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# Enteric nervous system development: A crest cell's journey from neural tube to colon

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## Abstract

The enteric nervous system (ENS) is comprised of a network of neurons and glial cells that are responsible for coordinating many aspects of gastrointestinal (GI) function. These cells arise from the neural crest, migrate to the gut, and then continue their journey to colonize the entire length of the GI tract. Our understanding of the molecular and cellular events that regulate these processes has advanced significantly over the past several decades, in large part facilitated by the use of rodents, avians, and zebrafish as model systems to dissect the signals and pathways involved. These studies have highlighted the highly dynamic nature of ENS development and the importance of carefully balancing migration, proliferation, and differentiation of enteric neural crest-derived cells (ENCCs). Proliferation, in particular, is critically important as it drives cell density and speed of migration, both of which are important for ensuring complete colonization of the gut. However, proliferation must be tempered by differentiation among cells that have reached their final destination and are ready to send axonal extensions, connect to effector cells, and begin to produce neurotransmitters or other signals. Abnormalities in the normal processes guiding ENCC development can lead to failure of ENS formation, as occurs in Hirschsprung disease, in which the distal intestine remains aganglionic. This review summarizes our current understanding of the factors involved in early development of the ENS and discusses areas in need of further investigation.

### Keywords

Enteric nervous system; Gut development; Neural crest; Hirschsprung disease

## 1. Introduction

The gastrointestinal (GI) tract is responsible for performing complex functions that are essential for host survival. These include (1) transport of food and waste, (2) digestion and

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absorption of nutrients, (3) secretion of water, electrolytes, mucus, signaling molecules, and antimicrobial substances, (4) preservation of intestinal barrier integrity, (5) maintenance of a healthy microbiota, and (6) protection from ingested pathogens, allergens, and toxins. Regulation of these critical processes relies principally on the GI tract's own intrinsic nervous system, referred to as the enteric nervous system (ENS). The ENS is the largest subdivision of the autonomic nervous system. While the sympathetic and parasympathetic subdivisions provide extrinsic innervation to the GI tract and can modulate ENS activity, the ENS is capable of completely autonomous function without input from the brain or spinal cord, earning its moniker as the "second brain" [1].

The ENS is comprised of enteric neurons and glial cells organized in two concentric ganglionated plexuses. The myenteric (Auerbach's) plexus, located between the circular and longitudinal muscle layers, is present along the entire length of the GI tract. The submucosal plexus, which is absent in the esophagus, has an external (Schabadasch's) and an internal (Meissner's) component [2] that are generally considered together since they interconnect extensively and no functional differences have been identified. The ENS contains >100 million neurons [3] and at least 18 functional subtypes [4] that comprise four major classes of neurons: motor neurons, intrinsic primary afferent neurons (IPANs), intestinofugal neurons, and interneurons [5]. The IPANs are sensory neurons that detect mechanical or chemical luminal stimuli and send signals via excitatory or inhibitory interneurons to effector cells, including epithelial cells and smooth muscle [4]. To achieve directional movement of luminal contents, this intrinsic neuronal circuitry is polarized. Luminal stretch, for example, activates ascending pathways that lead to smooth muscle contraction above the stimulus and descending pathways that relax smooth muscle below the stimulus, leading to aboral propulsion of luminal contents. This fundamental process was first described by Bayliss and Starling in 1899 [6], who referred to it as the "law of the intestine" and recent work has provided some of its cellular and molecular underpinnings [7].

Given the essential role of the ENS in maintaining normal GI function, abnormalities of the ENS can lead to serious health consequences. These include congenital disorders caused by abnormal embryologic development of the ENS, and acquired conditions due to inflammatory, infectious, or immune-mediated etiologies. The resulting diseases typically manifest with severe GI motor dysfunction, including esophageal achalasia, gastroparesis, intestinal pseudo-obstruction, and colonic dysmotility (reviewed in [8]). The classic enteric neuropathy, and the one whose etiology and pathophysiology are best understood, is Hirschsprung disease (HSCR). HSCR is a congenital disease that affects 1 in 5000 children and is characterized by the absence of enteric ganglia along variable lengths of the bowel, typically involving only the distal colon but occasionally extending more proximally, even involving in the entire GI tract in a minority of cases [9]. The resulting aganglionosis leads to severe functional intestinal obstruction and requires surgical removal of the aganglionic segment. Extensive research over the past few decades has provided important insights into the development of the ENS and the causes of HSCR. This review summarizes our current understanding of the molecular and cellular regulation of ENS formation and discusses how this knowledge can be applied to developing stem cell-based therapies for the treatment of neurointestinal diseases.

## 2. Neural crest origin of the ENS

During embryonic development a multipotent and highly migratory mesenchymal-like cell type, the neural crest cell (NCC), delaminates from the closing cranial neural folds and from the closed folds of the trunk neural tube through an epithelial-mesenchymal transition [10]. These newly formed cells arise from specific axial levels of the neural tube (cranial, cardiac, vagal, trunk, and sacral), migrate extensively throughout the embryo to colonize multiple organ primordia, and differentiate into a large variety of cell types, including connective tissue of the head, endocrine cells, melanocytes, and the glia and neurons of the peripheral nervous system, including the ENS [10–14]. While ENS development has been studied in numerous embryologic model systems, including zebrafish, avians, and rodents, the avian embryo has been a particularly valuable experimental system for this work [15]. Neural crest cells were first described in the avian embryo in 1868 [16]. In 1954, Yntema and Hammond published a classic paper where they showed that removal of the dorsal neural tube from chick embryos resulted in complete absence of an ENS in the gut, thus establishing over 60 years ago the neural crest origin of the ENS [11]. These results were further refined using inter- and intra-species chimeras in which segments of neural tube were transplanted into host chick embryos and showed that the majority of the ENS arises from vagal neural crest [13,17–19], specifically from the segment of neural tube located at the level of the postotic hindbrain adjacent to somites 1–7, which represents the junction between brain and spinal cord [20]. Within that region, fate mapping experiments in avian embryos further defined regional differences in the contribution of NCCs from specific axial levels [20–23]. Replacing the chick neural tube at somite level 1–2 with age-matched neural tube from quail results in quail-derived ENCCs only in the esophagus, whereas levels 3–5 contribute ENS cells from the stomach to the hindgut, and levels 6–7 give rise to precursors restricted to the hindgut [23]. Among these, somite level 3 is particularly critical for ENS formation, as this level is able to compensate for the removal of the entire vagal region [22]. Interestingly, in mice, DiI labeling of NCCs from somite level 6–7 resulted in labeling of ENS cells only in the esophagus [21], but whether this represents a true species-specific difference in foregut ENS origin remains to be determined.

After delamination, vagal NCCs migrate along two separate pathways. First, at the 10-somite stage (10ss; embryonic day 8.5 (E8.5) in mouse and early E2 in chick), NCCs from the level of somites 1–3 migrate dorsolaterally under the ectoderm to colonize the pharyngeal arches and the cardiac outflow tract. By 13ss, a second population arises from somites 1–3 and follows a ventral path, with some cells going on to form the sympathetic and dorsal root ganglia and others entering the proximal foregut to give rise to the ENS [20]. Although the anterior vagal NC domain adjacent to somites 1–3 contains NCCs destined for both the heart and the gut, specific guidance cues segregate these two populations. Unlike vagal NCCs destined for the foregut, cardiac NCCs express CXCR4 and therefore migrate toward the ligand, SDF1, expressed by the pharyngeal mesoderm and the conotruncal mesenchyme of the developing heart [24]. In contrast, NCCs from somites 4–7 migrate only ventrally and join the migration stream into the foregut. Posterior to the vagal region are trunk NCCs, which do not enter the foregut. This is likely due to Slit-Robo interactions. The foregut expresses Slit2, which is repulsive to Robo-expressing trunk NCCs, but not to the

Robo-negative vagal NC-derived cells [25,26]. Entry of NCCs into the foregut occurs quite early in embryogenesis: 32 h post-fertilization in zebrafish [27], E2.5 in chick and quail [17], E9.5 in mouse [28], and week 4 of gestation in humans [29] (Table 1; Fig. 1).

An early critical event for ENS formation occurs as vagal NCCs migrate away from the neural tube toward the foregut. They travel through the anterior half of the somites, in the mesoderm between the dermomyotome and sclerotome [30,31]. Recent studies suggest that the commitment of NCCs to an enteric lineage occurs within that somitic environment, where retinoic acid (RA) produced locally by the paraxial mesoderm acts on the migrating NCCs, which express retinoic acid receptors α and γ (RARα, RARα2, RARγ) [32]. This interaction activates NCC expression of the receptor tyrosine kinase, RET [33], a critically important protein for ENS development. Mice deficient in retinaldehyde dehydrogenase (RALDH2), which is required for RA production, exhibit agenesis of the ENS due to downregulation of RET in vagal NC-derived cells [34].

Once NCCs enter the foregut mesenchyme, at which point they are referred to as enteric neural crest-derived cells (ENCCs), Ret-expressing ENCCs travel anteroposteriorly to colonize the entire length of the gut tube, progressing at a migratory speed of about 40 μm/h in both avians and rodents [35,36]. The timing of arrival of ENCCs to specific landmarks along the gut in multiple model systems is summarized in Table 1. Careful immunohistochemical analyses have established the patterns of ENCC migration and differentiation, and highlighted interesting differences across species [19,29,35,37–42] (Fig. 2). In zebrafish, ENCCs fully colonize the developing gut as two parallel chains on either side of the mesenchyme by 66 h postfertilization (hpf), followed by a circumferential migration around the gut tube to completely colonize the bowel [38]. In avians and rodents, migrating ENCCs are randomly distributed in the outer mesenchyme of the foregut and midgut, where the smooth muscle has not yet differentiated [17,35,40,43,44]. As the wavefront cells progress toward the cecum, the circular smooth muscle begins to differentiate and ENCCs become limited to the outermost layer of the gut wall, between the smooth muscle and serosa, where myenteric ganglia will later form. In the midgut, myenteric ENCCs undergo a secondary migration, radially inward from the myenteric plexus toward the epithelium to colonize the submucosal mesenchyme and eventually give rise to the submucosal plexus [45], and this is described in greater detail below. In all species examined, the myenteric plexus develops before the submucosal plexus, with the only exception being the avian colorectum, where the sub-mucosal plexus develops first [19,46]. In both avian and human colorectum, both plexuses develop early in development, whereas in mouse the colorectal submucosal plexus develops only postnatally [18,19,40,47]. Zebrafish have no submucosal plexus, while amphibians and reptiles possess modest submucosal plexuses only in the esophagus and stomach [48–50].

In addition to the major contribution of vagal-derived NCCs, a second, more caudal region of the neural tube, the sacral NC, also contributes to the ENS. NC ablation and transplantation studies [13,17,19,51], organotypic cultures, genetic-labeling studies [52,53], and time-lapse live-cell imaging in chick embryos revealed that NC caudal to somite 28 contributes to the ENS, principally in the colorectum. Similar to vagal NC, sacral-derived NC follow a ventral pathway, migrate through the somites and tail bud mesenchyme, and

initially accumulate on either side of the cloaca and distal hindgut to form the prospective pelvic plexus, a rich ganglionated network. Sacral NCCs emigrate from the neural tube at E3 in chick and E9.5 in mouse, arriving at the cloacal region at E5 and E11.5, respectively [54,55]. A few days later, a subpopulation migrates into the distal hindgut along nerve fibers extending from the pelvic plexus to contribute enteric neurons and glial cells [52,54,55]. Interestingly, sacral NCCs enter the hindgut only after vagal ENCCs have already arrived [17]. Chick-quail sacral neural tube chimeras [17] revealed that sacral-derived ENCCs contribute to the hindgut ENS in a posterior-to-anterior gradient, accounting for up to 17% of enteric neurons in the terminal hindgut and only 0.3% in the proximal hindgut. In zebrafish, the sacral crest does not appear to contribute to the ENS [38], while in humans this remains unknown.

Vagal- and sacral-derived ENCCs are intrinsically different from each other, with the former being far more invasive. When the sacral neural tube is replaced by vagal neural tube, the vagal-derived ENCCs migrate into the distal bowel along normal pathways, but do so earlier in development and in much greater numbers. They also colonize far more proximally than sacral-derived ENCCs normally do [56]. Comparative microarray analysis performed in order to identify differences between avian vagal and sacral NCCs observed a 4-fold higher expression of RET transcript in vagal NC-derived cells, possibly accounting for their greater migratory invasiveness. Consistent with this hypothesis, overexpressing RET in sacral NCCs increases their colonization potential, providing a molecular explanation for intrinsic differences in the relative contributions of the vagal and sacral ENCCS to the ENS [57].

In addition to the wave of vagal and sacral ENCCs colonizing the gut, recent genetic tracing experiments in mice suggest that a subpopulation of Schwann cell precursors populate the hindgut by migrating along extrinsic nerve fibers. This source of cells contributes up to 20% of the ENS in the colorectum [58] and suggests that a distinct NCC population, one that does not follow the traditional anteroposterior or posteroanterior migratory paths, contributes to the ENS. Another study from the same group showed that a subpopulation of ENCCs in the mouse midgut avoids the cecum and crosses through the mesentery adjacent to the distal midgut and hindgut, structures which are juxtaposed transiently at the time when ENCCs are migrating down the small intestine. This *trans*-mesenteric migration appears to contribute the majority of the ENS in the distal two-thirds of the hindgut [59], again suggesting the presence of alternative pathways for ENCCs to reach their target.

## 3. Molecular and cellular control of ENS development

In order to form a structurally and functionally intact ENS, ENCCs must perform several key functions. These include cell proliferation to generate enough precursors to populate the entire gut, cell survival, directed migration, patterning into concentric plexuses, differentiation into neuronal and glial cells and their subtypes, ganglion formation, axon extension, and synaptogenesis. Each of these essential processes is regulated by a number of transcription factors, cell surface receptors, and neurotrophic signals whose activation must be highly regulated both spatially and temporally during ENS formation in order to complete this complex and highly dynamic process. For example, while ENCCs at the migratory wavefront need to be highly proliferative and invasive, a subpopulation of those cells needs

to stop migrating and begin differentiating, while more proximal cells start to form ganglia and extend fibers to other neurons and effector cells. Precise coordination of these processes, especially proliferation, migration, and differentiation, is thus critically important and, as described below, failure to properly regulate them leads to the aganglionosis that occurs in HSCR.

### 3.1. Proliferation and the importance of the size of the progenitor cell pool

One of the most important determinants of normal ENS development is the size of the population of ENCC progenitors available to colonize the gut. Reducing the size of the vagal neural crest in chick embryos results in variable lengths of distal aganglionosis [22,23,60]. Similarly, inhibiting the normal apoptotic cell death that occurs as vagal NCCs migrate toward the foregut leads to hyper-ganglionosis in the foregut [61]. A critical density of ENCCs at the wavefront is required for the formation of the cellular strands that drive their migration [22,36]. Evidence suggests that the highly migratory behavior of ENCCs at the wavefront relies on “chain migration,” a process in which cell migration is enhanced when ENCCs are in contact with each other [36,62,63]. Isolated ENCCs do not migrate as quickly or as directionally as chains of cells [64], emphasizing the importance of cell–cell contact during ENS formation. Experimental support for this hypothesis comes from mutations in L1 cell adhesion molecule (L1CAM), a protein that maintains such cell–cell contacts. L1CAM mutations reduce ENCC contact and lead to HSCR [65,66]. Since ENCCs at low density migrate more slowly, this may result in those wavefront cells being unable to colonize a microenvironment that is no longer permissive by the time they arrive [67]. Cell proliferation, which is the main driver that maintains cell density and therefore migratory speed, is thus critically important to ENS development [68,69].

### 3.2. Migration and its reliance on ENCC density

Another fundamental aspect of ENS development is that cells at the wavefront migrate directionally while cells behind the wavefront stay put. This process can also be understood in the context of cell proliferation and density. As ENCC density increases, the gut wall reaches a maximal carrying capacity, which may reflect, for example, a limited availability of specific neurotrophic factors (e.g. GDNF, discussed below). The wavefront cells thus move toward an unpopulated region of the bowel, where a greater concentration of neurotrophic signals is present. In this manner, cell proliferation can be expected to lead to anteroposterior migration without the need for any directional signals from the environment [69,70]. This model is consistent with experiments in avians in which vagal neural tube grafted into the sacral-level neural tube results in ENCCs migrating in the oral direction, into gut devoid of ENCCs [56]. Another potential mechanism linking cell density with wavefront migration is “contact inhibition of locomotion,” a process whereby cells that come into contact with each other stop migrating, repolarize, and migrate away from each other [71]. This has been observed when two neural crest cells meet, leading them to collapse their protrusions and alter their migratory direction [72], but has not been demonstrated in vagal ENCCs.

### 3.3. Differentiation, a critical event that must be carefully timed

ENS migration thus relies heavily on maintaining sufficient ENCC proliferation, but this must be carefully balanced by the cessation of proliferation and the onset of cellular differentiation. Inadequate ENCC proliferation delays migration and leaves an insufficient number of cells to complete colonization of the bowel. Premature neuronal differentiation leads to a similar effect by producing differentiated neurons, which are no longer able to proliferate. Delayed differentiation, however, is also problematic since it results in an excessive number of progenitor cells which will need to differentiate in an older mesenchymal environment that may no longer support normal neuronal and glial development. Coordination of these processes depends on both intrinsic factors expressed by ENCCs and on signaling interactions between ENCCs and their surrounding microenvironment. The role of those factors that have a major role during ENS formation is described below, with an emphasis on how each impacts ENCC survival, proliferation, migration, and differentiation.

### 3.4. Signaling molecules

**3.4.1. RET signaling—**One of the most important pathways involved in ENS development is the RET signaling pathway, which has pleiotropic effects on multiple aspects of ENS development. Almost all families affected with HSCR show linkage with *Ret* [73,74]. Coding mutations are found in about 50% of familial HSCR cases and in one-third of sporadic cases [75–77]. Almost all remaining cases have a non-coding *Ret* variant, located in a conserved enhancer-like sequence in intron 1, that significantly reduces its expression [78]. RET is a tyrosine kinase transmembrane receptor expressed by ENCCs. Its expression requires the transcription factors SOX10 [79] and PHOX2B [80], the loss of either of which leads to total intestinal aganglionosis. RET is activated by glial-derived neurotrophic factor (GDNF), a factor expressed in the gut mesenchyme. GDNF binds to a receptor complex comprised of RET and its co-receptor, GDNF family receptor α1 (GFRα1). This binding leads to phosphorylation of RET and activation of several downstream pathways, including RAS/mitogen-activated protein kinase (MAPK), Jun-associated N-terminal kinase (JNK) pathways, and phosphatidylinositol-3 kinase (PI3K) [81]. Of these, PI3K signaling is especially important, as it mediates GDNF-induced ENCC proliferation [82], survival [83], and migration. PI3K promotes migration by activating expression of RAC1 at the migratory wavefront, which is critical for lamellipodia formation and cell motility [84]. Inhibiting PI3K, which normally converts PIP2 to PIP3, thereby reduces ENCC migration and leads to distal aganglionosis [85]. The protein phosphatase and tensin homolog (PTEN) inhibits PI3K activity by converting PIP3 back to PIP2, and thus serves to regulate the extent of ENCC proliferation, survival, and migration. As a result, overexpression of PTEN reduces ENCC migration *in vitro* [86] and ENCC-specific PTEN deletion results in hyperplasia of the ENS [87]. PTEN is specifically absent from the leading edge of ENCC migration. This local inhibition is due to RA receptor signaling and is important in order to maintain sufficient PI3K activity in the highly migratory sub-population of ENCCs at the wavefront [86]. RA signaling thus not only induces RET expression in pre-enteric NCCs [33,88] but also maintains PI3K activity in migrating ENCCs. Interestingly, PTEN is expressed by enteric neurons just proximal to the wavefront,

which may explain how those cells make the decision to stop migrating and start differentiating. The crucial role of RA led to the hypothesis that Vitamin A deficiency may contribute to the etiology of HSCR [86]. Two other negative regulators of GDNF-RET activity include SPRY-2 [89] and KIF26A [90], and the loss of either gene leads to enteric nerve hyperplasia due to unregulated RET signaling.

In addition to its role in promoting migration, activation of RET also promotes proliferation and survival of ENCC precursors [91–96]. RET-deficient mice exhibit extensive apoptosis of ENCCs in the foregut [94], diminishing the number of available progenitors and contributing to the intestinal aganglionosis observed in mice deficient in RET, GDNF, or GFRα1 [97–101]. Proliferation and survival of ENCCs is essential in order to generate the significant numbers of progenitors that are needed to populate the entire length of the GI tract. An insufficient pool of progenitor cells can lead to distal intestinal aganglionosis, as occurs in HSCR. In support of this, the effect of RET on ENS development has been shown to be dose-dependent. While mice with homozygous deletion of RET exhibit near-total aganglionosis, heterozygous mutants have a normal ENS [97]. When RET expression is reduced to about one-third of normal levels, colorectal aganglionosis occurs [102], suggesting that the level of RET expression can predict the length of aganglionosis.

GDNF also has a potent chemoattractive role which has been proposed to promote the directional migration of ENCCs along the gut [85,96,103]. GDNF is first expressed in the stomach while ENCCs are migrating through the foregut, and later it is in the cecum when ENCCs are in the midgut [85], presumably attracting the cells to continue their anteroposterior migration. Deletion of GFRa1 has been shown to significantly slow the velocity of ENS precursor cell migration [45], offering yet another contributing mechanism for the aganglionosis associated with loss of Ret signaling. RET activation by GDNF is also required for the secondary radial migration that gives rise to the submucosal plexus [45].

In addition to its effects on ENCC migration, proliferation, and survival, GDNF-RET signaling also promotes enteric neuronal differentiation *in vitro* [91,92]. However, its effect on differentiation *in vivo* is controversial. Retroviral-mediated silencing of GDNF in avian embryonic gut leads to premature neuronal differentiation [96], and mice heterozygous for GDNF also exhibit enhanced differentiation [104]. In contrast, other data suggests that lack of GDNF signaling inhibits neuronal differentiation and maintains ENCCs in a precursor state [45]. This apparent contradiction reflects the complexity of this signaling pathway, including the pleiotropic effects of RET activity and its dose-dependency [96].

Even after colonization of the gut is complete, RET signaling remains important for enteric neuron survival at later stages of ENS maturation. Conditional ablation of RET [102] or GFRα1 [105] during late gestation, for example, leads to the death of postmigratory enteric neurons. Interestingly, this cell death is limited to the colon, suggesting another mechanism whereby loss of RET signaling could lead to HSCR.

**3.4.2. EDNRB signaling—**Endothelin receptor B (EDNRB) is a G protein-coupled receptor expressed by ENCCs. Its ligand, endothelin-3 (ET3) is a 21 amino acid peptide expressed in the gut mesenchyme. Deletion of either gene, or of endothelin converting

enzyme-1 (ECE1), leads to colorectal aganglionosis [106,107]. Mutations in these genes have been identified in human HSCR [77], with homozygous mutation occurring in Shah-Waardenburg's syndrome, which includes pigmentation abnormalities due to deficient melanocytes, congenital deafness, and HSCR [108].

EDNRB signaling promotes the proliferation of ENCCs [95,109] and inhibits their differentiation into neurons [92,109,110], thus maintaining them in an uncommitted and proliferative state. Loss of this signaling thereby leads to diminished ENCC proliferation and premature differentiation, resulting in cells that are unable to divide or migrate and leading to failure to complete ENS colonization of the gut [111]. Interestingly, EDNRB signaling does not appear to be required until ENCCs reach the cecum [112], explaining why deletion of this pathway results specifically in colorectal aganglionosis.

Several lines of evidence support genetic and molecular interactions between RET and EDNRB pathways during ENS development. For example, while RET heterozygous mice and homozygous EDNRB$^{s}$ mice (a hypomorphic EDNRB allele) both have a normal ENS, the combination of the two mutations leads to aganglionosis, confirming a genetic interaction [113,114]. ET3 has been shown to enhance synergistically the proliferation of ENCCs induced by GDNF [95]. In contrast, these pathways have antagonistic roles with respect to neuronal differentiation, with GDNF increasing neuronal numbers in cultured ENCCs and ET3 inhibiting this effect [92]. These observations highlight the critical balance between cell proliferation and differentiation in the ENS, a balance that ensures creation of an adequate population of dividing progenitor cells while allowing neuronal differentiation to occur at the proper time and place along the length of the intestine.

**3.4.3. Transcription factors—**SOX10, an HMG box-containing transcription factor, is expressed by NCCs as they delaminate from the neural tube, and remains expressed in migrating ENCCs. It is required for the survival of ENCCs, and homozygous SOX10 mutants exhibit total intestinal aganglionosis due to NCC apoptosis prior to their arrival in the foregut [115]. In addition to being a survival factor, SOX10 is also required to maintain ENCCs in an undifferentiated and proliferative state [116–118], similar to EDNRB [117]. While a null mutation results in total aganglionosis, [115], heterozygous Sox10 mutants exhibit premature enteric neurogenesis [119], a decrease in the number of ENCC progenitors, and distal aganglionosis [79]. Interactions among ENS signaling pathways also play a role in SOX10 mutants since SOX10 directly activates expression of RET [120] and EDNRB [121]. SOX10 is ultimately turned off by ENCCs once they differentiate into neurons, but remains expressed by enteric glial cells.

The paired-like homeobox 2b gene (PHOX2B) is a transcription factor expressed by ENCCs once they enter the gut mesenchyme [28]. PHOX2B is essential for the formation of all autonomic ganglia, including enteric ganglia. Its role is to promote ENCC proliferation and survival, and deletion leads to total intestinal aganglionosis [122]. Like SOX10, PHOX2B is required for RET expression [122]. Both SOX10 and PHOX2B are expressed by undifferentiated ENCCs. These two transcription factors suppress each other, and this is an important mechanism for maintaining the proper balance of neurons versus glial cells in the gut. While cells that form neurons turn off SOX10, PHOX2B is turned off in glial cells

[123]. This was shown experimentally using a mutant PHOX2B that was both unable to suppress SOX10 and also led to transactivation of SOX10, biasing ENCCs toward a glial lineage [118].

Another important transcription factor in ENS development is the mammalian achaetescute homolog 1 (MASH1; ASCL1), a basic helix-loop-helix DNA binding protein expressed by ENCCs upon arriving in the foregut. Deletion of *Ascl1* leads to loss of enteric neurons specifically in the esophagus [124]. Recent evidence shows that *Ascl1*−/− mice also exhibit delayed neurogenesis with a reduction in neurons expressing calbindin, tyrosine hydroxylase, and vasoactive intestinal peptide (VIP), supporting a role in enteric neuronal subtype specification [125]. Interestingly, ASCL1 normally suppresses SOX10, a signal critical for maintaining ENCCs in a progenitor state [126]. Loss of ASCL1 therefore increases SOX10 expression and could explain the delayed neurogenesis observed. However, if ASCL1 expression were left unregulated, SOX10 would be suppressed in all ENCCs, which would reduce their proliferation and lead to early and extensive neurogenesis. Fortunately, Notch signaling acts to suppress ASCL1 in a subset of cells and thereby SOX10 remains expressed and the pool of ENS progenitors is maintained [119].

HAND2, another basic helix-loop-helix transcription factor, is expressed by ENCCs once they colonize the intestine. Its expression is required for later stages of neurogenesis [127] and neuro-transmitter specification [128]. Decreases in HAND2 expression therefore lead to relatively subtle effects on ENS morphogenesis but have significant physiologic consequences by disrupting intestinal motility [129]. Mice with HAND2 deletion are able to complete ENS colonization, but exhibit impaired neuronal differentiation, with a reduction in enteric neuron numbers and specific loss of nNOS-, calretinin-, and VIP-expressing neurons [127,129,130].

**3.4.4. Additional molecular factors—**Bone morphogenetic proteins, particularly BMP2 and BMP4, have pleiotropic effects during ENS development [131]. Their activity regulates ENCC migration [132,133] as well as enteric neuronal [134] and glial [135] differentiation. BMPs appear to have an important role in determining the ratio of neurons to glia in the gut. Inhibition of BMP signaling with NOGGIN, for example, increases total neuronal numbers [134] while decreasing glial cell density [135], whereas BMP overexpression increases the glia-to-neuron ratio [135]. BMPs also promote ganglionogenesis, the aggregation of ganglion cells to form clusters, by a mechanism that involves modification of neural cell adhesion molecule (NCAM) [132,133,136]. In the sympathetic nervous system, BMP activates expression of PHOX2B, ASCL1, and HAND2 [137], but whether these transcription factors mediate some of the effects of BMP signaling in the ENS is not known. While BMPs limit total enteric neuronal density, they influence neuronal subtype diversity by specifically promoting the development of enteric neurons that express TrkC [134]. TrkC is a tyrosine kinase receptor expressed by a subpopulation ENS cells. Binding of neurotrophin-3 (NT-3) to this receptor promotes the survival and differentiation of those neurons, and deletion of NT-3 or TrkC reduces enteric neuron number in the ENS [138].

### 3.5. Extracellular matrix

In addition to growth factors, the gut mesenchyme produces many extracellular matrix (ECM) proteins that have been implicated in ENS development. Early studies in ENS development noted abnormal ECM expression in mouse models of HSCR. ET3-null mice were found to have increased expression of laminin, collagen type IV, perlecan, and other proteoglycans in the aganglionic segment, raising the possibility that altered ECM composition might contribute to the aganglionosis [139–141]. A similar hypothesis was suggested based on studies of human HSCR material showing elevated laminin expression in the aganglionic segment [142]. Indeed it has been shown recently that ECM molecules form a complex and dynamic molecular scaffold that provides both a physical surface for ENCC migration and important signals that regulate multiple aspects of ENS development. The intestinal ECM includes glycoproteins (e.g., laminins, fibronectin, tenascins), collagens, and proteoglycans that influence ENCCs via receptors expressed on their surface [139,143]. Precisely patterned ECMs modulate ENCC polarity, migration, proliferation, differentiation, aggregation into ganglia, and patterning into plexuses. The ECM composition of the gut thus has a significant effect on ENS development.

Laminin [144], fibronectin [145], vitronectin [146], and collagen type I [109] all support ENCC migration *in vitro*. In contrast, collagen type VI inhibits GDNF-mediated ENCC migration [147]. Chondroitin sulfate proteoglycans (CSPGs), including versican and collagen type IX, may also be inhibitory to ENCC migration [148], similar to their effect on NCC *in vitro* [149–151]. Most pro-migratory ECM molecules are widely distributed along the length of the developing gut, including the inner mesenchyme, where ENCCs normally do not colonize. Therefore, the concentric positioning of enteric plexuses must be controlled by other ECM molecules that are non-permissive or inhibitory to migrating ENCCs. For example, avian and mouse ENCCs do not enter mesenchyme that contains high level of versican or collagen type IX [148]. The boundaries between permissive and non-permissive ECM proteins appear to serve an important role in establishing the radial pattern of plexus development.

A recent report identified an interesting mechanical property of ECM that influences ENCC migration. They noted that the stiffness of the gut ECM dynamically changes during the period of ENCC colonization. Tensile stretching of chick and mouse embryonic guts studied in combination with atomic force and second-harmonic generation microscopy revealed that during ENCC colonization the gut wall gradually stiffens in association with an increased density of collagen type I fibers. This increased stiffness affects the speed of ENCC migration. The inverse relationship between migratory speed and ECM stiffness was observed both in the midgut and hindgut [152].

While the ECM clearly influences ENS development, ENCCs also affect ECM composition. Chick-rat intestinal coelomic chimeras and *in vitro* cultured gut explants showed that vagal-derived ENCCs produce tenascin-C, an ECM protein that promotes ENCC migration during ENS development [145]. Interestingly, sacral-derived ENCCs do not produce tenascin-C, which may partly explain their decreased invasiveness [145]. A recent study using insertional mutagenesis generated mice with ENCC-specific upregulation of *collagen-6a4* gene expression. The increased level of collagen VI protein made by the ENCCs interfered

with the pro-migratory effect of fibronectin and significantly reduced the speed of ENCC migration [147]. ENCCs also secrete matrix metalloproteases (MMP) into the extracellular microenvironment to degrade and remodel the ECM. The importance of ENCC-derived MMPs was confirmed in a study in which MMP2 activity was inhibited in explanted embryonic gut, which led to severe disruption of collective ENCC migration and network formation [153]. These observations show that ENCCs are able to alter their microenvironment in order to promote their own migration.

ENCCs express on their surface a large repertoire of cell adhesion molecules that modulate their interactions with the ECM. These include L1CAM [19,66,154], N-cadherin [19,154], and members of the integrin family (α4β1, α5β1, α6β1, αVβ1, αVβ3, αVβ5) [144,146,155–158]. Conditional deletion or functional blocking of β1 integrin leads to abnormal cellular adhesion and migration and results in a Hirschsprung-like phenotype [144,146,155]. Similarly, exposure to L1CAM function-blocking antibody decreases the rate of ENCC migration by disrupting the chain of cells at the migratory wavefront [66]. Mice deficient in either L1CAM or N-cadherin exhibit significantly delayed ENCC colonization of the gut [66,156]. ENCC development is thus highly dependent on the local environment surrounding the migrating cell and on the profile of ECM receptors it expresses. For example, while laminin is diffusely expressed by the gut mesenchyme early in development, it becomes concentrated in the basal lamina of epithelial, endothelial, and smooth muscle cells [144,159]. This localized expression is critically important for ENS formation. In avians, laminin-expressing endothelial cells form two concentric capillary plexuses that predict the position of the later-arriving ENCCs. As the ENCC wavefront colonizes the gut, the cells are directly in contact with these laminin-expressing endothelial cells [144]. A similar relationship is seen in zebrafish [144]. While the role of endothelial cells in ENCC migration is debated [36,160–162], disrupting enteric capillary development in mouse and avian embryos arrests ENCC colonization and leads to distal aganglionosis [144,163], suggesting that endothelial cells serve as a substrate to guide ENCC migration. The mechanism for this interaction involves the ECM receptor, β1 integrin, since ENCCs are able to migrate *in vitro* on cultured endothelial cells, but do not in the presence of β1 integrin blockade [144].

### 3.6. Signals from the epithelium

Epithelial-derived signals are essential for development of the gut [164], including the ENS. The epithelium secretes morphogens that act either directly on ENCCs or indirectly via effects on mesenchymal components. In a recent classical embryology experiment, avian hindgut mesenchyme was recombined with non-intestinal epithelium and this led to abnormally large and ectopically positioned ganglia [148], confirming the important role of inductive epithelium-ENS interactions. Members of the Netrin and Hedgehog (Hh) families, both produced by intestinal epithelial cells, have been shown to be key players in this interaction.

Netrins, known for their role in axon guidance, are secreted by the intestinal epithelium. They are chemoattractive to cells expressing the protein deleted in colon cancer (DCC), which is expressed by early enteric neurons. Netrins promote the centripetal migration of

neurons from the myenteric to the submucosal region [165,166]. In DCC-mutant mice, the submucosal plexus is absent [166]. Although netrin protein is highly expressed in the subepithelial mesenchyme, ENCCs do not migrate there, presumably due to the presence of other inhibitory factors, including ECM components, that prevent them from colonizing that region.

HH proteins, including sonic hedgehog (SHH) and indian hedgehog (IHH) are diffusible morphogens produced by the gut epithelium from the earliest stages of intestinal organogenesis. SHH is essential for regulating proliferation and differentiation of the mesenchyme and for establishing concentric patterning along its radial axis [164,167]. HH signaling directly regulates BMP4 expression, which inhibits smooth muscle differentiation in the mesenchyme underlying the gut epithelium, regulates ENCC migration, and helps to define the radial position of the forming submucosal plexus [132,133,167,168]. HH proteins also activate mesenchymal expression of forkhead transcription factors (FOXF1 and FOXF2). Inactivation of *Foxf* expression leads to a severe reduction of collagen types I and IV and causes colorectal aganglionosis [169].

HH proteins target the subepithelial mesenchyme through the transmembrane receptor Patched-1 (PTC1), which subsequently activates downstream genes via the Smoothened-Gli cascade. Recent sequencing of DNA and genome-wide association studies identified mutations in *Ptc1* and *Gli* genes in human HSCR [170,171]. In *Shh*-deficient mice, ENCCs colonize the gut but develop ectopic ganglia in the subepithelial mesenchyme, increased neuronal cell numbers, and abnormal neurite projections [172,173]. Inhibiting SHH signaling using cyclopamine or function-blocking antibody resulted in similar large, ectopic ganglia adjacent to the epithelium, while SHH overexpression led to intestinal aganglionosis [148]. These results support an inhibitory role for SHH in ENS development, which may counteract the chemoattractant role of Netrin described above. Mutations of the *Ihh* gene are associated with segmental aganglionosis in rodents [172], but the precise role of this gene in ENS formation has not been characterized.

Expression studies of SHH receptors in various model systems have yielded conflicting results. Expression of *Ptc1* transcript in zebrafish suggests expression of this gene by ENCCs [174], and immunohistochemistry in mice supports this finding [170,175]. However, expression analysis using *Ptc1-LacZ* and *Gli1-LacZ* transgenic mice show no expression of HH receptors by ENCCs [176,177]. Furthermore, *in situ* hybridization studies in avian gut find *Ptc1* expression in the gut mesenchyme but not in the ENS [148]. These latter findings suggest that SHH does not act directly on ENCCs, which appear to lack the HH receptors, but rather indirectly. Overexpression of SHH in the mesenchymal compartment induces ectopic expression of chondroitin sulfate proteoglycans (versican, collagen type IX), known negative regulators of NCC migration in both avian and mouse [149–151], and likely accounts for the inhibition of ENCC migration and intestinal aganglionosis observed in these animals [148].

## 4. Summary and future directions

Neural crest-derived cells undergo an extensive journey that begins at the vagal (and sacral) level of the neural tube and ends in the distal hindgut. The journey involves a lengthy migration during which cell survival, proliferation, and differentiation must be tightly regulated to ensure that the entire GI tract is colonized by the proper density and diversity of enteric neurons and glia. This review has highlighted many of the cellular and molecular factors that control this dynamic process during embryonic development. An understanding of basic ENS biology and development informs our knowledge of neurointestinal diseases and furthers our ability to treat them. As discussed throughout this review, HSCR is due to the failure of ENCCs to complete their anteroposterior migration to the end of the bowel. The lessons learned from ENS developmental studies have enhanced our understanding of the etiopathogenesis of this disease. Similarly, what we have learned about the control of ENCC proliferation and patterning can provide important insights into the causes of other congenital enteric neuropathies, including hypoganglionosis, hyperganglionosis [178], and intestinal neuronal dysplasia (IND), a condition characterized by hyperplastic and ectopic enteric ganglia [8]. Recently, investigators successfully recombined pluripotent stem cell-derived ENCCs with human intestinal organoids and demonstrated the formation of functional neuroglial networks. Importantly, they use this system to study the effect of PHOX2B mutations on ENS development and thus establish a new way to model genetic causes of neurointestinal diseases [179].

While substantial progress has been made in understanding early ENS formation, many fundamental questions remain. For example, development of the ENS continues well after enteric neurons and glial cells colonize the bowel. These cells need to form functional neural and glial circuits which requires that they extend neurites and make proper synaptic connections to innervate target cells within the gut wall. Only recently are the mechanisms underlying development of this functional circuitry beginning to be elucidated [7]. Moreover, these developmental processes continue postnatally, with both morphologic and electrophysiologic changes occurring after birth [180,181], emphasizing that ENS development is not limited to embryonic and fetal stages. Understanding how these complex processes develop prenatally and postnatally to lead to formation of functional neuroglial networks is an important area of ongoing investigation.

Another demonstration of the importance of postnatal ENS development is the fact that ENSCs persist after birth [182]. These cells undergo constitutive neurogenesis in mice during the first 4 months of life [183], likely in order to generate new neurons as the gut grows, but then stop. In adults, enteric neurogenesis has also been identified, but only following injury, for example due to chemical denervation of the gut [184] or to experimentally induced inflammation [185]. We still don’t know why a subpopulation of progenitors remains dormant in the postnatal intestine, what prevents them from differentiating in the steady state, and what signals activate their neurogenic response. These answers will shed important light on the pathophysiology of certain enteric neuropathies characterized by injury to enteric neurons.

The presence of ENSCs in the adult intestine is being actively investigated for their potential utility as novel cell-based therapies to replace missing or abnormal enteric neurons in neurointestinal diseases [186,187]. ENSCs have been successfully isolated from postnatal intestine and grown in culture to generate large numbers of progenitor cells for transplantation into animal models of Hirschsprung disease [188]. However, these studies have generally demonstrated limited engraftment, migration, and proliferation of the transplanted cells. Applying what we learn about ENS development has the potential to improve significantly the success of these cell-based therapies. By understanding the molecular and microenvironmental factors that promote ENCC survival, proliferation, and migration during embryogenesis, we can apply that knowledge to engineer enteric neuronal stem cells (ENSCs) and the gut environment to enhance the success of cell transplantation protocols.

Given the central role of the ENS in regulating nearly all aspects of GI structure and function, it is important to consider the impact of abnormal ENS development not only on gut motility but also on a myriad of other essential aspects of GI homeostasis. Understanding the potentially diverse effects of abnormal ENS development will help to broaden our appreciation of the symptomatology and clinical consequences of neurointestinal diseases. For example, abnormalities in enteric neurons and glia have been shown to alter epithelial barrier function [189] and thus may contribute to inflammatory and infectious enteropathies. Enteric neurons, particularly in the submucosal plexus, regulate visceral sensation in the gut and therefore perturbations of this sensory function contribute to the symptoms associated with irritable bowel syndrome [190]. The ENS also plays an important role in modulating intestinal immune function [191], with ENS abnormalities likely contributing to the risk of inflammatory bowel disease. Finally, congenital aganglionosis has been associated with major microbial dysbiosis [192], potentially contributing to the risk of enterocolitis associated with HSCR. Abundant evidence supports the conclusion that understanding the development of the ENS is essential to enhancing our knowledge of intestinal pathology and to informing our treatments of these conditions.

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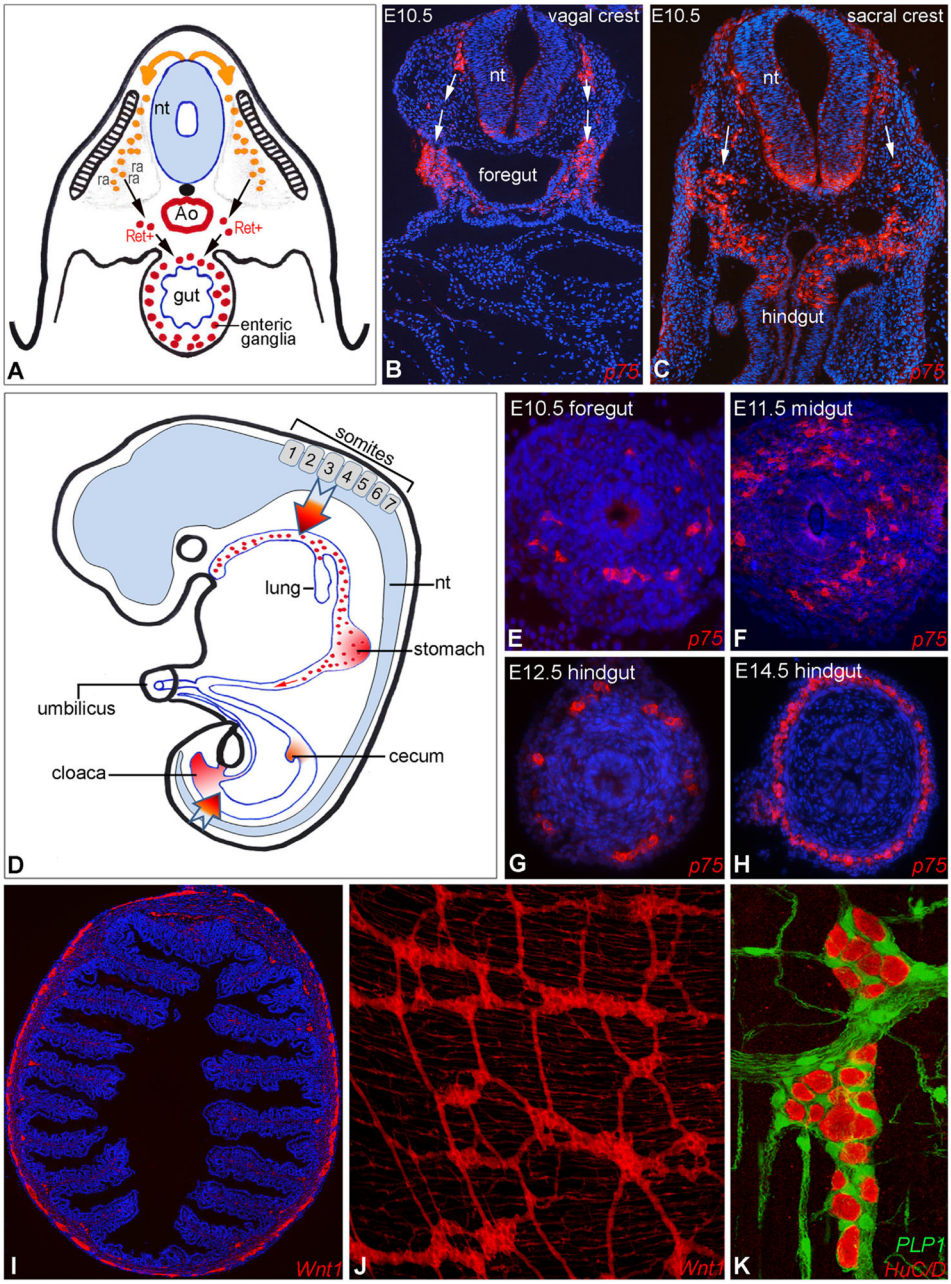


**Fig. 1.**

Migration, patterning, and differentiation of enteric neural crest-derived cells (ENCCs) in the mouse embryo. NCCs (orange dots) delaminate from the neural tube and migrate through the somites, where they are exposed to retinoic acid (ra) and begin to express Ret prior to entering the foregut mesenchyme (A). Serial sections through an E10.5 mouse embryo show vagal (B) and sacral (C) p75+ NCCs (red) migrating from the neural tube to the gut (white arrows show migratory path). The schematic illustration (D) shows vagal- and sacral-derived (arrows) contributions to the ENS. Red dots represent migrating ENCCs and shaded areas in the stomach, ceca, and cloaca denote high concentrations of ET3 and GDNF

[95,109,193–195]. At E10.5, ENCCs are present in the foregut (E), and 1 day later they have reached the distal midgut (F).

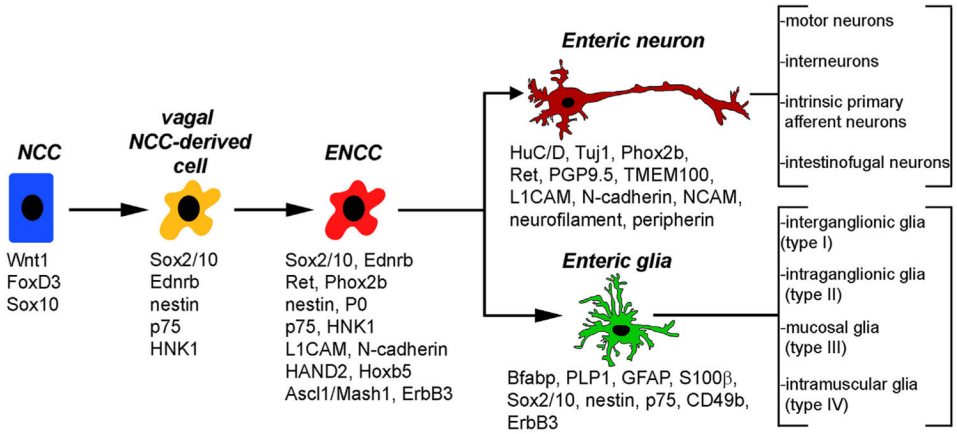


**Fig. 2.**

Differentiation of enteric neurons and glia. Vagal neural crest-derived cells undergo progressive lineage restriction from NCC to ENCC to enteric neurons and glial cells. At each stage, specific markers label these cells. Commonly used markers for each cell type are shown. Based on functional and morphologic analyses, four general types of neurons and glial cells have been described in the gut [5,196].

*NC, neural crest cell; ENCC, enteric neural crest cell

## Table 1

Comparative embryology of ENS development.

| Time of vagal ENCC arrival at designated sites | | | | |
|---|---|---|---|---|
| | **Proximal foregut** | **Stomach** | **Cecal region** | **Distal end of hindgut** |
| zebrafish | 32 hpf | – | – | 66 hpf |
| quail | E2.5 | E4 | E5 | E7 |
| chick | E2.5 | E4.5 | E5.5 | E8 |
| mouse | E9.5 | E10.5 | E11.5 | E14.5 |
| human | Week 3 | Week 4 | Week 6 | Week 7 |

*hpf, hours post-fertilization; E, embryonic day.