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Fine scale differences within the vagal neural crest for enteric nervous system formation

Johanna E. Simkin[a,1,2], Dongcheng Zhang[a,2], Lincon A. Stamp[b], Donald F. Newgreen[a,*]

[a] Murdoch Children's Research Institute, Royal Children's Hospital, Parkville 3052, Victoria, Australia
[b] Dept Anatomy and Neuroscience, University of Melbourne, Parkville 3010, Victoria, Australia

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ABSTRACT

The enteric nervous system is mostly derived from vagal neural crest (NC) cells adjacent to somites (s)1–7. We used *in ovo* focal fluorescent vital dyes and focal electroporation of fluorophore-encoding plasmids in quail embryos to investigate NC cell migration to the foregut initially and later throughout the entire gut. NC cells of different somite-level origins were largely separate until reaching the foregut at about QE2.5, when all routes converged. By QE3.5, NC cells of different somite-levels became mixed, although s1-s2 NC cells were mainly confined to rostral foregut. Mid-vagal NC-derived cells (s3 and s4 level) arrived earliest at the foregut, and occurred in greatest number. By QE6.5 ENS was present from foregut to hindgut. Mid-vagal NC-derived cells occurred in greatest numbers from foregut to distal hindgut. NC-derived cells of s2, s5, and s6 levels were fewer and were widely distributed but were never observed in the distal hindgut. Rostro-vagal (s1) and caudo-vagal (s7) levels were few and restricted to the foregut. Single somite levels of quail neural tube/NC from s1 to s8 were combined with chick aneural ChE4.5 midgut and hindgut and the ensemble was grown on the chorio-allantoic membrane for 6 days. This tests ENS-forming competence in the absence of intra-segmental competition between NC cells, of differential influences of segmental paraxial tissues, and of positional advantage. All vagal NC-levels, but not s8 level, furnished enteric plexuses in the recipient gut, but the density of both ENS cells in total and neurons was highest from mid-vagal level donors, as was the length colonised. We conclude that the fate *and* competence for ENS formation of vagal NC sub-levels is not uniform over the vagal level but is biased to favour mid-vagal levels. Overviewing this and prior studies suggests the vagal region is, as in its traditional sense, a natural unit but with complex sub-divisions.

1. Introduction

For almost a century the autonomic nervous system has been viewed as having 3 division: the parasympathetic nervous system (with cranial and sacral divisions), the sympathetic nervous system and the enteric nervous system (ENS). Prior to Langley (1921) the ENS was regarded as a part of the parasympathetic nervous system. Recently a revision of the sacral parasympathetic nervous system based on its development was proposed (Espinosa-Medina et al., 2016) and this has been controversial (Ernsberger and Rohrer, 2018; Espinosa-Medina et al., 2018; Horn, 2018; Janig et al., 2017). Even more recently, and also based on developmental studies, a reclassification of the origin of the ENS has been proposed (Espinosa-Medina et al., 2017).

The ENS is the largest and most complex division of the neural crest (NC)-derived autonomic nervous system. Enteric neuropathies are relatively common and the prospect of investigating and treating them using human pluripotent cells induced to differentiate into ENS lineages is approaching (Burns et al., 2016). Many methods have been described for producing human NC-like cells (Zhu et al., 2016), but developmental studies suggest that certain spatially defined NC cells are optimal for later ENS differentiation (Newgreen et al., 1980; Zhang et al., 2010). The properties of these pro-ENS NC cells would need to be known and replicated for optimal ENS differentiation (Fattahi et al., 2016).

1.1. The spatial origin of ENS cells

'Post-otic and anterior spinal' NC was accepted as the major source of the avian ENS (Kuntz, 1922; Le Douarin and Teillet, 1973; Tucker

* Corresponding author.
E-mail addresses: jsimkin@museum.vic.gov.au (J.E. Simkin), don.newgreen@mcri.edu.au (D.F. Newgreen).
URL: https://bit.ly/2AJGvaz (J.E. Simkin).
[1] Present address: Human Biology and Medicine, Museums Victoria, Australia.
[2] Equal contribution.

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et al., 1986; Yntema and Hammond, 1954). This NC was bounded by the levels of somite 1 (s1) and somite 7 (s7), and for convenience this was termed the 'vagal' NC; this level will be referred to here as 'vagal$^{1-7}$'. This overlaps the cranial level (as defined by the occipital somites) and the rostral trunk NC (Kuo and Erickson, 2011). The cranial-trunk interface in the NC is thought to occur at the s3/4 border (Ferguson and Graham, 2004). No contribution to the ENS was described from more cranial NC, that is, rostral to the vagal$^{1-7}$ level or from trunk NC from s8 to s27 level. However trunk NC at sacral-levels (at and caudal to s28) contributes to the distal ENS, mainly in the colo-rectum, although numerically this is less than the vagal$^{1-7}$ NC's contribution (Burns and Le Douarin, 1998).

The origin of the mouse ENS is broadly similar: vagal NC and sacral NC (Cheung et al., 2003; Durbec et al., 1996; Kapur, 2000; Wang et al., 2011) but axial levels of origin are less well defined, and Durbec and colleagues refer to the s1-s5 level as vagal and the s6-s7 level as anterior cervical (ie. trunk). Recently in the mouse a postnatal-appearing ENS sub-population has been identified, deriving from Schwann cell precursors (SCPs). The identity of these SCPs as distinct from early NC cells is a matter of intense discussion (Furlan and Adameyko, 2018). The presumption is that these originate from mid-trunk and sacral NC (Uesaka et al., 2015), but it is also possible that these cells are at least partially derived from vagal$^{1-7}$ trans-mesenteric enteric NC cells (Nishiyama et al., 2012) that have lingered in the mesentery. Whether this source has a parallel in other vertebrates is not yet known.

1.2. Sub-divisions of the vagal$^{1-7}$ NC

There are several axial levels in the NC, but their anatomical domains are not agreed upon and show some species differences (Hutchins et al., 2018). NC terminologies are also diverse, and may reflect the position of origin, and this may be broad (eg. cranial NC), or fine (eg. somite 1-level NC). Other names imply the final location (eg. circumpharyngeal NC) or fate (eg. cardiac NC, adreno-medullary NC). These naming systems are not always congruent, often overlap and assigning precise borders and functions to different levels has been controversial (Burke, 2000; Couly et al., 1993; Ferguson and Graham, 2004; Kuo and Erickson, 2011; Kuratani, 1997; Lumsden et al., 1991; Maschner et al., 2016; Shigetani et al., 1995).

The vagal$^{1-7}$ NC was tacitly assumed to be a natural unit with respect to origin of the ENS, but this level was sub-divided by Durbec and colleagues (Durbec et al., 1996), who identified s1-s5 level NC in the mouse embryo as providing Ret-dependent ENS cells (i.e. ENS cells which are lacking in *Ret*$^{-/-}$ mice) throughout the gastro-intestinal tract; they termed this NC level the *sympatho-enteric* NC since it also produced the most rostral sympathetic ganglion, the superior cervical ganglion (SCG), which is also Ret-dependent. Ret is the NC cell surface receptor for GDNF family signalling which is vital for ENS formation (Taraviras et al., 1999). NC cells of s6-s7 level (ie. cervical trunk NC) co-contributed 'a large (albeit reduced compared to the wildtype) number of cells' to the ENS in the foregut but not to the more distal ENS. These cells were Ret-independent since they persisted in *Ret*$^{-/-}$ mice, as did the sympathetic chain ganglia. Durbec and colleagues regarded this level as the rostral end of the *sympatho-adrenal* or trunk NC (Carnahan et al., 1991). It was known that there were differences in ENS-forming capacity between avian vagal$^{1-7}$ NC and trunk NC (Newgreen et al., 1980), but Durbec et al.'s study was important in that it showed for the first time that there were differences *within* the traditional vagal$^{1-7}$ NC.

The concept of the unitary vagal$^{1-7}$ NC has also been challenged as being a pseudo-unit (Espinosa-Medina et al., 2017). Instead, these authors defined *two* post-otic ENS-forming NC levels which are contiguous but differ in properties. These are a NC region adjacent to s1 and s2 (*sensu stricto* the region of vagus nerve outflow from the hindbrain): this NC – termed here vagal$^{1-2}$ – supplies many ENS cells to the foregut and in lesser numbers to the midgut and hindgut. These cells are dependent on neuregulin1 signalling via the ErbB3 receptor (Britsch et al., 1998). These NC cells migrate in concert with, and require, vagal axons and are classed as SCPs. Secondly they identified the NC adjacent to s3 to s7 whose cells migrate to the sympathetic ganglia but many cells 'overshoot' the sympathetic chain and extensively colonise all levels of the gut. These cells, in contrast to the vagal$^{1-2}$–derived SCPs, migrate in the gut in advance of and are independent of vagal axons and are ErbB3-dependent only to a degree, with the degree decreasing for progressively more distal-located ENS cells. They equated this vagal$^{3-7}$-level to the rostral-most trunk NC, although it extends one segment into the cranial NC domain as defined by Ferguson and Graham (2004). Espinosa-Medina et al. (2017) redefined the term *sympatho-enteric* (see above for usage by Durbec and colleagues) to refer to NC caudal from the s2/3 border to reflect their findings of functional difference between vagal$^{1-2}$ NC and vagal$^{3-7}$ NC.

1.3. Intra-vagal NC axial-level ENS fate differences

Avian vagal$^{1-7}$ NC cells of s1, s2 and, decliningly, s3 and s4-levels mainly contribute the circumpharyngeal ectomesenchyme (Kuratani and Kirby, 1992; Shigetani et al., 1995) but also provide some ENS cells. Kuo and Erickson (2011) observed that some s1-s2-level NC cells reach the future oesophageal/tracheal region (i.e. proximal foregut) by E4 whereas NC cells from s3 and s4-level contribute most to the ENS down to and including the stomach. NC cells of s5 and s6-level also reached the stomach, but they did not observe s7-level NC cells in the gut by this stage. These enteric NC cells later colonise the rest of the gut as a timetabled rostro-caudal wave within the gut mesoderm (Allan and Newgreen, 1980), being joined later in the hindgut by sacral NC cells (Burns and Le Douarin, 1998). Similar events occur in the mouse (Kapur, 2000; Wang et al., 2011; Young et al., 1998).

The later fate of sub-regions of the vagal$^{1-7}$ NC has also been explored with *in ovo* orthotopic transplants carried through to stages when colonisation is complete (Burns et al., 2000; Espinosa-Medina et al., 2017). While there was over-representation in the ENS of mid-vagal$^{1-7}$ NC-derived cells and considerable mixing, there was a tendency for the rostro-caudal order along the vagal$^{1-7}$ axis to be translated later to the ENS derivatives placed rostro-caudally along the gastro-intestinal tract. This raised the intriguing possibility of rostro-caudal co-linearity between the vagal$^{1-7}$ NC and the ENS.

The same question of later fate was the subject of a large and detailed *in ovo* lacZ viral labelling study by Epstein's group (Epstein et al., 1994), who tracked the fate of single-somite levels of the vagal$^{1-7}$ NC in the chicken embryo ENS. Rostral vagal$^{1-7}$ NC cells (s1, s2 levels) contributed strongly to the cardiac outflow and pharyngeal arches but produced few ENS cells which were restricted to the oesophagus, crop, proventriculus and gizzard and duodenum (i.e. foregut derivatives). NC cells of s3-s6-levels made the greatest contribution to the ENS in all regions of the gastro-intestinal tract although the number in the oesophagus was low. The caudo-vagal$^{1-7}$ NC (s7) also produced few ENS cells, but these were restricted to the proximal digestive tract. Paralleling this, Durbec et al. (1996) has described that in mouse the rostral cervical NC (i.e. caudo-vagal$^{1-7}$) contributed only to the oesophageal ENS. These results are not consistent with NC/ENS co-linearity described above.

1.4. Intra-vagal NC axial-level functional differences

Epstein et al. (1994) concluded from the degree of overlap in the fate-map that there were no differences of competence of vagal$^{1-7}$ sub-regions to supply the ENS of specific gut regions. Kuo and Erickson (2011) concluded that there were great similarities but also some inherent differences between rostro- and caudo-vagal$^{1-7}$ NC cells, displayed as pathway preferences and choice of cardiac or ENS fate. They drew the line of difference as falling between s3 and s5 level. As

described above, Durbec et al. (1996) also suggested two regions within the vagal[1–7] NC, with the division falling at about s5/6. Espinosa-Medina et al. (2017) also concluded that there were two different regions within the vagal[1–7] NC, with the division at s2/s3, the caudal region being the rostral extreme of the trunk NC.

Gross axial level differences in ENS competence have been tested with experimental ablation and recombination approaches. Chick/quail embryo heterotopic transplants and combination grafts showed that trunk NC (caudal to s7) can contribute ENS experimentally (Le Douarin and Teillet, 1974; Smith et al., 1977) but is far inferior numerically to vagal[1–7] NC (Newgreen et al., 1980; Peters-van der Sanden et al., 1993b; Zhang et al., 2010). In contrast, cranial NC cells from levels rostral to the vagal[1–7] level, though not fated to form ENS, were capable of ENS formation when given the opportunity (Zhang et al., 2010).

Attention has been turned to finer scale differences of competence within the vagal[1–7] NC region. Ablation of s3-s5 level NC *in ovo* resulted in absence of ENS only in the hindgut, with the consequent suggestion that s3-s5 level vagal NC was spatially specialised beforehand for hindgut ENS formation (Peters-van der Sanden et al., 1993a). These results were confirmed later (Barlow et al., 2008; Burns and Le Douarin, 2001), and further experiments in Barlow et al.'s study pointed to fine scale differences of ENS competence within the vagal[1–7] NC. Following ablation of the entire vagal[1–7] neural *anlage*, s3-level neural tube with NC cells was re-inserted at s3-level. This restored ENS colonisation of the entire gut. In contrast, re-insertion of s1-level NC cells at this s3-level gave incomplete colonisation, in that only the foregut was colonised. Moreover s3-level NC cells implanted at s1-level did not give complete colonisation. This was interpreted as demonstrating two processes: i) intrinsically different ENS development potentials exist along the rostro-caudal neuraxis within the vagal[1–7] level, with s3-level NC having greater ENS forming ability than s1-level; ii) certain NC cell migration routes (e.g. adjacent to s3) are more favourable than others (e.g. adjacent to s1) for ENS colonisation.

1.5. Comparison with the origin of the rostral sympathetic ganglia

The most rostral sympathetic ganglia can be compared with the ENS as they are also produced by the NC and lie proximal to the gut. Avian explant cultures suggested that the SCG catecholaminergic neuron precursors lie in the distal vagal[1–7] NC (about s6) domain (Newgreen, 1979). In mouse Durbec et al. (1996) found s4 level NC cells contributed to the SCG and more recent *in vivo* labelling studies show that s3-level NC cells (but not vagal[1–2] level) contribute to the SCG (Espinosa-Medina et al., 2017) where, interestingly, the s3 NC cells produced not neurons but ganglionic glia. A resolution of the axial level difference in these studies may be that most SCG neurons arise several somite-widths caudal to the origin of at least some of the ganglionic glial cells. This would require that sympathetic cells migrate several segments from their level of origin, which has been observed elsewhere in the sympathetic chain (Kasemeier-Kulesa et al., 2005; Yip, 1986).

1.6. Scope of this study

There are some differences reported in ENS fate-mapping of the post-otic NC, so we attempt to clarify this with short-term and long-term fate mapping studies using focal labelling of single-somite levels of avian pre-migratory vagal[1–7] NC by vital dyes (Lumsden et al., 1991) and electroporation of fluorescent markers (Sato et al., 2007; Simkin et al., 2009). Then, ENS-forming ability was evaluated by combining the neural *anlage* (including pre-migratory NC) with aneural mid and hindgut, the ensemble grafted to the chorio-allantoic membrane (CAM). The distinction from previous CAM graft studies (Zhang et al., 2010) is that single somite-lengths of the neural *anlage* were used.

The results suggest that there are relative but important gradations in ENS fate along the vagal[1–7] axis; this confirmed most previous results but did not support the idea of NC/ENS 'co-linearity'. This gradation in fate was exactly matched by differences in efficiency of vagal[1–7] NC sub-levels to form ENS in the mid and hindgut.

Given the emergence of functional differences within the vagal[1–7] zone for ENS generation, the recent denial of this traditional zone as a natural entity and the proposal of new sub-divisions within this zone (Espinosa-Medina et al., 2017), we re-assess the new categorisations and terminologies of the post-otic enteric NC. The results carry implications for optimal NC cell identity for ENS stem/progenitor cell therapies.

2. Materials and methods

2.1. Ethics statement

This study used early stage avian embryos so is deemed non-reportable; it was approved by the Royal Children's Hospital Animal Ethics Committee (AEC A596 and A650).

2.2. Avian embryos

White Leghorn/Black Australorp cross chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were obtained from Research Poultry Farm and Lago Game Supplies Vic., Australia, respectively. Eggs were incubated at 38 °C and 60% humidity and embryos were staged by embryonic days (E), Hamburger and Hamilton stages (HH)(Hamburger and Hamilton, 1951) and, for embryos of less than E2.5, by somite stage (ss) counts.

2.3. Identification of somite levels

The first one or two somites lose clear segment boundaries (Hammond, 1965; Huang et al., 1997; Williams, 1910) so it is important to be able to identify somite levels after this has occurred. DiI (SP-DiI C18, D7777, Invitrogen) was prepared at 2 μg/mL and microinjected into s1 at HH9 (7 ss) *in ovo*, then the embryos (N = 4) were allowed to continue developing for 1–2 days. The embryos were fixed in 4% PFA in PBS and the DiI-labelled somite structure identified (Suppl. Fig. 1).

2.4. Single segment short term labelling of vagal NC cells

Short term focal labelling was achieved *in ovo* by vital staining. DiI and DiA (D3911 and D3883 respectively; Invitrogen) were dissolved in ethanol at 2 mg/mL. Several agarose beads (diam. about 100 μm; Cibacron blue 3GA beads, C1535, Sigma, St. Louis, MO) in distilled water were transferred as a drop to a sterile non-tissue culture dish and allowed to partially dry, then a small drop of dye solution was added and the ethanol allowed to evaporate. Conventional techniques as used for *in ovo* grafting (Le Douarin et al., 2000) were used to insert beads in the vagal[1–7] neural tube of QE1.5 (ss5–10, HH8+ to HH10) embryos with a tungsten needle, after which the eggs were sealed with adhesive tape and returned to the incubator for 1 and 2 days. When several beads were inserted in the one host, a separation of one or more somite-widths was maintained. It is important to place beads precisely initially; nudging them into the desired final position results in a trail of labelled cells. The embryo age and somite level of insertion was recorded.

Focal labelling was also performed by *in ovo* electroporation. The transposon-containing pT2K-CAGGS-GFP expression construct encoding EGFP, and the pCAGGS-T2TP expression construct encoding transposase (Sato et al., 2007) was provided by Dr. Yoshiko Takahashi (Nara, Japan). Dr. Catherine Krull (Ann Arbor, MI, USA) supplied the pMes GFP construct (Swartz et al., 2001). The dsRed-Express2 construct pdsRed-Express2-N1 was from BD Biosciences Clontech (Franklin Lakes, NJ, USA). These were prepared using a

QIAfilter Plasmid Midi kit (12243; QIAGEN, Hilden, Germany), and all were resuspended in water at 2.4–4 μg/mL. Agarose beads were dried then a few μL of construct solution was added as a standing drop. The beads immediately imbibed the construct solution, swelling to their previous size. These were inserted in the host neural tube as above. In some cases beads with different fluorophore plasmids were inserted at different sites in the neural tube of the one host. For neural tube/NC electroporation at vagal$^{1-7}$ level in HH9-HH10 (7–10 ss) embryos (Kadison and Krull, 2008), parameters were three 10–12 V, 50 ms pulses delivered unilaterally (right side) at one second intervals, using a BTX ECM 830 square-wave electroporator (Fisher Biotech, Melbourne, Australia) with gold electrodes. The eggs were sealed and re-incubated for 1 and 2 days. For details see Simkin et al. (2009).

2.5. Single segment long term labelling of vagal$^{1-7}$ NC cells

Only the transposon-based system was used for long term expression since this stably integrates into the genome of the transfected cells (Sato et al., 2007). The same electroporation system was used on embryos of QE1.5 (HH9-HH10; 7–10 ss). Incubation was continued for a further 5 days. Details are given elsewhere (Binder et al., 2012).

2.6. CAM grafting

Vagal$^{1-7}$ NC cell donors consisted of transverse slices of QE1.5 (ss7–11) one segment wide from the levels of s1 to s8. Somites, ectoderm, endoderm, notochord and lateral plate were removed using Dispase II digestion (Zhang et al., 2010). As positive control for the ability of vagal NC cells to form ENS, the proximal midgut of QE4 embryos were used as donor; these contain the enteric NC cell wavefront in the gut mesoderm (Simpson et al., 2007) and efficiently form ENS in aneural gut (Zhang et al., 2018).

The CAM of chick hosts of ChE7–8 were prepared as in Zhang et al. (2010). Aneural mid and hindgut from the umbilicus to the cloaca (Allan and Newgreen, 1980) was dissected from ChE4.5 (HH25 or younger) embryos in Ham's F12 medium (Thermo Electron, Melbourne, Australia) using tungsten needles. Guts were staged according to Southwell (2006). The quail NC donor tissues were placed in contact with the rostral end of the chick aneural gut on a small square of sterile Millipore HA paper (Hearn and Newgreen, 2000) for growth on the host CAM for 6 days.

2.7. Fixation, fluorescent labelling, imaging and evaluation

Embryos and CAM grafts were retrieved from the egg and fixed for 1 h in 4% PFA in PBS for wholemount preparations. QE2.5 to QE3.5 embryos were sagittally or transversely sliced with tungsten needles during the first minutes of fixation. For QE6.5 embryos and CAM graft specimens, the gastro-intestinal tract was microdissected free and treated with 0.05% trypsin (Roche, Aust.) for 10 min before fixation. Antigen retrieval of fixed specimens used 10 mM citrate buffer pH 6.0 for 20 min at 95 °C. Specimens were washed in PBS 3 times for 10 min, permeabilized and blocked with 0.1% Triton X-100% and 1% horse serum in PBS for 30 min, then incubated overnight or for 2 days at 6 °C sequentially in primary and secondary antibodies (Suppl. Table 1) in blocking solution. Specimens were washed > 6 h in PBS between treatments. Specimens were mounted in glycerol with 200 mM DABCO (Sigma-Aldrich) antifade reagent between two coverslips with coverslip spacers. Antibodies are listed in Supplementary Table 1.

Samples were visualized using an Olympus IX70 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 Image-Pro Plus 4.5 (MediaCybernetics, Silver Spring, MD, USA). Confocal imaging was performed with a Leica TCS SP2. Image processing was via Leicalite, ImageJ and Image-Pro–Analyser 6.1 (MediaCybernetics).

2.8. Cell counts

In QE6.5 embryos focally transfected by electroporation, GFP+ cells were counted in gastrointestinal tract wholemounts after GFP antibody (see above) for seven major region of the gastro-intestinal tract: 1. oesophagus and proventriculus, 2. gizzard, 3. duodenum and pre-umbilical midgut (termed mg1), 4. post-umbilical midgut (mg2), 5. caecal region, 6. proximal hindgut (hg1), 7. distal hindgut (hg2). The number of GFP+ cells over 50 and up to 199 were rounded to the nearest 50. GFP+ cell numbers from and above 200 were rounded to the nearest 100.

For NC cell density estimates in CAM grafts, the ENS was counted in wholemounts similarly to previously (Rollo et al., 2015; Zhang et al., 2018). In brief, the chick midgut of each graft was imaged at x20 at multiple focal planes confined to the myenteric (outer) plexus. In each segment QCPN+ and HuC/D+ cells were then counted in six 100 × 100 μm squares. (Note that all non-neuronal NC-derived cells in the plexuses were SoxE+, but QCPN immunoreactivity was more suitable for counting). Neural cells in the submucosal (inner) plexus or in the muscle layers or lamina propria were not counted.

2.9. Statistics

Data are expressed as mean ± standard error of the mean (sem). All statistical tests were performed using Excel and GraphPad Prism version 6. A difference among multiple groups, statistical comparisons were performed using one-way ANOVA followed by Fisher's LSD post-test. A P value of less than 0.05 was considered significant.

3. Results

3.1. Short term tracking of single segment vagal NC cells in vivo

NC cells of s1 (N = 7) and s2 level (N = 7) labelled with DiI, DiA or by electroporation left the neural tube in the dorso-lateral route over their somite of origin, under the ectoderm. Upon reaching the lateral-most part of the embryo adjacent to s1 and s2 about 12 h after labelling (QE2, HH13), labelled cells then continued to migrate under the ectoderm with a slightly caudal drift to be present at the rostral base of s3 by about QE2.25 (HH14). Some cells were placed more ventrally and angled towards the midline near the foregut subjacent to s3 by QE2.5 (HH15–16). However most labelled cells from s1 and s2 levels became crowded into pharyngeal arches 3–6 (Suppl. Fig. 2). By QE3.5 (HH17) most ENS cells of this origin were in the most proximal gut between the pharyngeal arches but proximal to the swelling of the future gizzard.

Labelled NC cells of s3 and s4 levels appeared in both the dorso-lateral route (s3 > s4) and the ventral hemisegmental route (s3 N = 16; overlapping s2 and s3 N = 3; s4 N = 9). Both pathways led to the base of s3-s4 (though the dorso-lateral stream was situated more laterally, and appeared to be in line for the outflow vessels of the heart). Labelled cells then were found more ventrally and caudally around the side of the dorsal aorta. Transverse slices showed these labelled NC cells surrounding the dorsal part of the foregut by QE2 (HH14) but were not in the ventral foregut at this stage. The s3 and s4-level NC cells were within the foregut mesoderm by QE2.5 (HH15), as early as or earlier than NC cells of other sub-regions, and were in greater numbers than NC cells of other levels, and spread further rostrally and caudally (Fig. 1A). By QE3.5 (HH17) these cells were found in large numbers throughout the foregut. Unlike NC cells of more caudal origin, these NC cells did not accumulate at the position of the sympathetic ganglia (Fig. 1A; Suppl. Fig. 2).

NC cells of s5 (N = 22), s6 (N = 12), s7 (N = 15) and s8 (N = 7) levels moved via the ventral route. Cells from s5 to s8 collected in sympathetic ganglia of the corresponding somite-level and with lesser contribution to 1 or 2 adjacent ganglia. Upon reaching the sympathetic

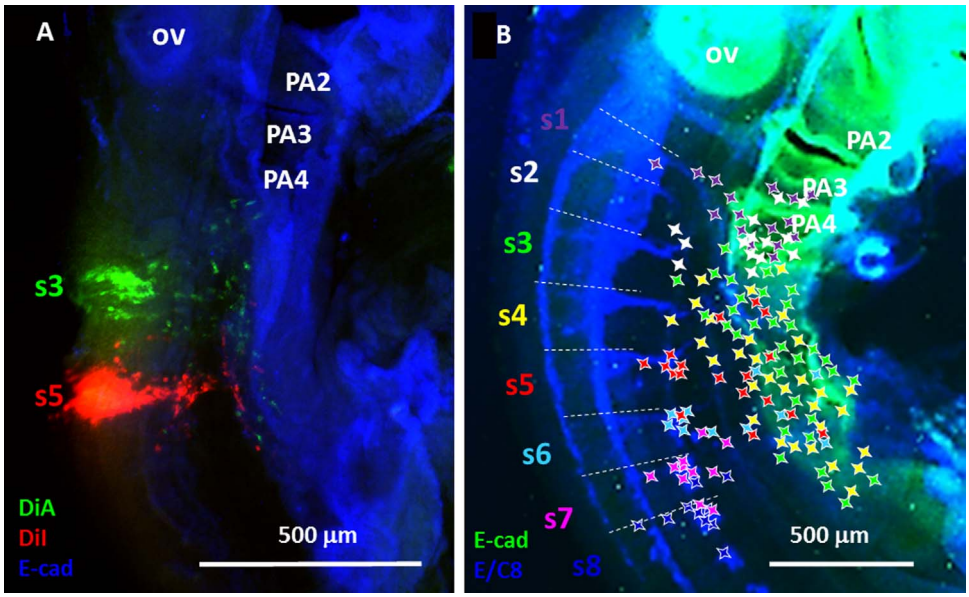

Fig. 1. Labelled vagal$^{1-7}$ NC cells of different somite levels migrate to the foregut in ovo. A. Quail embryo (HH15, 27 ss; 24 h post labelling; sagittal slice) with vagal$^{1-7}$ NC cells of s3 level labelled with DiA, and s5 NC cells labelled with DiI. Many s3 NC cells and fewer s5 NC cells have reached the foregut (endoderm E-cadherin), and have spread along it. B. Scheme of location of vagal$^{1-7}$ NC cells ~24–48 h after focal DiI/A and electroporation labelling at s1 to s8-levels of NC at the 5–9 ss. Cells of s1-s6 origin were plotted at ~30 ss and s6-s8 at ~35 ss. Cells are projected onto an HH17 embryo sagittal slice wholemount labelled for axons (E/C8) and endoderm (E-cadherin). Not all cells are shown. PA: pharyngeal arch; OV: otic vesicle.

position of their corresponding somite's ventral margin, labelled cells appeared further ventrally on either side of the dorsal aorta on an angle towards the midline. The labelled cells eventually reached the dorsal mesentery to reach the gut. Labelled s5 and s6-level NC cells approached the gut opposite their somite of origin at about QE2.5 (Fig. 1A) but did not migrate greatly in the rostral direction. NC cells of s7 level were found directly ventral in that somite but were not detected in the foregut, and s8-level cells did not extend past the dorsal aortal level.

Distally the tight streams of migrating NC cells through the somites s3 to s7, became more rostro-caudally dispersed from the bottom of the somites, dorsal to the foregut. The NC cells appear to swarm around the dorsal half of the foregut (i.e. many vagal$^{1-7}$ NC cells were at this area and NC cells of different somite levels intermingled). This was particularly noticeable with s3 and s4 vagal NC cells where there were more labelled cells. These vagal$^{1-7}$ NC cell distribution patterns are shown schematically in Fig. 1B.

3.2. Long term tracking of single segment vagal NC cells in vivo

To investigate vagal$^{1-7}$ NC enteric distributions over the longer term, individual sub-populations were labelled via *in ovo* focal electroporation. At QE6.5 (HH30–31), guts were harvested (N = 23) for immunolabelling. GFP+ gut specimens were immunostained for GFP, the neuronal marker HuC/D and the enteric NC/glial marker SoxE.

GFP-labelled vagal$^{1-7}$ NC sub-populations contributed to all or most regions of the gut (Fig. 2) in the form of isolated patches with variable numbers of GFP+ cells interspersed with regions with few or no GFP+ cells. The number of GFP+ cells varied greatly between gut regions and between specimens, even from the same focal sub-region (Fig. 3). Despite this uneven distribution of GFP+ cells, total ENS colonisation and differentiation was normal in all specimens since SoxE+ enteric NC cells and HuC/D+ enteric neurons were found profusely along the gut.

The s3 and s4-level of vagal$^{1-7}$ NC contributed moderate to large numbers of GFP+ cells; often in all 7 major gut regions. When the GFP + contributions of s2, s5 or s6-level vagal NC were considered individually, GFP+ cells were spread over several adjacent regions. However when each of these somite levels were summed, all contributed GFP+ cells to 6 of the 7 major gut regions, the exception being the distal hindgut. The number of GFP+ cells from these levels was smaller than from s3 and s4-levels.

The most rostro-vagal$^{1-7}$ NC (s1) and most caudo-vagal$^{1-7}$ NC (s7) levels of origin provided small numbers of GFP+ cells with a spread restricted to one or two major gut regions centred on the gizzard and the next region rostral, or the next region caudal. Neither s1 nor s7 levels contributed labelled cells to the ENS distal to the duodenum. The numerical variability between individuals was highlighted by one embryo labelled at the extreme caudo-vagal region, overlapping s7 and s8. This showed over 150 GFP+ cells in the gizzard, the third highest GFP cell number recorded in this region. This distribution of GFP+ cells in various segments of the gastro-intestinal tract is shown in Fig. 3.

3.3. Competence of single segment vagal NC cells to populate aneural midgut and hindgut in co-grafts

In CAM grafts, chick aneural post-umbilical midgut, caeca and hindgut co-grafted with donor single-somite quail vagal$^{1-7}$ NC cells placed at the rostral end of the recipient gut (N = 65) developed ENS plexuses consisting of donor (QCPN+) cells which were all either HuC/D+ neurons or SoxE+ enteric NC cells or glia. ENS cells were distributed in the midgut and often into the caeca and the hindgut (Fig. 4A). Distally ENS cells in total and especially neurons were fewer (Suppl. Fig. 3). This is expected since distal hindgut and cloaca is normally slow to be colonised (Allan and Newgreen, 1980; O'Donnell et al., 2004).

As controls for cell counts to assess colonisation ability and ENS cell differentiation, newly colonised QE4 proximal midgut segments were used as 'gold standard' enteric NC cell donors (Fig. 4B). In all cases (N = 6) donor midgut-derived QCPN+ cells colonised the recipient gut rapidly and to high density in the recipient midgut myenteric plexus (almost 70 ENS cells/0.01 mm^2) with almost half differentiating as neurons by 6 days growth (Figs. 4B, 5A-D). In all control specimens the ENS extended into the hindgut.

Single somite level quail neural tube/NC donors showed more variable ability to form ENS in the CAM graft assays. The NC-derived

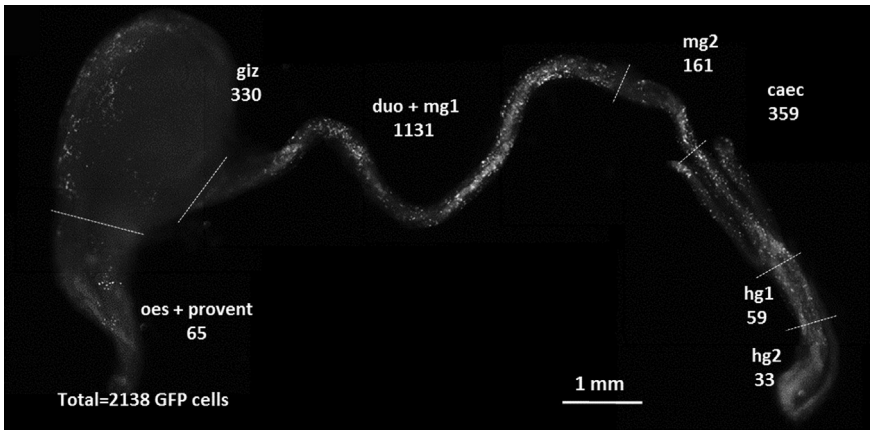

Fig. 2. Mid-vagal$^{1-7}$ NC cells migrate extensively throughout the gastro-intestinal tract in ovo. Cells labelled with GFP at s4-level at QE1.5 are widely but patchily distributed in the gastro-intestinal tract at QE6.5. The number of GFP+ cells in each gut region is shown. Gut regions: oesophagus and proventriculus (oes+prov), gizzard (giz), duodenum and pre-umbilical midgut (duo+mg1), post-umbilical midgut (mg2), caeca (caec), proximal hindgut (hg1), distal hindgut (hg2).

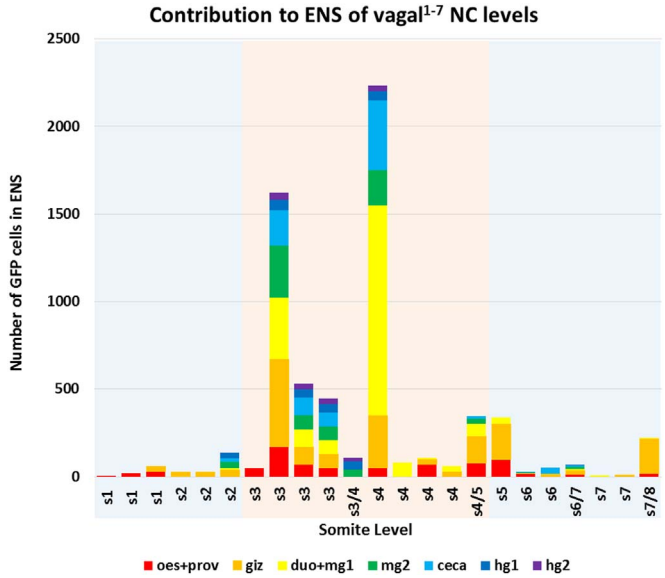

Fig. 3. S3 and s4-level NC has greater efficiency than rostral-vagal$^{1-7}$ NC and caudal-vagal$^{1-7}$ NC in colonising the quail gastro-intestinal tract *in ovo*. The number of GFP+ cells in gut segments at QE6.5 (y-axis) plotted against each level of origin (s1 level, s2 level etc.) focally electroporated at QE1.5 (x-axis). The seven major gut segments are colour-coded: oesophagus and proventriculus (oes+prov), gizzard (giz), duodenum and pre-umbilical midgut (duo+mg1), post-umbilical midgut (mg2), caeca, proximal hindgut (hg1), distal hindgut (hg2).

QCPN+ cells in the myenteric plexus from donors of s3 (N = 8) and s4 (N = 9) levels were similar to each other (nearly 80 ENS cells/0.01 mm^2), and to the QCPN+ cells from control midgut donors (Fig. 5A). However the density of non-neuronal myenteric ENS cells (over 50 cells/0.01 mm^2) was significantly higher than with midgut control donors (Fig. 5B). The density of ENS cells that had differentiated as neurons was not significantly different between s3, s4-level and midgut control donors (Fig. 5C) but the proportion of myenteric ENS cells that were neurons was lower than that of the midgut donor controls (Figs. 4C, 5D) and this difference was significant for the s4 level donor (midgut 45% vs s4 28%). The ENS in 12 of 17 s3 and s4-level donor grafts extended into the hindgut after 6 days growth on the CAM.

NC cell donors of s1 level (N = 7) produced a less dense ENS in the myenteric plexus (32 ENS cells/0.01 mm^2) with far fewer neurons (8 ENS cells/0.01 mm^2) compared to both the control donors and the s3-level donors. The ENS extended into the hindgut in only 2 of 7 s1-level grafts. The ENS furnished by s2 level donors (N = 10) was, compared to s1 level, significantly more dense in total (55 ENS cells/0.01 mm^2) and for non-neuronal cells. However, these densities were significantly less than those of midgut and s3-level donors. The ENS extended into the hindgut in 5 of 10 s2-level grafts. Caudal to s4 the ENS formation ability declined. Donors of s5 (N = 6), s6 (N = 8) and s7 (N = 7) levels provided an ENS cell density in the myenteric plexus of about 40–45 ENS cells/0.01 mm^2 which was significantly less than that achieved by control and s3-level donors (Fig. 5). The density of ENS neurons was also relatively low; this was significantly less than the density from control and s3-level donors (Figs. 4D, 5). The ENS extended into the hindgut in only 3 of 21 grafts of s5, s6 and s7 donor levels.

Donors of s8 level (N = 10) were significantly different from the controls and from each of the s1 to s7 levels; they furnished few cells in the myenteric plexus (9.4 ENS cells/0.01 mm^2), of which only about 3% were neurons, and three of these grafts included ectopic enteric melanocytes of donor origin (Figs. 4E, 5).

3.4. Relationship between ENS cell density and neuron differentiation in CAM grafts

Taking all the grafted intestines together, regardless of the somite-level of origin of the ENS donor, there was in the myenteric plexus of the midgut, a highly significant positive correlation between the proportion of myenteric plexus cells that had differentiated into HuC/D+ neurons and the density of all myenteric QCPN+ cells, indicated by fitting a linear regression equation and trendline and calculating the coefficient of determination R^2 (Suppl. Fig. 4).

4. Discussion

4.1. Vagal$^{1-7}$ NC somite level sub-populations initially migrate separately but mix at the foregut in vivo

The fate-mapping showed that cells from each vagal$^{1-7}$ NC somite-level separately reach the foregut (Kuo and Erickson, 2011; Kuratani and Kirby, 1991, 1992; Shigetani et al., 1995), but with different efficiencies as judged by the pathways and cell number at E2.5–3.5. On reaching the foregut, the s1 and s2-level ENS cells occurred chiefly in pharyngeal arches and in the foregut immediately caudal to the pharynx, with few s2 cells further distal (eg. to gizzard *anlage*). However NC cells of other levels of origin became mixed over the foregut by E3.5 with labelled s3 and s4-level NC cells being most numerous. These results confirm in detail those of Erickson's laboratory (Kuo and Erickson, 2010, 2011).

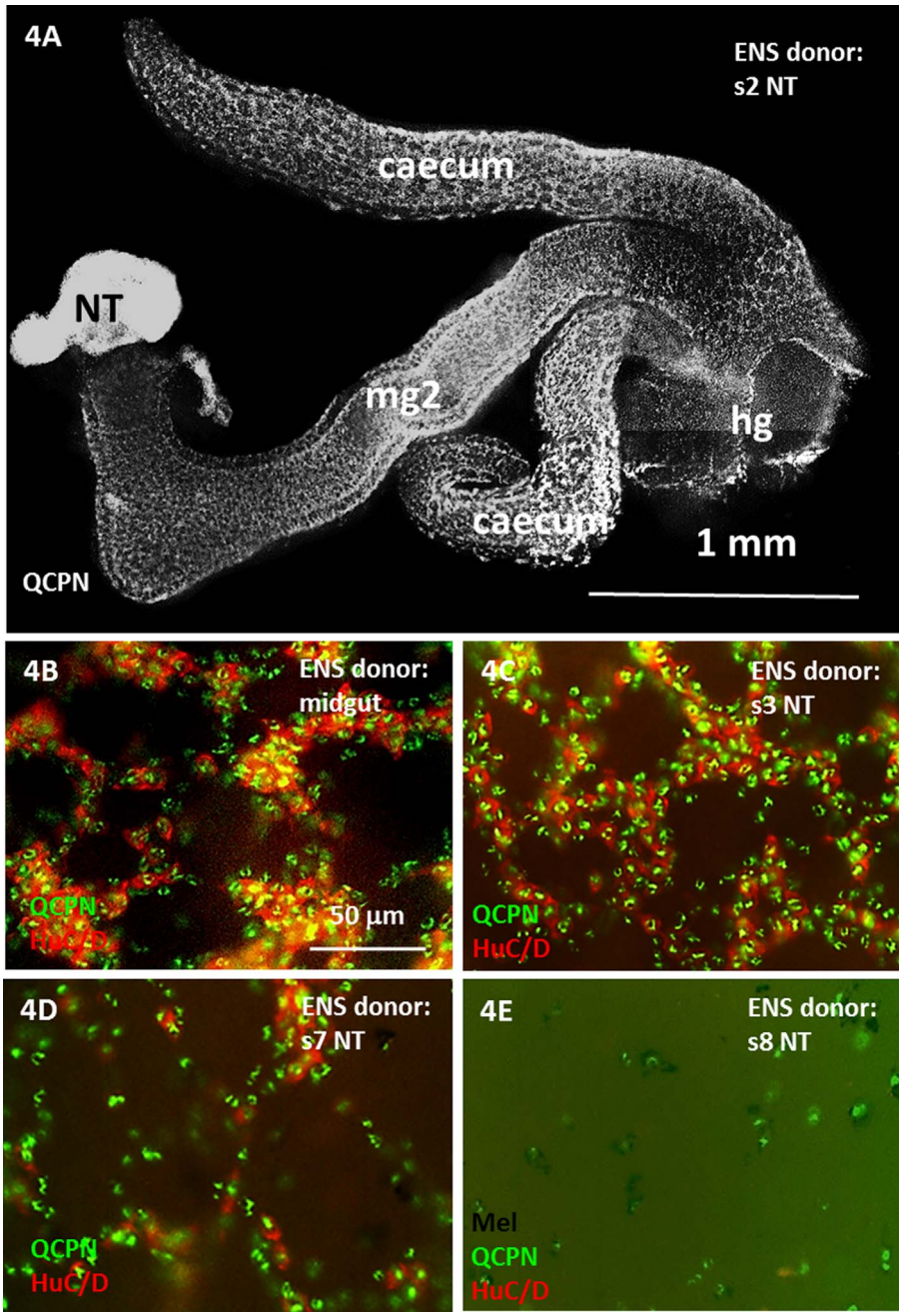

Fig. 4. Vagal$^{1-7}$ NC cells populates the midgut and hindgut in CAM grafts with efficiency that varies with somite-level of origin. A. Wholemount of a 6-day CAM graft of chick post-umbilical midgut (mg2) to hindgut (hg) labelled for QCPN+ quail cells derived from somite-2 level quail neural tube (NT) donor. B-E. En face images of mg2 myenteric plexus derived from donor NC cells. All quail NC-derived cells in the recipient gut are QCPN+ (green) and neurons are HuC/D+ (red). B. ENS cell donor is control QE4 proximal midgut. Neurons have differentiated and formed ganglia. C. ENS cell donor is s3 level QE1.5 NT; these form dense ENS. D. ENS cell donor is s7 level QE1.5 NT; the ENS is sparser with fewer neurons. E. The NC cell donor is s8 (post-vagal) level QE1.5 NT. The NC cells colonise the gut very sparsely and neurons fail to differentiate, but many of NC cells form ectopic melanocytes identified by melanin (Mel) in the cytoplasm.

4.2. Vagal NC somite level sub-populations distribute widely, patchily and unpredictably along the gut in vivo

Following electroporated specimens out to when the enteric NC cell colonisation extends into the distal hindgut (Allan and Newgreen, 1980) showed a wide variation in distribution of GFP-labelled ENS cells between individuals with the same somite-level of vagal$^{1-7}$ NC labelled. In each individual the labelled ENS consisted of irregular loose patches of GFP+ cells within a uniform unlabelled ENS. This is characteristic of the labelling of a small proportion of ENS precursor cells whose migration, distribution and proliferation have stochastic elements marked by major differences in clonal expansions (Binder et al., 2012; Cheeseman et al., 2014).

NC cells of s3 and s4-levels supplied most ENS cells but adjacent levels could supply at least some ENS cells at all or most gut regions. The most rostro-vagal$^{1-7}$ and most caudo-vagal$^{1-7}$ enteric NC cells were fewer and mostly restricted to the foregut. This confirms the distribution observed with viral lacZ labelling (Epstein et al., 1994).

4.3. Are vagal$^{1-7}$ NC somite levels co-linear with the ENS along the digestive tract in vivo?

Using orthotopic grafts, Burns and colleagues have reported a rostro-caudal co-linearity of cells within the vagal$^{1-7}$ NC and later within the ENS along the gastro-intestinal tract (Burns et al., 2000). Like Epstein et al. (1994), we observed that rostro-vagal$^{1-7}$ NC cells

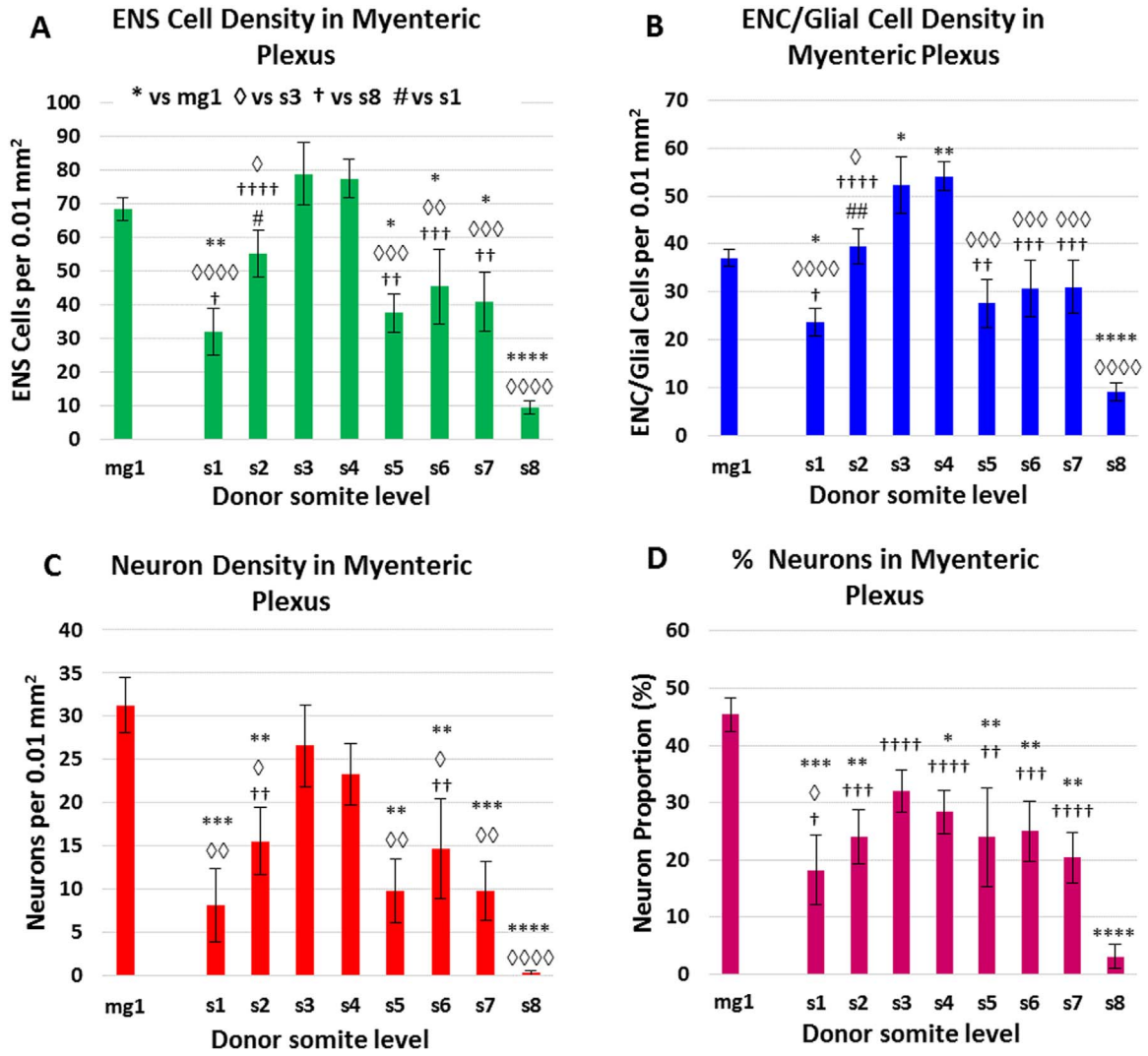

Fig. 5. Mid-vagal[1–7] NC has greater efficiency in producing ENS in aneural gut in CAM grafts, compared to rostro-vagal and caudo-vagal levels. Quail NC-derived cells in the myenteric plexus of the recipient chick midgut in CAM grafts plotted against the quail NC donors: control QE4 rostral midgut (mg1; N = 6) and single-somite level (s1, s2 etc.; N each=6–10, N total=65) vagal[1–7]neural primordium. A. Only s2, s3 and s4 level NC donors matched the midgut ENS-derived donor in terms of ENS cell density. B. s3 and s4 level NC donors exceeded the midgut ENS-derived donor in enteric NC/glial density. C. s3 and s4 level NC donors matched the midgut ENS-derived donor for neuron density. D. The proportion of vagal[1–7] NC cells that had differentiated as ENS neurons was below that of midgut-derived ENS for all levels other than s3. (enteric NC and glial cells: QCPN+/HuCD-; Neurons: QCPN+/HuCD+. Error SEM; 1 symbol $p < 0.05$, 2 symbols $p < 0.01$, 3 symbols $p < 0.001$, 4 symbols $p < 0.0001$).

distributed mainly to the foregut and the mid-vagal[1–7] NC cells occurred in the rostral ENS but were more prevalent in the mid and hindgut ENS but, contrary to the co-linearity notion, s7-level NC cells distributed to the foregut. We found, especially for NC cells of s3 to s6 origin, little or no propensity for any sub-level to map to a particular region of ENS. In general there was a co-distribution of NC cells of all levels along the gastro-intestinal tract in agreement with Epstein et al. (1994). This is likely to be a consequence of the mixing of these cells in the earliest stage of colonisation (see above). However the rostro-vagal[1–7] NC showed a distribution biased to the rostral gut and this level of NC was inferior to the s3 and s4 levels in forming ENS in mid and hindgut CAM grafts. This may represent the s1-s2-level SCP moiety identified by Espinosa-Medina et al. (2017).

4.4. Are all vagal[1–7] NC somite level sub-populations equal or are some more equal than others in vivo?

Notwithstanding the above conclusion, and despite the number and pattern of labelled ENS cells varying from specimen to specimen, the number and breadth of distribution of ENS cells derived from mid-vagal[1–7] level, especially s3 and s4-level, was usually the greatest. On the other hand, the number of s1 and s7 level vagal NC progeny in the ENS was least, and these cells were restricted to rostral gut. The same mid-vagal[1–7] dominance was recognised by Epstein et al. (1994), as was the correlation between fewer progeny and restriction to the proximal digestive tract.

4.5. Could the numerical differences between vagal[1–7] NC sub-populations in vivo arise non-specifically?

Before invoking specific differences between vagal[1–7] sub-regions (discussed below) to account for the numerical differences in contributions to the ENS, it is possible to suggest non-specific morphogenetic explanations. The mid-vagal[1–7] NC cells have a more direct ventral route to the foregut than do the cells of s1 and s2-level origin, whose numbers are depleted by diversion of cells to the pharyngeal arches and cardiac outflow tract (Shigetani et al., 1995). On the other hand the more caudal somite-level vagal[1–7] NC cells commence migration later than those of s3 and s4 (Newgreen and Erickson, 1986), and significant numbers accumulate proximal to the foregut at the sympathetic zone.

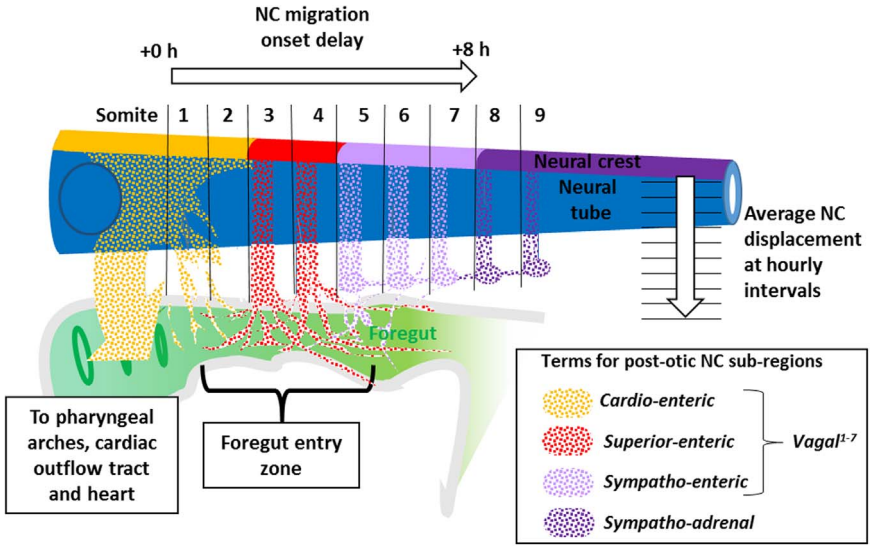

Fig. 6. Scheme for s3 and s4 level NC non-specific advantage for colonisation of foregut, and suggested terminology for post-otic NC. The NC cells of s3 and s4-levels are advantageously positioned adjacent to foregut entry zone. NC of s1 and s2-level start migration several hours before s3 and s4 NC, but their pathway dorso-laterally is longer, and most cells move into the pharyngeal/cardiac migration tract. NC of s5-7-levels NC start migration progressively later than s3 and s4 NC, and many arrest at sympathetic position. In the post-otic NC, the vagal$^{1-7}$ region retains its validity for ENS fate and competency, but terminology for sub-regions representing developmental biases within this region is based on a combination of cell fate, ENS-forming competence, and ErbB3 and Ret-dependency.

The outcome of this could be that mid-vagal$^{1-7}$ NC cells arrive in the foregut as 'first with the most'. The carrying capacity and frontal expansion hypothesis of enteric NC cell colonisation described by Simpson et al. (2006, 2007) favours the progeny of the first cells to arrive. We propose that this exaggerates a slight mid-vagal$^{1-7}$-level advantage in the foregut if high enteric NC cell proliferation pertains, and BrdU labelling shows that it does (Barlow et al., 2008). A scheme for this idea is shown in Fig. 6

4.6. Could the spatial differences between vagal$^{1-7}$ NC sub-populations in vivo arise non-specifically?

Focally labelled NC populations from s2 to s6-level contributed some GFP+ cells that had, by E6.5 *in vivo*, advanced beyond the region of initial foregut entry. Since only wavefront NC cells advance significantly (Druckenbrod and Epstein, 2005; Nishiyama et al., 2012; Simpson et al., 2007; Young et al., 2004), this implies that some cells from all these levels must have resided in the wavefront, at least for a time. If so, why do rostro-vagal$^{1-7}$ and caudo-vagal$^{1-7}$ NC cells rarely contribute to distal-most ENS?

Mathematical models predict that continued distal colonisation requires that NC cells must remain in the wavefront (Simpson et al., 2007), yet the probability of any cell falling behind the wavefront is high, and once lost, a wavefront position is almost impossible to regain. Inevitably, larger cell numbers achieved by earliest arrival, highest arrival number and consequent greater proliferation (see above) increases the probability that at least some progeny of the initial mid-vagal$^{1-7}$ NC cells remain at the wavefront, and it is only these cells that continue to contribute distally (Landman et al., 2007; Simpson et al., 2006). In contrast, cells arriving behind (that is, rostrally), such as s1 and s2 NC cells, and those that arrive later, such as s7 NC cells, remain confined to the proximal gut derivatives, that is, to the region of their arrival, because of the competitive advantage gained by the resident mid-vagal$^{1-7}$ NC cells, as first suggested by Kuo and Erickson (2011). This competitive exclusion is seen elsewhere in the gut experimentally (Simpson et al., 2007). These outcomes are consistent with contact inhibition of locomotion, with growth factor competition and/or with population pressure as operative mechanisms.

4.7. Could differences in the local environment contribute to the differences between vagal$^{1-7}$ NC sub-populations in vivo?

The somitic environments encountered by NC cells *en route* to the gut have important effects of ENS generation (Simkin et al., 2013). There are gene expression differences between the post-otic somites and the general trunk somites (Blentic et al., 2003), and there are differences related to the somite level that affect NC cell migration (Barlow et al., 2008; Ferguson and Graham, 2004; Kuo and Erickson, 2011). In addition, avian post-otic somites, with a functional border at about s5/6, fail to elicit sympathetic neuron differentiation (Newgreen, 1979; Newgreen et al., 1980) and dorsal root ganglion formation and survival (Lim et al., 1987). Thus different segment-level environments could also differentially influence the spatial and numerical contributions of vagal$^{1-7}$ NC cells to the ENS, but this requires further investigation.

4.8. Do vagal$^{1-7}$ NC somite level sub-populations have innately different ENS-forming competence?

Additional to non-specific differences affecting NC levels (discussed above), some innate regionalisation of ENS potential must also be present in the NC since there is a marked decrease in ENS-forming ability caudal to the s7/8 border (Newgreen et al., 1980; Zhang et al., 2010). We tested this with finer spatial detail by using single-somite lengths of quail vagal$^{1-7}$ neural tube as NC cell donors for aneural chicken mid/hindgut. This was similar to previous experiments (Newgreen et al., 1980; Peters-van der Sanden et al., 1993a; Zhang et al., 2010), with the NC donor moiety reduced to a single somite-length. This also removed competition between vagal$^{1-7}$ NC somite level sub-populations, a mechanism proposed by Kuo and Erickson (2011) to account for the spatial restriction of rostro-vagal$^{1-7}$ NC cells. In addition possible confounding factors due to different levels of adjacent tissues (somites etc.) were avoided by omitting these tissues. Additionally, because of the considerable reserve capacity of NC populations to form ENS (Zhang et al., 2018), to further challenge their ability, we increased the length of aneural gut to be colonised and reduced the time available for colonisation and differentiation.

NC of s3 and s4-level formed ENS that was similar in distribution, cell density and neuron differentiation to that achieved by enteric NC

cells from donor midgut controls. Vagal$^{1-7}$ NC populations rostral and caudal to this had lower (but not negligible) capacity to colonise the intestine and form ENS in similar assays, but this capacity dropped abruptly to almost zero at s8 level. This provides much greater spatial detail of ENS-forming competence than previous studies (Newgreen et al., 1980; Zhang et al., 2010). Regional differences favouring the s3-level compared to the s1-level have also been observed in *in ovo* transplant experiments by Barlow et al. (2008). Other studies have noted that when s6 and s7-level NC was included as ENS donors in intestine CAM grafts, ectopic melanocytes were found instead of neurons in the gut plexuses (Peters-van der Sanden et al., 1993a), thereby resembling trunk NC (Newgreen et al., 1980; Peters-van der Sanden et al., 1993b; Zhang et al., 2010). This fits with the view of Kuo and Erickson (2010) of the vagal$^{1-7}$ region being a transition between cranial and trunk levels.

We conclude that, in addition to a position of origin advantage, there is along the vagal$^{1-7}$ level an innate gradation in ability to form ENS which peaks at mid-vagal$^{1-7}$ NC levels and this mirrors the spatial map of vagal$^{1-7}$ NC fate.

4.9. Why is neuron differentiation deficient when vagal$^{1-7}$ NC cells are supplied directly in CAM grafted gut?

In CAM grafted gut, NC-derived cells from 'gold standard' midgut donors not only populated the enteric plexuses richly but the level of neuron differentiation was high, almost half the ENS cells (Zhang et al., 2018), similar to the ENS *in vivo* (Rollo et al., 2015). In contrast, when vagal$^{1-7}$ NC cells were derived from single-segment neural tube donors, the proportion of ENS neurons was lower, particularly for rostro- and caudo-vagal$^{1-7}$ levels. Taking all the grafts together, the higher the ENS cell density, the higher the proportion of neurons. This suggests that, as enteric NC cells increase in density, neuron differentiation rather than proliferation becomes increasingly probable (Hackett-Jones et al., 2011).

However, this is not a complete explanation, because total ENS cell density and density of non-neuronal cells was higher from s3 and s4-level donors than that achieved by midgut ENS 'gold standard' donors, yet the neuron proportion was still somewhat lower, significantly lower for s4 level. One possible contribution to this relative dearth of neurons is that pre-migratory NC cells (starting age QE1.5) have had less time for neuronal differentiation, compared to that achieved by older midgut enteric NC cells (starting age QE4). Another possibility is that vagal$^{1-7}$ NC cells normally acquire some innate differentiation capacity in the normal migration environment in the somites *en route* to the foregut (Simkin et al., 2013), and this is lacking in grafts where vagal$^{1-7}$ NC cells were supplied directly to aneural gut. These possibilities could be tested simply by, respectively, increasing the duration of the graft and by interposing vagal somites in the graft combinations.

4.10. Post-otic/vagal NC terminology

The vagal$^{1-7}$ region has been regarded as a functional unit (Yntema and Hammond, 1954). Combining previous gene expression, molecular dependency and ENS-forming capability data suggests there are potentially functionally important sub-divisions assigned variously to s0/s1, s2/s3, s3 to s5, s5/s6 and s7/s8 levels (Durbec et al., 1996; Espinosa-Medina et al., 2017; Kuo and Erickson, 2011; Robertson and Mason, 1995; Zhang et al., 2010). We present here a harmonising definition of the post-otic ENS-forming and sympathetic–forming region of NC (Fig. 6).

It should be noted that where borders (e.g. somite levels) are stated, strict cut-offs may be implied but cell tracking at one-somite resolution eg. Shigetani et al. (1995) and Kuo and Erickson (2011), indicates that these borders are often 'fuzzy'. Also, these borders are derived from studies of populations of cells so where NC populations across a sub-divisions are not equivalent, it is not possible to decide whether this is because each NC population has a different mix of pre-specified (or pre-biased) cells, or has one type of NC cell with differently weighted multiple potency biases in different sub-divisions. In addition, data on spatial origin, on gene expression and dependency and on experimental competencies are melded from avian and rodent sources; it is not clear in all instances whether this is justified (Hutchins et al., 2018).

We suggest, contrary to Espinosa-Medina et al. (2017), that the vagal$^{1-7}$ term be retained as it has reality in regard to ENS fate and competence, while acknowledging that its caudal extent does not contribute to the vagus nerve. This region is not merely the rostral extent of the sympatho-adrenal or trunk NC since the s7/8 border marks a real step-wise difference in ENS-forming ability (see also Zhang et al., 2010). It hardly seems accidental that this region maps exactly to the transient expression of *Ret* in chick embryo NC at pre-migratory stages (Robertson and Mason, 1995); this may be a response to RA signals derived from adjacent somites (Blentic et al., 2003; Patrone et al., 1997).

Nested within this vagal$^{1-7}$ region of the NC we propose (Fig. 6) that there are several sub-levels which encompass the two recently proposed units (Espinosa-Medina et al., 2017) but are more complex to accommodate additional ENS data.

1. The rostral of the dual regions of Espinosa-Medina et al. (2017) we suggest be termed *cardio-enteric*, because this region also contains most of the previously defined cardiac NC and circumpharyngeal NC levels (Kuratani and Kirby, 1991; Shigetani et al., 1995), whose cells are in nature ectomesenchymal, although they can form ENS experimentally (Ciment and Weston, 1983). The ENS-fated NC here (Epstein et al., 1994) is as defined by Espinosa-Medina and colleagues. This cell population includes the ErbB3-dependent SCP contribution of about half the foregut ENS. It may be reasonable to revert to a century-old view and regard these vagus-associated ENS cells as part of the cranial parasympathetic system. It is possible that these are the foregut ENS cells that are retained in *Ret* null mice (Durbec et al., 1996), consistent with the suggestion of Espinosa-Medina and co-authors that ErbB3 signalling may ameliorate the effect of loss of Ret signalling, and *vice versa*. The foregut ENS cells that are ErbB3-independent may be the rostral-overlapping extension of the *Ret*-dependent ENS-competent cells continuous with the next segment-levels since, although fated to have a slight contribution to distal ENS normally, NC cells of this level have considerable capacity to colonise distal gut and form ENS experimentally.
2. Caudal to this (s3 and s4 level) we propose a sub-division we term *superior-enteric*: cells of this level contribute glia to the SCG (Espinosa-Medina et al., 2017) and can and do form most efficiently the bulk of the ENS throughout the gastro-intestinal tract. These are fully (SCG) and partially (ENS) dependent on ErBB3 signalling and both are *Ret*-dependent (Durbec et al., 1996; Espinosa-Medina et al., 2017).
3. The term *sympatho-enteric* was previously used for s1-5 NC (Durbec et al., 1996) and re-purposed for s3-7 NC (Espinosa-Medina et al., 2017). We propose to re-purpose this term yet again to describe the NC region from s5 to s7, since this forms *Ret*-dependent ENS and *Ret*-independent sympathetic chain ganglia (Durbec et al., 1996). Experimentally these cell populations have ENS-forming fate and capacity in mid/hindgut although less than the immediate-rostral level.
4. Beyond the vagal$^{1-7}$ zone, beginning at s8-level, we revert to the term *sympatho-adrenal,* but restricting this from its more rostral original extent (Carnahan et al., 1991) since it does not contribute to the ENS normally (Epstein et al., 1994; Le Douarin and Teillet, 1973) and has limited (but not zero) capacity to do so experimentally. This term is synonymous with trunk NC. The NC at the sacral level does provide ENS distally (Burns and Le Douarin, 1998) but in heterotopic transplants and in co-cultures the sacral NC has modest ENS-forming capacity. Barlow et al. (2008) suggest that this capacity, while inferior

to vagal$^{1-7}$ NC, is superior to mid-trunk NC. Other studies find the sacral NC similar to the rest of the trunk NC, and it cannot compensate for lack of the vagal$^{1-7}$ source (Burns et al., 2000; Hearn and Newgreen, 2000; Newgreen et al., 1980). Its contribution to the ENS was suggested to stem simply from its placement advantageously close to the hindgut rather than from possessing higher ENS-forming capacity than the rest of the trunk NC (Erickson and Goins, 2000). Part of the functional difference between vagal$^{1-7}$ NC cells and sacral NC cells can be attributed to a lower Ret expression level in the latter (Delalande et al., 2008). Until further information is available, we suggest that the sacral NC be included as a sub-division of the trunk NC (cf. Hutchins et al.'s usage)

4.11. Implications for stem cell replacement therapies for ENS neuropathies

Stem/progenitor cell approaches for investigating and even treating disorders of the ENS are being explored (Burns et al., 2016; Fattahi et al., 2016; Stamp et al., 2017). If there are intrinsic differences in the NC that give rise to various elements in the ENS, it will be necessary to consider this in choice of cell donors (Stamp, 2017). For example, some enteric neuropathologies such as achalasia and gastroparesis affect the ENS in the foregut, some such as forms of chronic intestinal pseudo-obstruction affect the midgut ENS and some like Hirschsprung disease affect the distal midgut and hindgut ENS. In normal development the ENS of each of these regions involves a different mix of enteric NC-derived cells. All have a major contribution from the *superior-enteric* NC, but in the foregut would be also *cardio-enteric* SCPs, in the midgut *sympatho-enteric* NC cells and perhaps *sympatho-adrenal* NC-derived SCPs, and in the hindgut also *sacral* NC. At present it is unclear whether each of these spatially coded contributors would be required to form a functional ENS, or whether just one type, such as the major *superior-enteric* NC, would be adequate.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.11.007.

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