QCPN-immunopositive cells were present within nerve tracts outside the gut wall (arrowheads), external (arrows) and internal (open arrowhead) to the circular muscle layer (CM) and within the circular muscle (open arrows). (E,F) HH38. Sacral NCC (arrows) occurred within myenteric (MY), and submucosal ganglia (SM). Bars: A,D, 100 μm; B,C, 50 μm; E,F, 25 μm.

Fairman et al. (1995) observed immunopositive cells within the developing nerve of Remak from E3.5, in both studies, immunoexpression within the gut wall followed the proximodistal temporal migration of vagal crest cells and consequently positive labelling was not observed within the colorectum until E7.5.

How do NCC follow precise migration pathways as they colonise the gut?

It is clear from the information obtained from vagal and sacral grafts that the gut is colonised in a complex manner involving intricate cell movements along precise pathways, some of which, particularly in the colorectum, appear to guide cells in opposite directions towards their final destination. Considerable evidence has been accumulated suggesting that it is the microenvironment encountered along the pathways leading to the target regions that determines the fate of migrating NCC and not their site of origin within the neural crest (Le Douarin and Dulac, 1992; Le Douarin and Kalcheim, 1998). Consequently, much effort has been directed towards the identification of the molecular signals likely to occur along these pathways. Although the distribution of some extracellular matrix (ECM) proteins, which are known to play an important role in NCC migration (for review see Le Douarin and Kalcheim, 1998), has been investigated (Tucker et al., 1986; Newgreen and Hartley, 1995), little overlap in the spatiotemporal relationship has been found between the distribution of NCC and ECM molecules along the length of the bowel.

With the recent advent of molecular biology and gene inactivation studies, numerous genes, cell lineages and signalling pathways have been implicated in the development of the ENS (Tennyson et al., 1998; for review see Gershon, 1997). For example, *ls/ls* mice have been shown to carry a point mutation in the gene encoding endothelin-3 (ET-3) (Baynash et al., 1994), the ligand for endothelin receptor-B (EDNRB), a G protein-coupled receptor responsible for the initiation of several intracellular signal transduction events (Arai et al.,

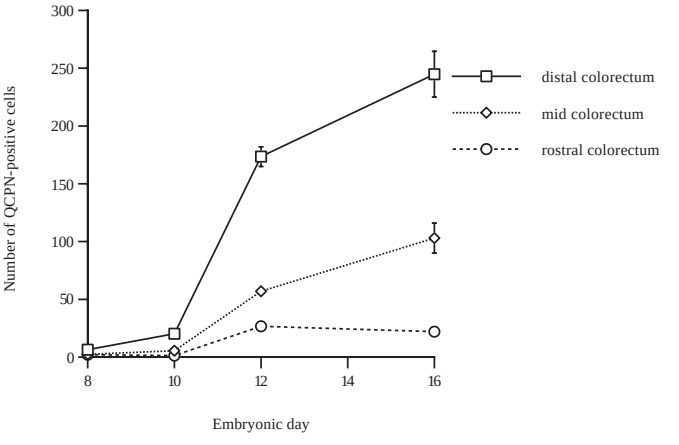

Fig. 8. Number (mean±s.e.m.) of sacral neural crest-derived cells per histological section within regions of the colorectum during development.

1993). Similarly, the gene encoding this endothelin receptor (*EDNRB*) has been shown to be deleted from the S^l chromosome in piebald lethal (S^l/S^l) mice (Hosoda et al., 1994), which also have a phenotype of aganglionic megacolon and spotted coat colour. It is significant that mutations in these genes have been reported in patients with Hirschsprung's disease (Puffenburger et al., 1994; Edery et al., 1996). Following gene cloning, the histological localisation of ET-3 and EDNRB expression has been determined in the chick. Nataf et al. (1996) described EDNRB expression in vagal NCC both during their migration within the gut and after the formation of the myenteric and submucosal plexuses. In contrast, ET-3 mRNA appears to be diffusely expressed within the mesenchyme of the gut (Nataf et al., 1998), but it does not appear that its expression pattern corresponds precisely with any of the pathways followed by NCC described in this study.

Among the most important signalling molecules during development of the ENS are GDNF and its functional receptor RET, as knockout of either of these genes causes a total loss of enteric neurons and glia within the gut caudal to the esophagus and cardiac stomach (Schuchardt et al., 1994; Pichel et al., 1996; Sanchez et al., 1996). RET is expressed in enteric neuroblasts (Pachnis et al., 1993), while GDNF is expressed within the gut mesenchyme (Hellmich et al., 1996). However, like ET-3, there does not appear to be a specific GDNF expression pattern corresponding to the distribution of NCC within the gut. As more growth factors and receptors/ligands involved in the development of the ENS are continually being identified, determination of their expression patterns may give an insight as to why NCC follow such specific pathways.

Why do sacral NCC fail to compensate for the absence of vagal NCC in aganglionic megacolon or Hirschsprung's disease?

As we have shown that the sacral neural crest contributes neurons and glia to the ENS within the distal hindgut, the question is raised why crest cells from this neuraxis do not compensate for the absence of vagal-derived ganglion cells in aganglionic megacolon in animals, or Hirschsprung's disease in humans. A possible explanation is that, in mammals, unlike the chick, the sacral neural crest does not contribute a significant number of precursors to the ENS. However, the work of Serbedzija et al. (1991) suggests that the sacral neural crest does contribute some cells to the hindgut of the mouse, although in their study labelled cells were few and the phenotypic derivatives were not determined. A second possibility is that, in order to colonise the hindgut, sacral NCC require an interaction with vagal neural-crest-derived precursors or neurons, or with factors produced by them. We found that although the sacral NCC formed the nerve of Remak adjacent to the gut wall early in development (from E4), sacral-crest-derived cells did not begin to migrate into the gut until E7.5 and were only present in significant numbers after E10, when vagal NCC had already extensively colonised the hindgut. Further support for this theory comes from the work of Meijers et al. (1989) and Peters-van der Sanden et al. (1993). In these studies, when the migration of vagal NCC was prevented by either severing the bowel proximal to the hindgut at E4, or by ablating the vagal neural crest, a contribution to the hindgut from the sacral neural crest was not observed. A third possible explanation for the failure of sacral NCC to compensate for the absence of vagal NCC in megacolon is that the region of the hindgut that remains aganglionic may not provide a permissive environment for the migration/proliferation/survival of either

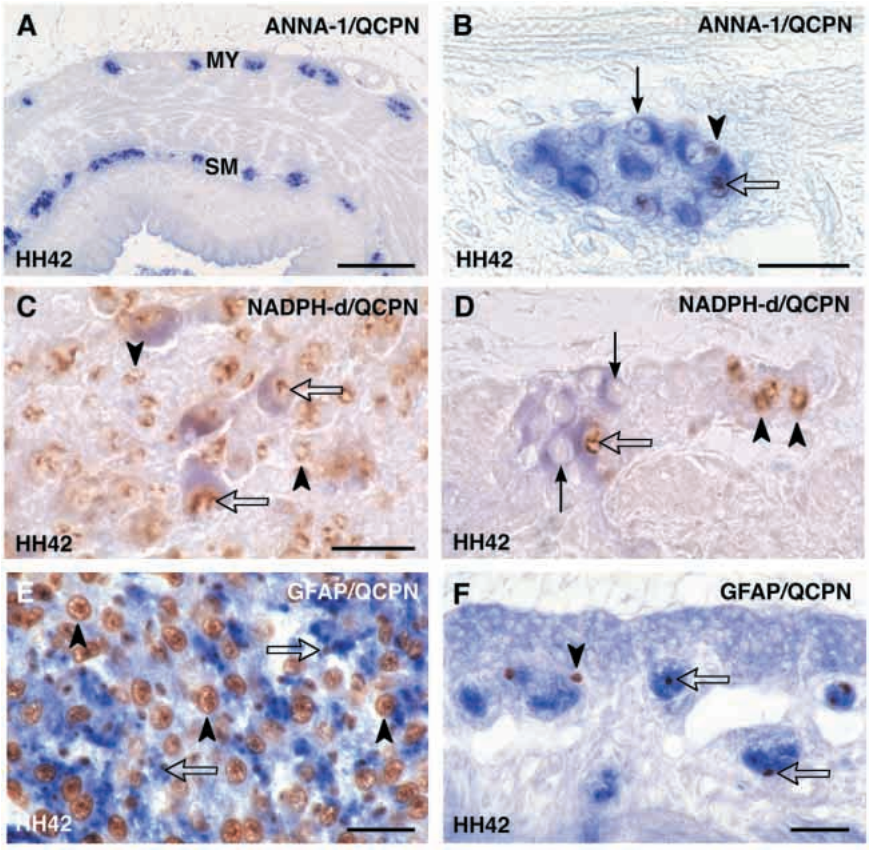

Fig. 9. Determination of the phenotype of sacral NCC within the hindgut. (A,B) ANNA-1 immunohistochemistry, HH42. ANNA-1-labelled neurons within the myenteric (MY) and submucosal (SM) plexuses. Immunopositive cells possessed densely stained (blue) cytoplasm and large unstained nuclei (arrow). The perinuclear cytoplasm of quail-derived cells was stained brown from the QCPN-DAB reaction product (arrowhead). Double-labelled cells had ANNA-1-positive (blue) cytoplasm and QCPN (brown) nuclei (open arrow). (C,D) NADPH-diaphorase histochemistry, HH42. Nerve of Remak and myenteric ganglion. NADPH-d stained the cytoplasm of neurons blue (panel D, arrows) while the nuclei of quail cells were stained brown (arrowheads). Double-labelled neurons possessed blue cytoplasm with brown nuclei (open arrows). (E,F) GFAP immunohistochemistry, HH42. Nerve of Remak and myenteric plexus. In the nerve of Remak (E), large brown-stained QCPN-positive neurons (arrowheads) were often surrounded by smaller GFAP/QCPN-positive cells (open arrows). In the myenteric plexus region (F), GFAP/QCPN double immunopositive cells were present within nerve tracts (open arrow) and enteric ganglia. Bars: A, 100 μm; B-F, 20 μm.

vagal- or sacral-derived NCC. This theory is supported by the presence, in *ls/ls* mice, of ectopic ganglia situated outside the aganglionic region of distal bowel (Rothman and Gershon, 1984; Payette et al., 1987). This observation may be explained by recent findings concerning the effect of ET-3 on NCC. Lahav et al. (1996) demonstrated that ET-3 dramatically increased the proliferation of trunk NCC in culture and may therefore have an important role in expanding the population of NCC as they leave the neural folds and begin to migrate. Furthermore, Hearn et al. (1998) examined the effects of ET-3 and GDNF on NCC and found that, in addition to promoting the proliferation of enteric neural precursors within the gut, GDNF was also important for the differentiation of these cells into non-migrating, non-proliferating enteric neurons. Although ET-3 was also found to promote proliferation of NCC, its primary effect was to modulate the action of GDNF by inhibiting the differention of NCC into enteric neurons, thus maintaining the precursor pool ensuring that sufficient cells are present to colonise the entire gut. Therefore in the lack of ET-3 in vivo, as in *ls/ls* mice, the pool of undifferentiated NCC may be reduced due to premature development of enteric neurons from both the vagal and sacral neural crest, resulting in an absence of neurons in the distal hindgut.

In conclusion, we have shown that vagal and sacral NCC follow very precise migration pathways within the developing gut of the avian embryo. Vagal NCC migrate in a proximodistal direction, colonising the entire length of the bowel, while sacral NCC migrate in a distal-proximal direction forming the entire nerve of Remak and contributing enteric neurons and glia to the ENS, distal to the umbilicus. The colorectum is populated in a complex manner as vagal-derived cells first colonise the submucosal region, then migrate outwards through the circular muscle layer to the myenteric plexus region. In contrast, sacral NCC enter the colorectum external to the circular muscle layer at the level of the myenteric plexus and subsequently migrate inwards to the submucosal ganglia. Thus, within the colorectum, cells from two distinct regions of the neural crest migrate in opposing directions to form enteric ganglia. It remains to be determined how neural crest cells are directed along such specific pathways during formation of the ENS.

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