Neural crest regionalisation for enteric nervous system formation: Implications for Hirschsprung’s disease and stem cell therapy

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ABSTRACT

Midbrain, hindbrain and vagal neural crest (NC) produced abundant enteric nervous system (ENS) in co-grafted aneural hindgut and midgut, using chick–quail chorio-allantoic membrane grafts, forming complete myenteric and submucosal plexuses. This ability dropped suddenly in cervical and thoracic NC levels, furnishing an incomplete ENS in one or both plexuses. Typically, one plexus was favoured over the other. This deficiency was not caused by lower initial trunk NC number, yet overloading the initial number decreased the deficiency. No qualitative difference in neuronal and glial differentiation between cranial and trunk levels was observed. All levels formed HuC/D+ve, NOS+ve, ChAT+ve, and TH-ve enteric neurons with SoxE+ve, GFAP+ve, and BFPAP+ve glial cells. We mathematically modelled a proliferative difference between NC populations, with a plexus preference hierarchy, in the context of intestinal growth. High proliferation achieved an outcome similar to cranial NC, while low proliferation described the trunk NC outcome of incomplete primary plexus and even more deficient secondary plexus. We conclude that cranial NC, relative to trunk NC, has a positionally-determined proliferation advantage favouring ENS formation. This has important implications for proposed NC stem cell therapy for Hirschsprung’s disease, since such cells may need to be optimised for positional identity.

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Introduction

The enteric nervous system (ENS) is the largest and most complex part of the autonomic nervous system. It is derived from the neural crest (NC), a population of migratory cells originating in the dorsal neural tube (Newgreen and Young, 2002b). Although the NC extends along virtually the entire neural axis, only a small length is fated to provide ENS cells. Most ENS cells in bird embryos are derived from the cranial region (hindbrain), level with somites 3 to 6 (Epstein et al., 1994; Hearn and Newgreen, 2000; Burns and Le Douarin, 2001). These cells traverse the adjacent paraxial tissue to reach the nearby foregut (Allan and Newgreen, 1980), then migrate along the midgut and hindgut (Allan and Newgreen, 1980). A second source of ENS cells lies in the lumbosacral NC caudal to somite 28, which provides a smaller number of ENS cells in the hindgut (Burns and Le Douarin, 1998; Burns et al., 2000; Hearn and Newgreen, 2000). These details of ENS origin are highly conserved in the rodent, with a major vagal NC origin of ENS cells (Burns et al., 2000; Natarajan et al., 1999) paired with a numerically minor sacral NC contribution (Kapur, 2000). The rest of the NC, that is, cranial NC rostral to the vagal level and trunk NC between the vagal and the sacral levels, makes no contribution to the ENS (Graham, 1994; Hammond and Yntema, 1947; Le Douarin and Teillet, 1973).

The neural tube shows rostro–caudal positional information from early stages, most dramatically illustrated by the staggered expression of Hox genes (Altmann and Brivanlou, 2001; Holland and Graham, 1995). Axial level differences in positional gene expression at early stages have consequences for latter differentiation of the central nervous system (Wu et al., 2008). The NC prior to migration shares this genetic axial patterning (Trainor and Krumlauf, 2001) and this also has consequences for NC differentiation (Abzhanov et al., 2003). For example, ectomesesenchyme is typically regarded as an NC-derived connective tissue lineage (but see Breau et al., 2008), and this competence is present at cranial levels and absent at trunk levels in amniotes (Leblanc et al., 1990; Lee et al., 2005; Lwigale et al., 2004).

Axial level differences in NC competence to form neural derivatives (as opposed to ectomesenchymal derivatives), including the ENS (Anderson et al., 2006), have not been fully clarified. In chick–quail grafts (Le Douarin and Teillet, 1974; Ziller et al., 1979), mid-trunk level (i.e. non-ENS producing) neural tube/NC was heterotopically grafted in place of vagal level neural tube/NC. The grafted trunk NC cells migrated to the gut and formed ENS, but on detailed examination the ENS did not develop identically to those in homotopic vagal neural
tube/NC grafts. In particular, trunk NC-derived ENS occurred in the rostral part of the gut, but was absent in the distal gut. In a nuanced study, a major (but not complete) deletion of the chick vagal NC in ovo and implantation of a small (one segment length) mid-trunk quail NC source was made (Barlow et al., 2008). These trunk NC cells did not contribute to the ENS at all unlike both large trunk grafts of Le Douarin and Teillet (1974) and small vagal grafts of Barlow et al. (2008), but improved the ENS-forming ability of the depleted host vagal cells.

Vagal/trunk level differences in ENS generation ability were first focussed on by Newgreen et al. (1980), by directly combining isolated mid-trunk level neural tube/NC with aneural hindgut and growing the combination on the chorio-allantoic membrane (CAM) of a chick host. The mid-trunk neural tube/NC furnished neurons in aneural hindgut (Ziller et al., 1979), but cell counts revealed far fewer neurons than those provided by the vagal neural tube/NC (Newgreen et al., 1980). Lumbosacral NC, which normally participates in ENS formation (Le Douarin and Teillet, 1973) had similarly low ENS-generating capacities to mid-trunk NC when the two levels were swapped in heterotopic chick–quail transplants (Erickson and Goin, 2000; Newgreen et al., 1980). The ENS-forming capacity of these two levels was also described as similar in the CAM graft assays when combined with aneural hindgut (Newgreen et al., 1980). However, in experiments testing the ability of short (one-segment) NC transplants to redress a major vagal NC ablation, the ability to form ENS was greater for the lumbosacral NC than the mid-trunk NC, though still far inferior to that of the vagal NC.

These results indicate a large innate superiority of vagal NC, and possibly a lesser superiority of lumbosacral NC, over mid-trunk NC in ENS-forming ability. Barlow et al. (2008) provided evidence for differences even within the vagal region. Whether ENS-forming ability occurs rostral to the vagal region has not been investigated, and whether it declines gradually or suddenly caudal to the vagal region is not known.

It has been argued (Barlow et al., 2008; Newgreen et al., 1996; Simpson et al., 2006) that a major determinant of the gut colonisation drive by enteric NC cells is the number or density of enteric NC cells. This has been systematised as the “frontal expansion” model, in which proliferation of NC cells at the wavefront in the gut is of particular importance (Simpson et al., 2007). Numerical differences acting via proliferation differences would therefore lie at the heart of the number or density of NC cells. It is therefore possible that the ability of the gut to elicit or support ENS differentiation varies along its rostro–caudal axis, and this could intersect with different ENS-forming capabilities of various axial levels of the NC. Consistent with differences between gut regions affecting the ENS, conditional transgenic ablation of the Ret gene caused loss of ENS in the colon but not in more rostral levels of the intestine (Uesaka et al., 2008). Thus, it could be argued that ENS-fated (i.e. vagal) NC can provide ENS efficiently in the foregut, midgut and hindgut but trunk levels of the NC can provide ENS in the more rostral gut but fails in the hindgut.

Combining neural tube/NC directly with aneural gut as CAM grafts can be used to analyse axial level differences of ENS-forming ability (Nagy et al., 2007; Newgreen et al., 1980). This avoids the undoubtedly complex phase of initial migration to reach the foregut. In this paper, we describe CAM experiments combining quail neural tissue with chick gut in which we compared the ENS-forming ability of donor neural tube/NC from closely spaced axial levels extending both rostral and caudal to the vagal region. We questioned whether the differences in ENS formation of different levels of the NC was dependent on axial level of the host gut, by using both midgut and hindgut recipients. We extended this to situations in which the donor NC cells of different axial origins faced competition from resident vagal NC cells. We also clarified whether ENS formation differences might simply reflect axial level-related differences in starting numbers of NC cells. We also investigated whether neuron and glial cell differentiation parameters varied in ENS of vagal and trunk origin.

Differences in ENS formation ability of different axial levels of NC were observed, as well as detailed variations in the development of the two plexus layers. These pointed to proliferative difference between NC cells of different positions of origin. To probe the degree to which proliferative differences could contribute to detailed differential outcomes, we turned to formal modelling. Mathematical models provide a useful framework to test ideas and also to guide our experimental programme. The results of these simulations are presented in the Discussion.

Materials and methods

Embryos

Fertilised quail (Coturnix coturnix japonica) and White Leghorn/Black Australorp cross chicken (Gallus gallus domesticus) eggs were obtained respectively from Lago Game Supplies and Research Poultry Farm, Vic., Australia. Embryos were staged according to the number of embryonic days (E), Hamburger and Hamilton stages (HH; Hamburger and Hamilton, 1992) and, for embryos of E2.5 and younger, by somite counts.

Intestinal tissues

Guts were staged according to Southwell (2006). Aneural hindgut from just caudal to the cecae to just rostral to the cloaca was dissected from E5 (HH26–27) chick embryos in Ham’s F12 medium (Thermo Electron, Melbourne, Australia) using tungsten needles. At this stage, the hindgut is yet to be invaded by NC-derived neural precursors (Allan and Newgreen, 1980; Nagy et al., 2007). To minimise contamination of the graft by chick sacral-derived NC cells (Nagy et al., 2007), the sacral NC-derived Nerve of Remak was reduced by cutting along the dorsal mesentery against the dorsal surface. However, it is not possible to remove all Nerve of Remak cells from the hindgut (Pomeranz and Gershon, 1990). The post-umbilical aneural midgut was dissected from E4.5 (HH25–25) chick embryos, from just caudal to the umbilicus to a point immediately caudal to where the endoderm of the cecae bud off. This has not been invaded by NC-derived neural precursors (Supplementary Fig. 1) (Allan and
Newgreen, 1980). A similar segment of “neural” midgut, that is, containing the wavefront vagal NC-derived cells (confirmed by whole-mount immunolabelling; Supplementary Fig. 1, and by dissociation, labelling and counting), was obtained by placing the rostral isolating cut about 200 µm rostral to the umbilicus.

**Neural and paraxial tissues; definition of levels**

Neural and adjacent tissues were obtained from many axial levels staged by reference to the NC migration timetable of Newgreen and Erickson (1986). Where paraxial tissues such as somites were removed, this was done with Dispase 2 (Boehringer-Mannheim) digestion (see Supplementary methods). Midbrain and rhombomere 4 tissue segments were dissected from E1.4 (HH8– to HH8+, 3–7 somite stages) quail embryos as described in Farlie et al. (1999). The segmental region spanning somite (s)1 to s4½ is classified as caudal hindbrain, and s4½ to s50 is termed trunk level. Within the segmental region, the vagal level tissue was dissected from E1.5 to 1.7 (HH9+ to HH11–12 somite stages) quail embryos. These included either the entire vagal level (from levels of somite s1–7) or smaller blocks of 3 somite-widths (s3–5, s5–7), or bisected single somite-widths (1/2 of level of s5). Cervical to rostral thoracic level tissue segments (from level of s8 to s20) were dissected from E1.7 (HH10+ to HH13, 11–19 somite stage) to E2.2 (HH11 to HH14, 13–22 somite stages) quail embryos. These included blocks of 7 segment and 3 segment length. Caudal cervical to thoracic level tissue in 3 or 4 segment lengths (from level of s18 to s24) were dissected from E2.5 (HH15+ to HH16–, 26–30 somite) quail embryos.

**Preparation of CAM grafts**

E8 chicken eggs were prepared as hosts for grafting experiments as previously described (Allan and Newgreen, 1980; Newgreen et al., 1980). The different combinations of quail neural tissue were placed in contact with the rostral end of the chick intestinal graft tissues on a 3 mm square piece of Millipore filter paper (type HA black, 0.45 µm pore; Millipore Corp., MA, USA) previously sterilised by brief exposure to 100% ethanol). In some cases multiple neural tissues were placed in contact with the rostral end of a single intestinal specimen. The filter was placed tissue-side down over the junction of blood vessels of the CAM and the eggs resealed. The grafts were incubated for 8 days (Supplementary Fig. 2).

**Section preparation**

Grafts were retrieved and fixed overnight in 4% paraformaldehyde (PFA) (Sigma-Aldrich Co., MO, USA) in PBS at 4 °C. In some cases where longitudinal sections were desired, the gut was dissected free from the investing CAM and straightened as far as possible before fixation. Grafts were frozen in Tissue-Tek (Sakura Finechemicals, Tokyo, Japan) as described in McKewon et al. (2005). Frozen sections were cut at 16 µm using a Leica CM1900 Cryostat (Leica Microsystems, Nussloch, Germany) and collected in 3 to 6 parallel series on Superfrost Plus slides (Biolab Scientific, Australia).

**Immunolabelling**

Antigen retrieval of sections used 10 mM citrate buffer pH 6, in which slides were held for 20 min at 95 °C. Slides were washed in PBS for 10 min, then blocked with 1% sheep, donkey or horse serum in PBS for 30 min. Sections were then incubated overnight at 4 °C sequentially in primary and secondary antibodies and other ligands prepared in block solution. Between treatments, the sections were washed extensively in PBS. Sections were mounted in Vectashield antifade reagent (Vector Laboratories, Inc., CA, USA) for microscopy. Antibodies are detailed in Supplementary Table 1.

**Imaging and evaluation**

Sections were analysed using an Olympus IX 70 microscope (Olympus Optical Co., Tokyo, Japan), under selective Texas Red, FITC and AMCA filters. Images were recorded using a Spot Monochrome camera model 2.1.1 with Spot Advanced 3.5 software (Diagnostic Instruments Inc., Sterling Heights, MI, USA), or with Image-Pro Plus 4.5 (MediaCybernetics, Silver Spring, MD, USA). Image-Pro-Analyzer 6.1 (MediaCybernetics) was used for analysis.

In general, grafts were only included for analysis if representative derivatives of all tissues implanted could be found and were in close proximity and well differentiated. However, all combinations with very small neural tube (half of s5 level) were evaluated even though the quail neural tube tissue was not always observed.

Typically, the graft intestine became tightly coiled so that each section had multiple profiles. ENS abundance clearly showed great variation in different parts of the gut. This was evaluated at low magnification (× 4 objective) with QCPN labelling by scoring ENS ganglion abundance on a scale of 0–3 at three widely separate sectional levels in each graft, so each graft had a maximum score of 3 × 3. The scores of all grafts in a category were averaged and expressed as a percentage. To further clarify the spatial variation in ENS, longitudinal sections of 24 straightened intestines (see Supplementary Figs. 2, 4) were examined.

**Estimation of NC cell numbers from neural tube explants**

It is not clear if there are numerical inequalities in NC cell number between different axial levels of the neural tube. To investigate this, neural tubes (N = 41) of 4 somite lengths were obtained as described above from rostral vagal to thoracic levels. Care was taken to capture essentially all the NC by using the region overlapping by just one somite’s width the onset of NC migration, as defined by Newgreen and Erickson (1986). The tissues were explanted into fibronectin coated dishes (Newgreen and Murphey, 2000). The neural tubes were cultured for 24 h before fixation in 4% PFA and nuclei were stained with DAPI. Images of the entire explants were made as above, and migrating NC cells were counted using Image-Pro-Analyzer 6.1 (see Supplementary methods).

**Estimation of the number of NC cells in neural intestine tissue**

Two methods were used to estimate the number of NC-derived cells initially included in the neural midgut grafts. Cells were counted in situ in wholemounts (N = 8; see Supplementary Fig. 1). Chick E4.5 (HH25– to 25) neural midguts as defined above were dissected out, fixed in 4% PFA, and wholemount-stained with SoxE antibody as in (Simpson et al., 2007). Specimens mounted in Vectashield between two coverslips were recorded with an IX70 microscope from both sides at various focal planes, and SoxE+ve cell nuclei were counted.

In addition, ENS cells in neural midgut specimens (N = 8) as above were counted after dissociation and plating on culture dishes (see Supplementary methods). After attachment for 5 h at 38 °C, these cultures were fixed in 4% PFA for 15 min, then double labelled with HNK-1 plus SoxE antibodies as described in (Simpson et al., 2007). Specimens mounted in Vectashield between two coverslips were recorded with an IX70 microscope from both sides at various focal planes, and SoxE+ve cell nuclei were counted.

In addition, ENS cells in neural midgut specimens (N = 8) as above were counted after dissociation and plating on culture dishes (see Supplementary methods). After attachment for 5 h at 38 °C, these cultures were fixed in 4% PFA for 15 min, then double labelled with HNK-1 plus SoxE antibodies as described in (Simpson et al., 2007). Specimens mounted in Vectashield with DAPI to stain all cell nuclei. The number of HNK-1+/SoxE+ cells to total DAPI-labelled nuclei were then counted in each culture using an IX70 microscope, and the total number was then calculated for each specimen.

**Mathematical formulation**

A cellular automaton (CA) model with domain growth, cell motility and cell proliferation, for cellular exclusion processes (Binder et al., 2008) is used and extended here.
**Gut tissue growth**

The growth of the developing three-dimensional gut tissues is idealised as a cylindrical shell. The thickening of the cross-sectional area and the radial expansion is small compared to the elongation (Binder et al., 2008) so that the overall growth can be approximated by the increase in the length of the outer cylindrical surface. The cylindrical shell is cut lengthwise, so becoming a flat rectangular plate with a fixed thickness and width. Therefore, we need only consider the elongation of a rectangular region in the Cartesian plane (with appropriate periodic boundary conditions along the length). The length of the tissue at time $t$ is denoted $L(t)$.

In the discrete CA model, the tissue consists of discrete integer sites, within a rectangular lattice $(L(t), Y)$, of fixed width $Y$, each occupied by a single tissue agent. During a time step from $t$ to $t + 1$ of the CA algorithm, for each row in the lattice, a number of tissue agents $n(t)$ are randomly selected to proliferate by mitotic division. Here $n(t)$ is the extension of the length in that time step, so equals the nearest integer value $L(t + 1) - L(t)$. If a tissue agent at $(x, y)$ proliferates, the original tissue agent moves to $(x + 1, y)$, and a new tissue agent is inserted at $(x, y)$. All tissue agents to the right of $(x, y)$ also move one unit in the positive $x$ direction (Supplementary Fig. 3A).

**NC cells on the growing tissue**

Within the growing tissue NC cellular agents can also occupy the lattice sites. Only one NC agent is permitted to occupy any one of the lattice sites at any time. Therefore, each site is either occupied by a single tissue agent or by a single tissue agent and a single NC cellular agent. If an NC cellular agent is occupying the same site as a tissue agent that moves to a new position in the lattice, due to tissue agent proliferation, then the NC cellular agent is transported or carried to this new position in lattice.

**NC cell motility and proliferation**

NC agents are also able to move and proliferate independently of the growing tissue. The tissue contains $m(t)$ NC agents at time $t$. During each time step, $m(t)$ NC cellular agents are randomly selected and given the opportunity to move, then $m(t)$ agents are randomly selected and given the opportunity to proliferate. An NC agent at $(x, y)$ that is chosen to be motile attempts to move with probability $P_m$ to one of the four nearest neighbours $(x ± 1, y ± 1)$ each with probability of 1/4 (Supplementary Fig. 3B). These rules were chosen since time-lapse data (Young et al., 2004) shows that the movement of an NC cell can be rostro-caudal, but also caudal-rostral, as well as in the transverse directions. However, we also simulated the opposite extreme by introducing a powerful inherent directional bias by nullifying all rostral movement. The outcome of this is shown in Supplementary Fig. 5. When considering a potential proliferation event a mother NC cellular agent attempts to divide with probability $P_p$ and the two daughter NC agents are either deposited onto $(x ± 1, y)$ or $(x, y ± 1)$ each with probability of 1/2 (Supplementary Fig. 3C). If the target site is occupied for any motility or proliferation event, then that event is aborted.

We consider more than one species of NC agents (e.g. trunk versus vagal). To vary the motility and/or proliferation rates between species, we vary $P_m$ and/or $P_p$, respectively.

**The donor**

The NC donor tissues, containing the reservoir of NC cells (in this case the neural tube) does not grow, unlike the gut to which they are attached. In the donor, NC cells follow the usual rules for proliferation. The donor tissue of fixed size is randomly provided with a chosen initial NC cell density (or in each column of the lattice). This is equivalent to randomly seeding each column with a chosen number ($n_0$) of NC cells. We explore the effect of varying the initial seeding density/number.

**Two layer model**

To consider the migration of NC agents (single species) from one tissue layer to second tissue layer, we modify the proliferation mechanism. If an NC agent is chosen to proliferate, we first assess whether all four nearest neighbour sites are occupied by NC agents. In this case the usual proliferation event would be aborted. Introducing a new mechanism gives the otherwise unsuccessful NC agent the opportunity to proliferate into a second layer (at $(x, y)$), provided there is no NC already there. Once a daughter agent is deposited into the second tissue layer it obeys the same proliferation and motility rules in the new layer as in the original tissue layer.

**Average density of NC cells in a single layer**

Each simulation of the discrete algorithm produces a single realisation. Each of these realisations will be different. We define some average properties of multiple realisations.

We define the density $d_i$ of NC agents in the growing tissue for the $i$th realisation as

$$d_i = \frac{M_i(P_p, P_m)}{LY}.$$  

where $M_i(P_p, P_m)$ is the number of NC cells for the $i$th realisation at the final simulation time using a fixed proliferation and motility rate $P_p$ and $P_m$ respectively. A good measure of the invasion success as a function of $P_p$ and $P_m$ is then the average density in the tissue over $N$ realisations:

$$\bar{d} = \frac{1}{N} \sum_{i=1}^{N} d_i.$$  

Here, by definition $0 \leq \bar{d} \leq 1$. To assess the variability of success between differing values of $\bar{d}$, we define the relative standard deviation as

$$s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} \left(\frac{d_i - \bar{d}}{\bar{d}}\right)^2}.$$  

We will fix $P_m$ and vary $P_p$, and determine the dependence of $\bar{d}$ and $s$ on $P_p$. Alternatively, we fix $P_p$ and vary $P_m$ and determine the dependence of $\bar{d}$ and $s$ on $P_m$.

**Results**

**Do cranial and trunk regions of NC not fated for ENS formation form ENS when allowed access to aneural hindgut?**

**Vagal neural tissues**

Combination on the CAM of chick aneural hindgut with quail vagal level neural tissue plus attached paraxial tissues (levels s1–7, $N = 6$; levels s5–7, $N = 4$) resulted in plentiful quail-derived (QCPN+ve) ENS cells. Aneural hindgut combined with isolated vagal level neural tissue levels s3–5, $N = 3$; Supplementary Fig. 4A) produced the same result. These positive controls confirm an earlier histological study (Newgreen et al., 1980), but also revealed previously undetected individual NC-derived cells in the plexuses between the ganglia and within the muscle layers. The ENS formed ganglia in two concentric layers in the gut wall. The identification of the layers as the myenteric and submucosal plexuses was made by their position relative to SMA+ve smooth muscle layers. The ENS cells were labelled by antibodies to the neuron marker HuC/D and to the undifferentiated NC and glial marker SoxE. These two labels were expressed mutually exclusively at the cell level, but each ganglion contained cells of both immunoreactivities. All cells and neurites in the ENS were labelled by...
HNK-1 (NC cell surface marker) and neurites were labelled by neurofilament antibody, Tuj-1 (β-tubulin) and E/C8 (NAPA73). Antibodies to GFAP and BFABP (glial cell markers) labelled some SoxE+ve non-neuronal cells in the ganglia, stretching between ganglia and traversing the muscle layers. Some groups of large QCPN-ve neurons (entirely chick origin) and support cells (mostly chick origin) were found outside the outer smooth muscle layers; these were probably Nerve of Remak remnants (Nagy et al., 2007).

In control CAM grafts of aneural hindgut (N = 9), large neurons and support cells were occasionally observed outside the intestinal muscle layers; these are likely to be Nerve of Remak remnants. Neurites probably of Nerve of Remak origin were present but not abundant in the intestinal wall. The gut itself was almost entirely lacking in NC-derived cells. The few SoxE+ve cells seen were sparse and were not neurons and some expressed glial markers.

Cranial neural tissues

Combination on the CAM of chick aneural hindgut with quail midbrain level neural tissue (N = 7 with paraxial tissue, N = 4 without paraxial tissue), and pre-otic hindbrain rhombomere 4 neural tissue (N = 8 without paraxial tissue) all resulted in plentiful quail-derived ENS throughout the host gut (Fig. 1A). This was similar in almost all details to the results with the vagal region combinations. However, in 3 of 19 specimens, a third concentric layer of ganglia occurred, internal to the other plexuses and with fewer cells. This was associated with thin SMA+ve strands of the muscularis mucosae.

Trunk neural tissues

Combination on the CAM of chick aneural hindgut with quail thoracic level neural tissue (N = 12 with paraxial tissue; N = 4 without paraxial tissue; levels s20–22 and s22–24), resulted in quail-derived...
ENS ganglia in the submucosal plexus (Fig. 1B). In comparison with cranial NC grafts, these ganglia were more variably spaced and sparser where they occurred, and occupied typically about half the entire intestinal graft (Figs. 1C, D), unlike the vagal combinations, confirming previous histological studies (Newgreen et al., 1980). Surprisingly, even in regions where submucosal plexus ganglia occurred, ENS ganglia were mostly absent in the myenteric layer (Figs. 1B, C), where scattered quail cells occurred. Cells in the ganglia included both neurons and glia. In addition, in combinations with trunk neural tissue, but only with those lacking paraxial tissues, melanocytes also occurred in the position of the intestinal plexuses (Fig. 1B).

Does ENS-forming competence of the NC differ in the midgut compared to the hindgut?

As mentioned in the Introduction, there is a possibility that, relative to vagal levels, the trunk NC has lower capacity to form an ENS in the hindgut, but can populate the more rostral intestine. To test the capacity of the midgut to elicit ENS formation, we combined on the CAM chick aneural midgut (post-umbilical small intestine to cecum, stages HH24 – 25) with quail vagal level neural tissue (levels s3–5; N = 11) and with upper cervical (level s8–10; N = 6), lower cervical (s16–18; N = 5) and thoracic (levels s20–22: N = 2) neural tissue. Five vagal and 6 lower cervical combination grafts were dissected free of the CAM and longitudinally sectioned to clarify the extent of NC colonisation.

The results were broadly similar to those in the hindgut (Figs. 1E, F, 2). Vagal level combination grafts showed a rich QCPN+ve ENS in both myenteric and submucosalplexuses throughout the graft midgut in 10/11 grafts (Fig. 1E). This included the full length of the cecae (Supplementary Fig. 4A), as revealed in 4 of the 5 longitudinally sectioned grafts.

Trunk combinations also had quail-derived neurons in ENS ganglia. However the ENS ganglion distribution in this was very variable and they never occupied the entire intestinal graft. When six of these graft midguts were longitudinally sectioned, the extent of the trunk-derived ENS ranged from dense ENS ganglia in all regions except the distal midgut and cecae (N = 1; Supplementary Fig. 4B) to very sparse neural cells with few ganglia (N = 2; Supplementary Fig. 4C), but the remainder (N = 3) had concentrically arranged ENS ganglia over part of the midgut (Supplementary Fig. 4D). Often this incomplete ENS preferentially lay in a single plexus, either the sub mucosal layer or more frequently the myenteric layer (Fig. 1F; Supplementary Fig. 4D). Despite the reduced number of ganglia compared to vagal combinations, quail trunk NC-derived cells occurred individually more extensively in the intestine particularly in the muscle layers of the cecal region (Fig. 7F). These cells were not neurons and some carried glial markers and some were melanocytes.

Does ENS-forming competence decline suddenly or gradually caudal to the vagal level?

CAM grafts with quail neural tissues (both with and without paraxial tissues) obtained from midbrain (N = 4), rhombomere 4 (N = 7) and vagal levels (total N = 18) were efficient in ENS generation. Similar grafts with post-vagal tissues in successive blocks from levels s8 to 10 (N = 10), s8 to 14 (N = 4), s11 to 13 (N = 10), s14 to 16 (N = 6), s16 to 18 (N = 5), s17 to 19 (N = 2), s20 to 22 (N = 2) and s22 to 24 (N = 5) and combined with aneural chick midgut or hindgut appeared similar. These levels showed a sharp decline immediately caudal to the vagal level in ability to furnish a complete ENS throughout the host chick aneural intestine (Fig. 3).

Does differential ENS-forming competence reflect different starting numbers of NC cells?

Explants of 4 somite lengths of neural tube produced a halo of NC cells by 24 h in vitro. Cell counts indicated that there were differences in NC cell numbers along the neural axis. Compared with vagal levels (N = 5, from within regions s1 to s7); the number of NC cells per explant at anterior cervical levels (N = 11, within regions s8 to s15) was 40% lower. However, the number of NC cells from thoracic levels (N = 10, within regions s19 to s27) was about 60% higher than from vagal levels. Counts from vagal–cervical overlap (N = 6, within regions s5 to s10) and posterior cervical (N = 9, within regions s13 to s19) levels were intermediate between the values of the flanking regions (Fig. 4). Interestingly, in these cultures on fibronectin, despite the difference in NC cell numbers, the cells from all axial derivations migrated out to reach similar cell densities (in cells/mm² ± sd; vagal: 1546.27 ± 74.32; trunk–vaginal overlap: 1577.12 ± 86.67; anterior cervical: 1518.98 ± 83.81; posterior cervical: 1390.83 ± 55.82; anterior thoracic: 1753.18 ± 93.66). The larger cell numbers were accommodated by greater areas of substrate occupation.

Can ENS formation by trunk level NC be improved by increasing the starting numbers of NC cells?

To increase the starting number of trunk NC cells, single aneural midguts were combined with 5 trunk neural tubes each of 5 somite lengths (N = 6). In longitudinal section, 5 of these grafts showed large numbers of ENS ganglia in two plexuses through most of the length of the gut (Supplementary Figs. 4E, F). However, in the distal (cecal region) midgut, numerous scattered quail cells were found within the muscle layers, with few or no quail NC-derived cells arranged in ganglia; this region therefore was never as well ganglionated as by the vagal neural tube/NC source. In one graft, scattered quail non-neuronal cells were present in the plexus and muscle layers over the entire gut but few neurons were present at any level. Unlike combinations with vagal neural tube, melanocytes were always present. Thus, increasing the initial number of trunk NC cells potentially...
available increases the ENS over that achieved by a single neural tube/NC donor, to approach that produced by a single vagal donor (Fig. 5).

Can ENS formation by vagal level NC be curtailed by decreasing the starting numbers of NC cells?

To decrease the starting number of vagal NC cells, neural tube level with a single somite, s5, was obtained. To further decrease the NC cell number, this neural tube segment was bisected longitudinally and one half combined with each aneural midgut (N = 7). Assuming a similarity to the cell culture assays described above, these small neural donors would provide around 250 NC cells in the first 24 h, compared to about 1500 NC cells by the standard 3-somite long donor. Quail NC cells formed an extensive ENS (Fig. 5), and longitudinal sections showed numerous quail ganglia in the myenteric and submucosal plexuses in over 80% of the gut length, including the cecal region, in 5/7 grafts (Supplementary Fig. 4G). However, the ganglia in these reduced NC grafts tended to be smaller than with the longer vagal combinations. Two grafts had a handful of quail cells in the plexus layers at the rostral end; the donor neural tube was not found in these. All these combination grafts were devoid of melanocytes.

Does competition with resident vagal NC-derived cells influence ENS formation of donor NC cells?

The previous combinations showed that all cranial levels of neural tissue provided a more extensive ENS in aneural gut than did all trunk levels. To test this differential more stringently, we repeated these combinations using host chick neural midgut with a small number of its own vagal ENS cells that had colonised the rostral end of the gut. To do this we obtained midgut HH25– to HH25 (chick E4.5) from slightly further rostral than previously. This captures the extreme front of the colonisation wave of NC-derived cells as confirmed by immunolabelling (see Supplementary Fig. 1).

Cell counts were made of these by wholemount staining with SoxE antibody (N = 8). One specimen, the youngest judged by cecal morphology, had no SoxE+ve cells. The average NC cell number was 62.0 ± 57.9 (range 0–163) SoxE+ve cells per specimen. Cell counts were also made by dissociating the intestine and plating the cells for 5 h on fibronectin islands in vitro, followed by staining with SoxE and HNK-1 and cell counting (N = 8). The number of NC-derived cells from this method was 47.5 ± 41.2 (range 3–122). These two calculations are in good agreement, the wide variation being expected due to the intentional inclusion only the extreme wavefront of NC cells. We conclude that nearly all “neural” intestines of this type will contain some enteric NC-derived cells, typically about 50–60, and not exceeding 200.

From the in vitro data, quail vagal level neural tissue (levels s3–5) would be expected to produce about 1500 NC cells. Combination of this neural tube with such early chick “neural” midgut with <200 vagal NC-derived cells (N = 4) resulted in plentiful bi-layered ENS throughout the graft intestine. This ENS was mostly made up of quai-derived (QCPN+ve) cells (Figs. 5, 6A). However, a lesser number of QCPN-ve chick ENS cells were found usually co-distributed in the same ganglia with quail cells. However, in small regions the chick-derived cells predominated, and in two grafts quail myenteric plexus coexisted with chick-derived submucosal plexus (Fig. 6B).

Similar combination with quai cervical level neural tissue (levels s8–10; N = 6; expected to produce about 900 NC cells) and quai thoracic neural tissue (levels s20–22; N = 7; expected to produce about 2000 NC cells) with chick “neural” midgut also had plentiful ENS ganglia throughout the graft, but most were of chick (i.e. vagal NC) origin and relatively few were QCPN-+ trunk NC origin (Figs. 5, 6C). ENS ganglia of mixed chick and quail origin, and scattered quial cells were found typically in a restricted region of the intestine, smaller than expected when trunk NC was combined with aneural gut. Unlike combinations of trunk neural tissue with aneural gut, melanocytes were never observed.

Fig. 4. A. Neural crest (NC) cells migrating from a 4-somite length neural tube (NT) explant at 24 h in vitro labelled with DAPI. B. Counts of NC cells (± sd) (y-axis) in cultures of this type showed that NC cells from the vagal level were more numerous than NC cells from vagal–cervical overlap and anterior and posterior cervical levels but less numerous than NC from anterior thoracic level.
Does ENS from different levels of the NC have similar differentiation patterns?

The morphology of ENS ganglia of vagal and trunk origin were similar, with small interlinked ganglia in the myenteric plexus and smaller ganglia in the submucosal plexus at midgut levels, and with larger ganglia in the hindgut (Figs. 7A, B, C). Each ganglion had both neurons and glial cells. Sub-populations of ENS cells of both vagal and trunk origin were labelled by antibodies to NOS and ChAT (Figs. 7D, E). TH+ve cell bodies (typical of trunk NC-derived sympathetic neurons) were never observed in the ENS in these graft combinations. The enteric plexuses also showed neurites labelled for neurofilament, β-tubulin (Tuj1) and NAPA73 (E/C8).

Two differences between cranial and trunk donors were repeatedly seen. Grafts with trunk neural tube/NC donors displayed more scattered SoxE+ cells in the gut muscle, particularly in the cecal region (Fig. 7F). In grafts of trunk donor plus aneural intestine, QCPN+ve melanocytes, which were visible with light field illumination due to black melanin, were scattered in the position of the ENS plexuses, either alone or associated with quail-derived neural cells (Fig. 1B). In contrast, in graft combinations with vagal neural tube/NC, melanocytes were rarely seen in the enteric plexuses. This confirms earlier observations on chick–chick combinations (Newgreen et al., 1980). However, when trunk neural tube/NC plus adjacent paraxial tissues was combined with aneural intestine, enteric melanocytes were never observed. In addition, as described above, when quail trunk neural tube/NC was combined with “neural” intestine containing small numbers of resident chick vagal NC cells, melanocytes were never observed among the predominantly chick vagal NC-derived ENS, even in regions where quail trunk NC cells occurred in the ENS layers.

Discussion

Cranial and trunk NC show differences in ENS-forming ability

The results presented here show that midbrain and rostral hind-brain level NC, although not fated to form ENS, had similar ENS-forming ability to vagal level NC cells, which are fated to produce ENS. In contrast, all trunk levels of the NC were inferior to all cranial (including vagal) levels of NC in forming ENS (in terms of ganglion density and distribution) in an initially aneural gut environment. By reducing vagal NC and increasing trunk NC, the ENS-forming potential of 1/2 somite length of vagal NC was found to roughly equal that of 25 somite lengths of trunk NC. This fold-difference is echoed in the similar fold-difference in final ENS neuron count after CAM grafting of single vagal versus single trunk neural tube combinations with aneural gut (Newgreen et al., 1980). This difference was exaggerated when there was competition with a smaller number of vagal NC cells already in the gut. The superiority of cranial level over trunk level NC in ENS-forming ability was manifest immediately caudal to the vagal level and did not seem to ramp down gradually along the axis.

We conclude that there is a difference in ENS-producing capability between different axial levels of the NC that is established prior to NC cell migration. This restriction did not precisely match the NC fate map. We conclude that rostral-to-vagal NC normally does not form ENS due to lack of opportunity not lack of competence whereas caudal-to-vagal NC indeed has lower competence. This caudal restriction of competence mirrors other differentiation capacities along the neuraxis. In particular, expression of no or low number (paralogous groups 1–5) Hox genes (Chan et al., 2005; Creuzet et al., 2002; Fu et al., 2003) correlates with efficient ENS production ability, provided
the cells have access to gut mesoderm. It is tempting to suggest that the progressive expression caudally of higher number Hox paralogues contributes to the reduction of ENS-forming efficiency in NC cells. Since overloading with thoracic NC cells at the initial stage was able to provide a more complete ENS, we conclude that the difference between cranial and trunk NC in ENS formation has a quantitative aspect. However, since the initial number of NC cells provided by vagal and trunk levels were broadly similar (at least in vitro), we suggest that the quantitative differences evolve from differential NC cell proliferation after reaching the gut.

Qualitative differences in ENS differentiation between trunk and cranial NC were subtle

Two elements of axial level-related qualitative difference were noted in the NC-derived cells in the gut. Trunk NC donors provided more non-ganglionic, non-neuronal cells in the gut muscle than did vagal NC. The reason for this difference is not clear. In addition, ectopic melanocyte differentiation ability in the enteric layers was confined to graft combinations with trunk level NC cells. It is possible that these cells represent differentiation of cells otherwise in the glial lineage (Adameyko et al., 2009). This might result simply from the high NC cell population density typically attained in vagal NC combinations acting to restrict melanocyte differentiation in the enteric environment. Consistent with this view, trunk NC cells present among numerous vagal NC-derived ENS cells failed to differentiate as melanocytes. However, since enteric melanocytes were still present in trunk NC overload experiments, innate qualitative difference in differentiation ability is likely to occur.

Notwithstanding the above qualitative differences, and the quantitative differences, ENS ganglion morphology and assemblage was similar for NC of all levels of origin. NC cell differentiation ability in the gut microenvironment was similar between cranial and trunk NC, in terms of appearance of HuC/D+ve neurons that were NOS+ve or ChAT+ve. The neurite patterns revealed by NAPA73, β-tubulin and neurofilament antibodies were similar and glial cells which were GFAP+ve and BFABP+ve were present in both. In addition, NC derivatives not normally found in the avian ENS, such as TH+ve adrenergic neurons (elsewhere normally derived from trunk and not cranial NC) were not observed.

We conclude that important differences in ENS generation capacity between various levels of the NC are quantitative. However, the differentiation repertoire in the ENS is much more extensive than tested here (Costa and Brookes, 2008), so other qualitative differences in differentiation between cranial and trunk NC in terms of neuronal and glial cells types cannot be ruled out.

Vagal and trunk NC have similar ENS-forming ability in midgut and hindgut

The formation of the ENS clearly involves interaction between NC-derived cells and gut mesoderm-derived cells. Different levels of the gut are innately positionally coded (Smith and Tabin, 2000) and it is possible that this interacts with regionalised NC abilities such that trunk NC can provide complete ENS in the more rostral gut yet fails to...
do so in the hindgut. However, when tested here, differential ability to elicit ENS differentiation by different levels of the intestine was not seen, since the superiority of vagal level NC over trunk level NC in ENS-forming ability was exhibited not only in the hindgut, but also in the midgut. It is still possible that the foregut is an environment favourable for efficient ENS formation from trunk level NC-derived cells.

There were some ENS differences related to the intestinal region. The ENS ganglia produced by trunk NC sometimes occupied just one plexus; in the midgut this was often (but not always) the myenteric plexus. In the hindgut the trunk NC-derived ENS was overwhelmingly the submucosal plexus. In avian development the first plexus to be colonised in the midgut is the myenteric, and in the hindgut it is the submucosal plexus ([Burns and Le Douarin, 1998; Conner et al., 2003]). Differences between the ENS plexuses have been reported elsewhere. In the colon of mice the myenteric plexus forms first ([McKeown et al., 2001]), and in ET-3−/−/− mice with distal colonic aganglionosis (Hirschsprung’s disease phenotype), a considerable length of colon lacks the submucosal plexus (Payette et al., 1987). Thus, when ENS formation capability is suboptimal, a difference in plexus preferences may emerge which favours the normally first-formed plexus.

A model for incomplete ENS formation by trunk NC based on NC regional proliferative differences: implications for Hirschsprung’s disease

The superiority for ENS generation of vagal over trunk NC observed by Newgreen et al. (1980) has been confirmed (Barlow et al., 2008; Burns et al., 2000, 2002; Hearn and Newgreen, 2000), and here extended to additional cranial and trunk levels. We propose that a proliferative advantage of the cranial (including vagal) NC over the trunk NC is chiefly responsible for the differences in ENS formation. Underlying this, mRNA for the ENS growth factor GDNF-receptor Ret was found to be much lower in the sacral-derived (i.e. trunk) NC than vagal-derived NC (Delalande et al., 2008). Given the mitogenic role of GDNF in the ENS ([Hearn et al., 1998]) and the fundamental role of proliferation of NC cells in ENS formation ([Simpson et al., 2006]), this differential expression level of Ret could at least in part account for the apparently qualitative differences between vagal and trunk NC. This was confirmed by over-expression of Ret in sacral NC cells, which conferred on them vagal NC-like ENS formation capabilities ([Delalande et al., 2008]). Intuitively, the proliferative advantage may interact with a growing gut domain ([Newgreen et al., 1996; Binder et al., 2008]) such that vagal NC can completely populate the growing gut, but trunk NC continually falls short.

Mathematical modelling was used to show that this is a viable and robust hypothesis. The invasion experimental system was simulated using the CA model set out in Fig. 8. We started with an initial NC density/cell number supplied by the donor neural tube, and determined the position and extent of the moving and proliferating NC cells in two layers on the growing gut tissue. Rules for transit between the layers are given in the Materials and methods and are discussed further in the next section. For ease of visualisation, in these simulations we had the gut growing linearly in time (\(L(t) = L(0) + \alpha t\)). The same results hold qualitatively for exponential growth. Given an elongation rate of the gut tissue and a given NC cell motility, we varied only the proliferation rate of the NC cells. Fig. 8 illustrates the results of a single realisation of the CA model for two proliferation rates. For a sufficiently large proliferation rate (Figs. 8A, B, C) the two layers were fully colonised, while for a sufficiently low proliferation rate (Figs. 8D, E), the second layer was only very sparsely colonised. These scenarios are shown in Supplementary Movies 1 and 2. The precise colonisation degree and pattern varies with each realisation as a result of the stochastic properties built into the proliferation and motility rules. Taking a large number of realisations, we were able to determine some measure of the colonisation ability of NC cells with differing proliferation rates.

The mean density over the single layer was evaluated as a function of the NC proliferation rate for different initial seeding cell density/numbers of NC cells from the donor. Intuitively, we expected that the colonisation to be more successful (large mean density) when the proliferation rate and/or the initial seeding density/cell number is increased, as shown in Fig. 9A. However, these curves were not linear. In addition, the relative variability of this measure decreased with increasing proliferation rate and with increasing initial seeding density/cell number (Fig. 9B).

Fig. 9A can also be used to make predictions. If the initial seeding density/cell number is sufficiently low for a fixed high proliferation rate, then the mean density may fall below a level that would characterise full colonisation. Alternatively, if the initial seeding density/cell number is sufficiently high for a low proliferation rate, then the mean density may increase above a level that would characterise full colonisation. Indeed this trend has been confirmed experimentally by combining 5 trunk neural tubes with one aneural gut (Fig. 5; Supplementary Figs. 4E, F).

Moreover, our modelling also explains why a small number of resident highly proliferative vagal NC cells will produce successful colonisation over a much greater number of donor trunk NC (Fig. 5). If both cell types have the same proliferative rate (i.e. both vagal), then no cell has an advantage, and the distribution of the colonised layer will be highly random, mixed between the two types, but broadly reflective of the relative starting abundance (Fig. 10A). However, if a few cells (i.e. vagal NC) have a proliferative advantage, then these cells fill the available space and restrict occupancy by a greater number of lower proliferating cells (i.e. trunk) (Fig. 10B). These outcomes are shown in Supplementary Movies 3 and 4.

We tested the alternative hypothesis that one cell type has a cell motility advantage over the other, instead of a proliferative advantage. Under this scenario, the mathematical modelling results (Figs. 5C, D) differ markedly from those reported above. We illustrate this by presenting the mean density and variance results evaluated as a function of the NC motility rate for different initial seeding cell density/numbers of NC cells from the donor. Intuitively we expected that the colonisation would be more successful (large mean density) when the motility rate increases, as shown in Fig. 9C. However, the change in the average density over the whole range of motility rate is significantly smaller than the change in the average density over the whole range of proliferation rates (Fig. 9A compared to Fig. 9C). In particular, when the motility rate is zero, the invasion can be deemed successful if the proliferation rate is high enough. Again, these results reinforce the importance of proliferation over motility ([Simpson et al. 2006]). Moreover, there was no evidence from in vitro cultures that trunk NC cells are less migratory than cranial NC cells since NC cells of all origins migrated to occupy substrate area to an equal degree defined by the population size, not by the level of origin. The results described here allow NC cells to have equal probability of moving in all four directions. We also tested an extremely biased case, where NC cells were not allowed to move in the rostral direction and moved preferentially in the caudal direction (probability of 1/2). The averaged results were little changed (Supplementary Fig. 5).

Our CA modelling and experimental work suggests that a proliferative advantage of cranial over trunk NC cells is a viable notion to explain the differences in ENS distribution especially in a growing domain. Many of the genes implicated in Hirschsprung’s disease directly or indirectly compromise NC cell proliferation ([Newgreen and Young, 2000a]), Therefore this hypothesis also provides a satisfactory model for the genesis of Hirschsprung’s disease, where the distal intestine lacks NC-derived ENS ganglia.

A model for ENS plexus formation based on hierarchical preference

The initial colonisation of the intestine is by extension of a single plexus layer of NC-derived cells, which then form a second layer
(Burns and Le Douarin, 1998) and in some regions in large mammals, a third plexus layer (Timmermans et al., 1990). We proposed previously a carrying capacity for ENS cells (Simpson et al., 2006) and now suggest that there is a hierarchy of preferences for layered ENS plexus positioning. As sub-proposals we suggest (i) that extending the occupation area in the existing plexus layer is the primary and preferred mode of accommodating increased ENS cell numbers; (ii) that, even when trans-plexus movement is possible, significant colonisation of the less preferred layer only commences when the preferred site is locally occupied to its maximum cell density, and (iii) that this hierarchy in birds differs between the midgut and hindgut, the myenteric layer being preferred in the midgut and the submucosal layer in the hindgut, whereas in mammals the myenteric layer is preferred at all regions. Proposal (i) is consistent with the in vitro data, where NC cells had a strong preference for a fixed cell density. Increased cell numbers were accommodated by occupation of more area rather than by crowding within existing area. Extension within an existing layer is also consistent with the observation that the two layers could develop independently (see Fig. 6B). Proposal (ii) is consistent with the unequal spatial extent of the two ENS layers when proliferation is limited. Proposal (iii) was suggested by the normal sequence of plexus formation. In the in vivo context, local

Fig. 8. Two tissue layer CA realisation with two proliferation rates. The first layer has donor tissue (neural tube) in light blue with NC cells (dark blue). Growing gut tissue agents are shown in yellow, where growth is linear as \( I(t) = I(0) + t \). The second layer is above and grows at the same rate. NC motility \( P_m = 1 \). (A–C) NC proliferation fixed at \( P_p = 1 \). A. Initial time \( t = 0 \), blue NC cells in the donor at initial density of 0.4. B. At \( t = 8 \) the red NC agents indicate those NC agents in the first layer which are chosen to proliferate and are at local carrying capacity. Their daughter is deposited into the second layer (if not already occupied), as shown by the red NC agents there. C. At \( t = 25 \), showing the colonisation of both layers, when NC cells have a sufficiently high proliferation rate. Red agents as in B above. (D–E) NC proliferation reduced to \( P_p = 0.3 \). D. At \( t = 0 \) with NC initial density in the donor at 0.4. E. At \( t = 25 \), showing the incomplete colonisation of the first layer, and even more extreme restriction in the second layer, when NC cells have a sufficiently reduced proliferation rate.
This proposal was tested with our CA model (Fig. 8). Since the aneural second layer emerges from the localised carrying capacity of the tissue, the already invaded region, which is undergoing local extension. An important mechanism is how quickly the NC agents can proliferate in this alternate case. The same outcome was observed in this alternative case. The locally crowded. Instead, we could have chosen the motility mechanism. A similar outcome was observed in this alternate case. The dependence on motility rate is much less than on proliferation rate, as seen in A. D. Measure of the relative variance versus the NC proliferation rate $P_p$ for different values of the initial density in the donor tissue (values and colours as in A). The lower the NC proliferation rate, the higher the variability for the same initial density. Also, the variability decreases as the initial density of NC cells increases, for each fixed proliferation rate. (C, D) NC proliferation fixed at $P_p = 1$. The mean density versus the NC motility rate $P_m$ for different values of the initial density in the donor tissue (values and colours as in C). The lower the NC motility rate, the higher the variability for the same initial density. Also, the variability decreases as the initial density of NC cells increases, for each fixed proliferation rate.

Crowding in the preferred plexus could be relieved by movement to the alternative plexus, provided that the local environment permits movement in this direction.

It must be emphasised that "crowding" and "occupation" refer to local conditions, they do not mean that the entire gut must be occupied by ENS cells at maximum density before any cells move to the less preferred layer. Note also that if there is room to proliferate and move in the existing layer, then this takes place. Note that we chose to use proliferation as the trigger for moving into the second layer, when locally crowded. Instead, we could have chosen the motility mechanism. A similar outcome was observed in this alternate case. The important mechanism is how quickly the NC agents can proliferate in the already invaded region, which is undergoing local extension.

A consistent mechanism for hindering the colonisation of the second layer emerges from the localised carrying capacity of the tissue. This proposal was tested with our CA model (Fig. 8). Since the aneural gut tissue is growing, the NC agents can continue to proliferate because more area is continually produced in their neighbourhood. Therefore, the less proliferative NC agents can proliferate in the new space made available, making trans-plexus movement into the new layer infrequent. Hence, the first layer may fill, but the second layer will be only sparsely occupied.

Highly proliferative NC agents are more likely to be able to fill their local neighbourhood; therefore if they are chosen to proliferate yet cannot proliferate within the existing layer, this provides impetus for one daughter cell to find its way to the second layer. If this occurs frequently, or early enough in the simulation, the highly proliferative NC agents within the second layer are able to proliferate freely and colonise this layer as well; that is, the second layer behaves like the first layer. Thus, the trans-plexus movement acts as a necessary seeding event, but most of the colonisation of both layers, even the less preferred, is by proliferation of cells already in that layer.

Therefore the CA modelling shows that crowding in the preferred plexus, combined with the proposal of proliferative inferiority of trunk level NC can translate into incomplete ENS formation in the most preferred plexus site and an even less extensive ENS plexus layer in the less preferred site, leading to the single plexus gut profiles frequently seen in sections of gut populated by trunk NC cells. The similarity of this outcome to the CAM grafts with trunk NC donor, and to the spatial discordance between myenteric and submucosal plexuses in the ET-3−/− mouse model (Payette et al., 1987) is striking. The molecular nature of plexus preference awaits further investigation.

Implications for somatic NC stem cell therapy if ENS ability is regionalised

Stem cell therapy is under intense investigation currently (Daley and Scadden, 2008) and optimization of outcomes is being sought through knowledge of normal embryonic developmental processes (Murry and Keller, 2008). NC stem cells (reviewed in Delfino-Machin et al., 2007) offer therapeutic potential for a variety of neurocristopathies (Jones and Trainor, 2004). The life-threatening birth defect Hirschsprung's disease is caused by a lack of ENS in the distal intestine, caused by failure of NC-derived cells to migrate to this region (Newgreen and Young, 2002a,b). Current surgical treatment leaves...
intestinal transit problems in two-thirds of patients (Catto-Smith et al., 1995). Hence to avoid bowel resection, NC stem cell therapy has been proposed at post-natal stages to build an ENS in the aganglionic distal intestine (Grundy and Schemann, 2005; Hotta et al., 2009a; Martucciello et al., 2007; Young, 2005; Young et al., 2006). NC stem and progenitor cells can be induced or isolated from a variety of somatic and ES sources (Almond et al., 2007; Brokhman et al., 2008; Hotta et al., 2009b; Hunt et al., 2008; Lee et al., 2007). If efficient ENS-generating capacity is regionally restricted very early in the NC, it may also be restricted in somatic NC stem-like cells, given that early-established positional information in other cells has remarkable longevity (Rinn et al., 2006). Positional information in stem cells has already been reported (Hitoshi et al., 2002; Temple, 2001; Klein et al., 2005; Leucht et al., 2008), though whether this is preserved is controversial (Jesús Santa-Olalla et al., 2003; Kelly et al., 2009). In particular, NC stem cells of different origins have different differentiation profiles (Mosher et al., 2007) which in the case of post-natal mouse hair follicle-derived NC stem cells, appears to relate best to embryonic axial level position of origin (Wong et al., 2006). Therefore, somatic stem cell therapy for Hirschsprung’s disease may need to reckon with achieving appropriate positional information for the replacement NC-like stem cells, as well as with achieving general NC lineage properties. The same principle of the importance of matching positional information also applies to all stem cell therapies that hope to build or repair tissues with specific locations and structures.

Conclusions

From our experimental work, from previously published work and from mathematical modelling, we conclude that major differences in ENS-forming ability do occur between all cranial and all trunk levels of the NC, and these rest to an important degree on greater proliferation of the cranial cells in the gut environment. In a system in which the gut environment is growing, this apparently simple differential proliferation is able to generate complete versus incomplete colonisation, differential and variable plexus development, and determine the outcome of competition between NC cells for ENS formation. In addition, because this position-of-origin difference in ENS competence is established prior to NC cell migration, it is carried with migratory cells and may be conserved after migration in somatic NC stem cells. This positional memory may impose previously unrecognised restrictions on the optimum NC stem cells to be used for future somatic stem cell therapies. A combined modelling and experimental approach is crucial in guiding further work to understand ENS formation and also stem cell therapy. The modelling approach is a powerful tool in testing hypotheses and designing experiments, as demonstrated here. Work on the molecular cues involved in ENS development, such as the plexus preference suggested by the modelling, is needed.

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