CHAPTER 11

Neural Crest and the Development of the Enteric Nervous System

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Abstract

The formation of the enteric nervous system (ENS) is a particularly interesting example of the migratory ability of the neural crest and of the complexity of structures to which neural crest cells contribute. The distance that neural crest cells migrate to colonize the entire length of the gastrointestinal tract exceeds that of any other neural crest cell population. Furthermore, this migration takes a long time—over 25% of the gestation period for mice and around 3 weeks in humans. After colonizing the gut, neural crest-derived cells within the gut wall then differentiate into glial cells plus many different types of neurons, and generate the most complex part of the peripheral nervous system.

The Enteric Nervous System

The ENS contains more neurons than the spinal cord.[1,2] There are many different classes of enteric neurons that differ in their neurotransmitters, electrophysiological properties, targets (circular muscle, longitudinal muscle, other neurons, blood vessels, epithelium etc), inputs and the direction along the gut in which their axons project.[3,4] The neurons are grouped into ganglia, and each ganglion contains many different types of neurons. Myenteric ganglia are located between the circular and longitudinal muscle layers, and submucosal ganglia are located internal to the circular muscle layer. The ENS plays a critical role in mediating motility reflexes, as well as regulating blood flow within the gut wall and water and electrolyte transport across the mucosal epithelium. The fact that some regions of the gastrointestinal tract can function autonomously (without CNS input),[5] and because of its high degree of complexity, the ENS has been termed "the second brain".[2]

Origins of the ENS

Although neural crest cells arise along the entire length of the body axis, studies by Yntema and Hammond (1954) and later by Le Douarin and Teillet (1973) showed that the ENS is derived from crest cells that originate from two specific regions of the neuraxis—the vagal (defined as post-otic hindbrain adjacent to somites 1-7) and sacral (caudal to somite 28 in chick embryos and caudal to somite 24 in embryonic mice and humans) levels[6] (Fig. 1). The vagal region includes the transition zone between the head and neck.[7]

By ablating neural crest cells from the chick post-otic hindbrain, Yntema and Hammond (1954) showed that enteric ganglia were absent from the esophagus, stomach and small and large intestine. In a series of elegant chick-quail transplantation studies, Le Douarin and Teillet (1973) later showed that vagal neural crest cells adjacent to somites1-7 are the major source of

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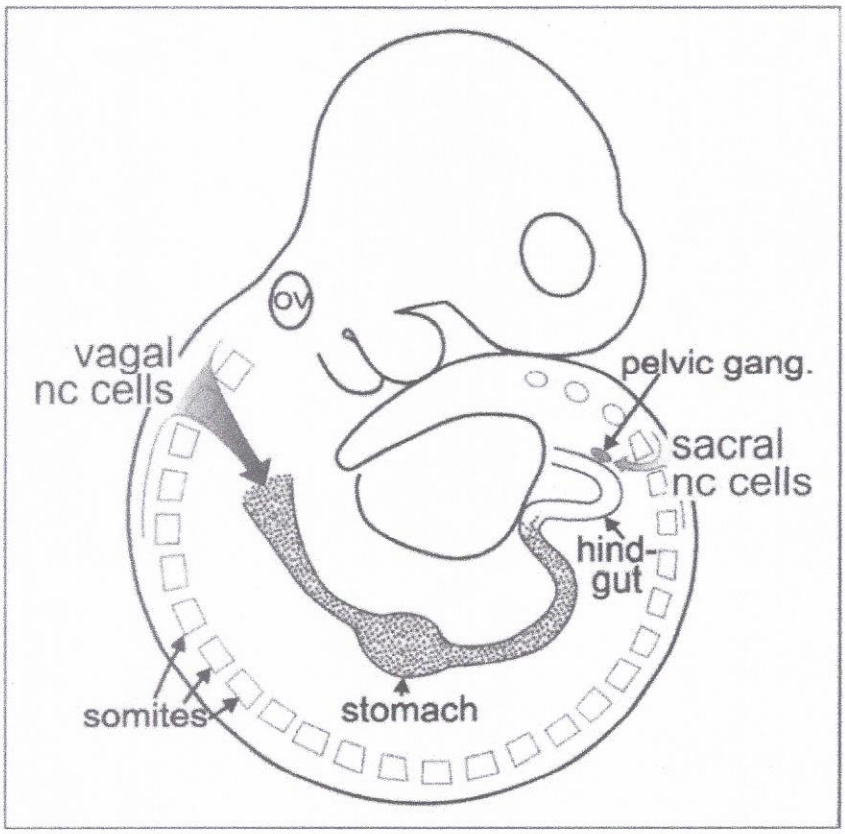

Figure 1. Diagram of an E10 embryo showing the origins of neural crest cells that colonize the developing gastrointestinal tract. nc—neural crest; OV—otic vesicle; pelvic gang.—pelvic ganglion primordia (note that pelvic ganglia are bilateral).

enteric neuron precursors, and that sacral level neural crest cells also contribute to the ENS, mostly in the hindgut. A sub-population of vagal neural crest cells also gives rise to major elements in the cardiac outflow tracts, as well as to neurons and support cells in intrinsic cardiac ganglia.[8-10]

More recent studies have further fine tuned the axial origins of the ENS. In chick embryos, vagal neural crest cells adjacent to somites 1-2 contribute predominantly to the esophagus and stomach; those adjacent to somites 3-6 contribute to the entire length of the gut; while neural crest cells adjacent to somites 6-7 are located mainly in the hindgut.[11-15] In mice, neural crest cell adjacent to somites 1-5 contribute enteric neurons along the entire length of the gut, while those adjacent to somites 6-7 give rise to cells only in the esophagus.[16] There therefore appears to be some species differences in the origin of esophageal neurons.

Vagal Neural Crest

The emigration of vagal level neural crest cells from the dorsal neural tube commences about the 7-10 somite stage. In avians, most vagal level neural crest cells migrate in a ventral route through the rostral half of somites 3, 4 and 5,[12] with some passing through branchial arch 6.[17,18] Chick vagal neural crest cells then migrate further ventrally to form the circumpharyngeal crest,[19,20] which is a complex pattern of crest cells that gives rise to a range of

different derivatives including the ENS.[21] The first vagal neural crest cells reach the vicinity of the chick foregut within 9 hours, at the 13 somite stage.[22] In mice, vagal neural crest cells migrate underneath the epithelium, through the somites, between the somites, and between the somites and the neural tube.[23] Neural crest cell that emigrate adjacent to somites 1-4 migrate around the pharynx along a pathway that is later followed by the vagus nerve, while neural crest cells adjacent to somites 4-7 form a continuous stream of cells that migrate ventrally beyond the dorsal aortae.[16,24] Near the foregut, neural crest cells form left and right strings along the route that is later followed by vagal axons from the hindbrain.[17,25] Vagal neural crest cells enter the nearby foregut mesenchyme at the 20-25 somite stage.[24]

Once in the foregut, vagal neural crest cells migrate caudally along the entire length of the gut (Figs. 1,2). Neural crest cells have been reported to migrate at speeds of between 35-40 μm/h in both birds and in explants from embryonic mice.[26,27] In chick, vagal neural crest cells enter the foregut around E2.5-3, reach the level of the umbilicus at E5, the cecal region at E6 and the colorectum at E7.5. The entire length of the chick gut is completely colonized by E8.5.[13] In mice (Fig. 2A-C), the colonization of the gut by vagal neural crest cells takes around 5 days; crest cells enter the foregut around E9.5; reach the cecal region at E11.5 (Fig. 2B); and completely colonize the gut by E14.5.[28] In humans, vagal neural crest cells enter the foregut at week 4 and reach the terminal hindgut by week 7.[29] Some of the crest cells that colonize the foregut emigrate out into the lung buds and give rise to ganglia within the airways,[30] and some of the crest-derived cells in the small intestine emigrate into the pancreas and give rise to pancreatic ganglia.[31,32]

In humans, birds and mice, vagal neural crest cells initially migrate in the outer half of the fore and midgut mesenchyme, which comprises an initially uniform population of cells that lie between the endoderm tube and the squamous epithelial serosal layer that surrounds the gut externally.[21,29] After each fore and midgut region is colonized, vagal neural crest cells then form a narrow layer in close proximity to the serosa, where myenteric ganglia will later form.[26,33] By the time vagal neural crest cells have reached the proximal hindgut in birds, the circular muscle layer has begun to develop, and the cells migrate internal to the circular muscle in the region where the submucosal plexus will form; vagal crest cells then undergo a secondary migration that gives rise to the myenteric plexus.[13] However in the hindgut of mice and humans, vagal crest cells migrate in the outer part of the mesenchyme and aggregrate in the region where myenteric ganglia will form (as they do in the fore and midgut); the submucosal plexus arises several days later from a secondary migration of neural crest cells from the myenteric plexus.[33,34] In mice, the netrin family of guidance molecules and their receptors has been shown to be required for the secondary migration of vagal crest cells to form the submucosal plexus.[32]

Sacral Neural Crest

Sacral neural crest cells migrate ventrally through the rostral halves of the adjacent somatic sclerotomes and congregate near the dorsal wall of the hindgut where they formed extramural ganglia (i.e., paired pelvic ganglia and, additionally in birds, the nerve of Remak)[13,24,35] (Fig. 2C). The avian-specific nerve of Remak is a large ganglionated chain running along the mesentery adjacent to the hindgut and distal midgut. Sacral neural crest cells form the nerve of Remak at E3.5 and remain in this structure until E7, when axons from neurons in the nerve of Remak project into the hindgut. Sacral neural crest cells then migrate into the hindgut along these extrinsic axons and colonize the hindgut in larger number from E10.[13] In mice, sacral neural crest cells form the pelvic plexus (Fig. 2C) adjacent to the distal hindgut from around E10.5 and do not enter the hindgut until E14.5.[24,35] In both chick and mice, sacral neural crest cells directly adjacent to the gut undergo a "waiting period" and only colonize the hindgut after it has been fully colonized by vagal neural crest cells. It was initially suggested that sacral neural crest cells might require the presence of vagal neural crest cell in the hindgut, in order for them to enter. However, this has subsequently been shown not to be the case. Following ablation of the vagal level neural tube in chick, the migration of sacral neural crest cells into the hindgut was

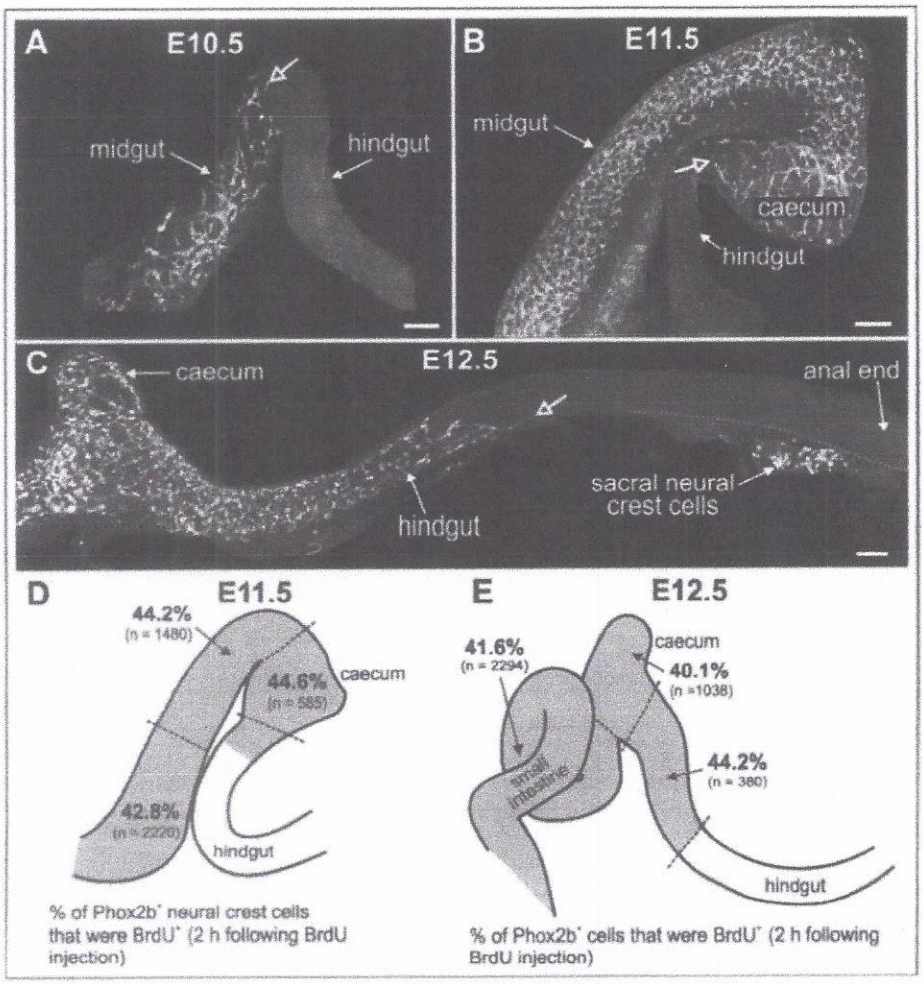

Figure 2. A-C. Micrographs of wholemount preparations of gut from E10.5 (A), E11.5 (B) and E12.5 (C) mice. The E10.5 and E11.5 (AB) preparations include the mid- and hindgut, whereas the E12.5 preparation (C) includes only the caecum and post-caecal hindgut. The preparations had been processed for immunohistochemistry using antisera to p75 (A,B) or Hu (C) to reveal neural crest-derived cells. The most caudal vagal crest-derived cell in each preparation is indicated with an open arrow. At E10.5, vagal neural crest cells have colonized most of the midgut, by E11.5, they have colonized the caecum and are entering the post-caecal hindgut, and at E12.5, crest cells have colonized the rostral half of the post-caecal hindgut. Sacral neural crest cells are present close to, but outside, the distal hindgut (C, dotted line indicates the border of gut). Scale bars: 100 µm. D, E. Diagrams of gut from E11.5 (G) and E12.5 (H) mice showing pooled data of the percentage of neural crest (Phox2b$^+$) cells that were also BrdU$^+$ from 6-8 embryos (E11.5) and 6 embryos (E12.5); BrdU had been administered to the mother two hours prior to sacrifice. The "n"s refer to the total number of crest-derived cells examined in each region (pooled from the different embryos). The regions of the gut that have been colonized by vagal crest-derived cells at these stages are shown in grey[54,141] (see also micrographs of E11.5 and E12.5 preparations above).

found to be unaffected,[14] and combination grafts of aneural hindgut with sacral cells, with and without vagal neural crest cells, have reported the same effect.[36] Similar finding have also been reported in mice, using *Wnt1-lacZ* transgene expression as an early marker of neural crest cells.[35]

The sacral "waiting period" could be due to an absence of attractive molecules (or their receptors on the sacral neural crest cells) and/or the transient expression of repulsive molecules in the distal hindgut. The chemorepulsive molecule Sema3A (formerly known as Collapsin-1) is expressed transiently in the distal hindgut of the embryonic chick, and is responsible for the delay in the ingrowth of axons from neurons in the nearby nerve of Remak.[37] Since sacral neural crest cells migrate into the hindgut along the axons of neurons in the nerve of Remak,[13] it is possible that the expression of Sema3A, either directly or indirectly, regulates the time of entry of the sacral neural crest cells into the hindgut.

Once sacral neural crest cells have entered the hindgut, they give rise to both neurons and glial cells.[13] The contribution of chick sacral neural crest cells was found to be confined predominantly to the distal hindgut, where they comprised up to 17% of myenteric neurons, and far fewer submucosal neurons.[13] Thus, even in the rectum, the vast majority of enteric neurons arise from precursors that originate in the hindbrain (vagal crest cells). In mice, the quantitative contribution of sacral neural crest cells to the ENS is still unclear. Mice in which the genes encoding members of the glial cell line-derived neurotrophic factor (GDNF) signaling pathway (GDNF, Ret or GFRα1) have been knocked out, lack enteric neurons in the gut caudal to the stomach[16,38] (Fig. 3). However, a small number of enteric neurons have been reported in the distal hindgut of these mice, which are almost certainly derived from sacral neural crest cells.[16,24,38] The small number of enteric neurons present in the hindgut of these mice is likely to be due to the fact that many of the sacral crest cells that normally enter the hindgut are dependent on the GDNF signaling pathway, but it is also possible that in mice, sacral neural crest cells contribute only a small number of enteric neurons.

Migratory Behavior of Neural Crest Cells

Images of fixed samples, and time-lapse imaging of living enteric crest-derived cells, have both shown that enteric crest cells migrate in chains, indicating the presence of cell-cell adhesive interactions between migrating neural crest cells.[28,39,40] The cell adhesion molecule, L1, is expressed by migratory and post-migratory crest cells in the embryonic mouse gut,[41,42] and in cultured gut explants, perturbing L1 activity with function blocking antibodies retards the rate of crest cell migration and increases the number of solitary cells (cells not in chains) near the migratory wavefront.[42] Other cell adhesion molecules, in addition to L1, are almost certainly involved in adhesive interactions between enteric crest-derived cells, as perturbing L1 function does not noticeably disrupt cell-cell contacts between post-migratory cells.[42] The gap junction protein, connexin 43, is also expressed by crest-derived cells in the embryonic mouse gut.[43] Direct cell-cell communication via gap junctions has been shown to be important for the migration and survival of trunk neural crest cells in culture,[44,45] but the role of gap junctions in enteric crest cells has yet to be determined.

Cell number can also influence the migratory ability of vagal crest cells. The partial ablation of vagal crest cells in chick embryos, results in aganglionosis of the distal gut.[11,14,46] Furthermore, when a small number of crest cells at the migratory wavefront in explants of embryonic mouse gut is isolated from the crest cells behind them, the isolated cells migrate more slowly.[40] These studies have lead to the idea of "population pressure" as a major driving force behind the colonization of the gut by crest-derived cells, and that high cell density and/or cell-cell contact stimulates migration and colonization.[47]

The migratory behaviour of crest-derived cells in the gut is also influenced by molecules expressed by the gut mesenchyme that could either promote or inhibit the motility of enteric crest-derived cells nondirectionally, or act as chemoattractive or chemorepulsive cues. GDNF, which is expressed by the gut mesenchyme, is chemoattractive to enteric crest-derived cells and appears to induce vagal cells to enter the gut and may also promote their rostrocaudal migration along the gut (see below).

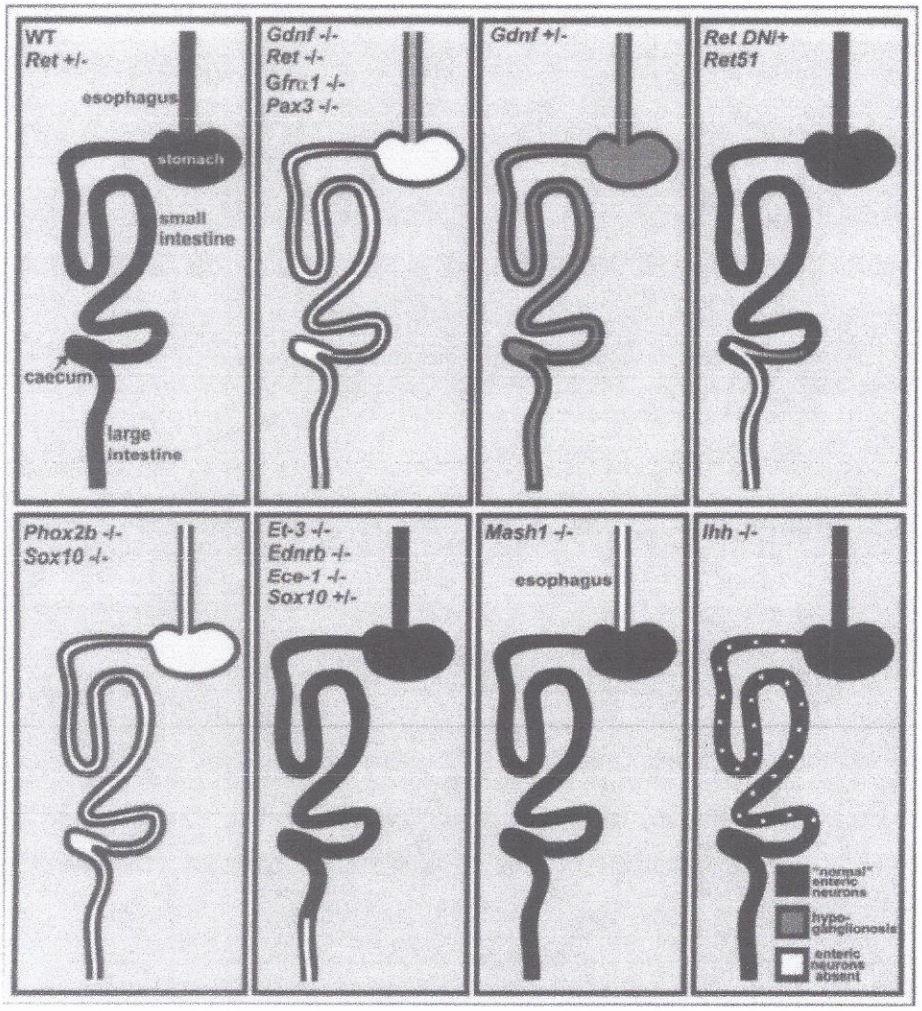

Figure 3. Diagram showing the gross phenotype of the best characterized genetically modified and naturally occurring mutant mice with enteric nervous system phenotypes. See text for details and references. *DN*—dominant negative; *Et-3*—endothelin-3; *Ednrb*—endothelin receptor B; *Gdnf*—glial cell line-derived neurotrophic factor; *Ihh*—Indian hedgehog.

Hirschsprung's Disease

Hirschsprung's disease (HSCR) is a congenital birth defect, with an incidence of around 1 in 5000 live births, in which enteric neurons are absent from variable lengths of the distal-most gut. Studies of mouse models have shown that HSCR is almost certainly caused by a failure of neural crest cells to colonize the distal regions of the gut, rather than a failure of crest cells to survive in the affected regions.[21,48] As enteric neurons are essential for propulsive activity in the gut, HSCR patients suffer from intestinal obstruction or severe constipation. The region lacking enteric neurons, termed the "aganglionic" zone, is usually contracted and devoid of contents. Although the region proximal to the aganglionic zone contains enteric neurons, it is distended (forming a "mega-colon") due to an accumulation of faecal contents. HSCR is treated by surgical resection of the aganglionic gut, although functional problems often persist. The

genetics of HSCR is complex.[48-51] Eleven "HSCR susceptibility genes" have been identified in humans, but mutations in known genes account for only about 50% of HSCR cases.[48,52,53] Many of the known susceptibility genes encode members of the RET- or EDNRB-signaling pathways or transcription factors that regulate *RET* or *EDNRB* expression. The importance of many of the known HSCR genes for normal ENS development is discussed below.

Proliferation, Cell Death and Differentiation in the Developing ENS

Proliferation

It is thought that somewhere in the order of 1000-2000 vagal neural crest cells enter the foregut of embryonic chicks and mice.[54] In small intestine and colon of adult mice, there are over one million neurons plus an equal number or higher number of glial cells.[55] Thus, crest-derived cells must undergo extensive proliferation in the gut during development. In fact, 2 hours following the administration of the thymidine analogue, BrdU, to pregnant female mice, over 40% of crest-derived cells in the gut of their E11.5 or E12.5 progeny are BrdU-labelled (Fig. 2C,D).[56] The expression of molecules that can influence the rate of crest cell proliferation (such as GDNF and endothelin-3) are higher in the caecum than in other regions of the developing gut. This has lead to the suggestion that the caecum may be a proliferative zone in which sufficient precursors to form the ENS of the colon are generated. However, a recent study has shown that the rate of proliferation of crest-derived cells does not vary in different regions of the embryonic mouse small and large intestine including the caecum, or at different distances from the migratory wavefront[56] (Fig. 2C,D). On the other hand, there does appear to be a lower rate of proliferation of crest-derived cells in the stomach of E12.5 mice compared to the midgut.[57]

Apoptosis

In most parts of the developing nervous system, excess neurons are generated, and then surplus neurons, usually those that have not projected to a target and have insufficient access to survival factors, are removed by programmed cell death (apoptosis). Surprisingly, studies of embryonic mouse and rat gut have failed to detect any evidence for apoptosis in the developing ENS.[55,58] Thus enteric neuron number appears to be regulated largely by the rate of proliferation.

Neuronal and Glial Differentiation

When crest-derived cells enter the mouse foregut at E10, a sub-population express pan-neuronal proteins (such as neurofilament, PGP9.5 and Hu).[25,59] By E14.5, around 50% of crest-derived cells in the small intestine express neuronal proteins, and the percentage of crest-derived cells that can be classified as neurons does not change much at later developmental stages.[60] Early differentiating neurons project their axons in the same direction (caudally) and along the same pathway through the mesenchyme as the migrating vagal cells.[61] Neural cells commonly migrate in close association with axons, for example in the development of peripheral nerves.[62] In the lateral line of the zebrafish, axons are the source of instructive cue(s) that guide migrating neural crest cells.[63] However, in the developing gut, it is still unknown whether axons are a source of guidance cue for migrating crest cells or vice versa, and hence whether crest cell migration can occur without axon growth.

The expression of molecules characteristic of particular neuron types (such as neurotransmitter synthesizing enzymes, etc) commences after the expression of pan neuronal proteins, and different neuron classes develop at very different developmental stages.[60] For example, in mice nitric oxide neurons develop at E12.5,[64] whereas cholinergic neurons do not develop in significant numbers until after birth.[65] In embryonic mice, cells expressing glial cell precursor markers are not detected until a day or so after crest cells have colonized a particular region of the gut, whereas in embryonic chicks, cells expressing glial markers are present close to the migratory wavefront.[39,60] Little is known about the molecular control of the differentiation of glial cells and different types of neurons from undifferentiated crest cells, but the transcription

factor, Sox10, appears to be required for the differentiation of enteric glial cells,[66] and another transcription factor, Mash1, is required for the differentiation of serotonin neurons.[67]

Molecules and Signaling Pathways Involved in ENS Development

A variety of signaling pathways and molecules have been shown to be required for normal enteric neural crest cell survival, proliferation, migration and differentiation. These genes and molecules have been identified from both basic research and from molecular genetic studies of patients with HSCR. Recent genetic and cell biological studies have shown that there are complex interactions between the different signaling pathways.[68-72]

GDNF Family

GDNF/Ret-GFRα1

GDNF is a secreted protein that is a distant member of the TGF-β superfamily. GDNF signals predominantly through the Ret receptor tyrosine kinase, but Ret is only activated if GDNF is bound to another protein, GFRα1, which is attached to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor.[73] GDNF is expressed by the gut mesenchyme prior to the entry of neural crest cells; Ret is expressed exclusively by neural crest-derived cells; and GFRα1 is expressed by both crest-derived cells and the gut mesenchyme.[74-77] GDNF-, GFRα1- or Ret-null mice die within 24 hours of birth, and lack neurons from most of the gastrointestinal tract, although they do have some neurons (but in greatly reduced numbers) in the esophagus, stomach and distal hindgut[78] (Fig. 3). *Gdnf*$^{+/-}$ mice have reduced numbers of enteric neurons throughout the gastrointestinal tract and motility defects.[79] Although *Ret*$^{+/-}$ mice have normal numbers of enteric neurons,[55] humans that are heterozygous for *RET* mutations can exhibit aganglionosis of the distal gut.[80]

There are two main isoforms of Ret, Ret9 and Ret51. Mice lacking Ret51 have neurons throughout the gut, while mice lacking Ret9 have no neurons in the colon (Fig. 3); this indicates that Ret9 is the most important isoform for the development of the ENS.[81] Recently, mice were generated that express a dominant negative mutation in Ret (*Ret*^{DN}) by inserting human cDNA encoding a mutant Ret9 (with two point mutations) into the first coding exon of the *Ret* gene. In contrast to *Ret*$^{+/-}$ mice, *Ret*$^{DN/+}$ mice have an aganglionic colon[82] (Fig. 3). Therefore, reduced Ret signaling in mice can be tolerated to some extent (as in *Ret*$^{+/-}$ mice), however a reduction in Ret signaling beyond a certain level results in defects in the ENS. The extent of aganglionosis is in all likelihood correlated with the degree to which Ret signaling is reduced. The developing ENS in humans appears to be more sensitive to reduced Ret signaling than the ENS of mice. In mice, it appears that excessive GDNF/Ret-GFRα1 signaling can also cause ENS defects. Mice lacking Sprouty2, an inhibitor of receptor tyrosine kinases, have ENS hyperplasia and intestinal pseudo-obstruction (delayed transit of intestinal contents) which can be corrected by inhibiting GDNF/Ret signaling.[83] Thus, the negative regulation of Ret by Sprouty2 appears to be important for maintaining appropriate levels of GDNF/Ret signaling during ENS development.

GDNF/Ret-GFRα1 signaling plays multiple and varied roles during ENS development. In mice lacking Ret, vagal neural crest cells die around the time at which they reach the foregut, suggesting that GDNF/Ret-GFRα1 signaling is required for survival.[16] Studies of enteric neural crest-derived cells grown in vitro have shown that GDNF also promotes their differentiation and proliferation.[76,84-87] Furthermore, in vitro assays have shown that GDNF is chemoattractive to migrating neural crest cells.[75,88] Therefore, GDNF expressed by the gut mesenchyme probably plays a role in inducing vagal neural crest cells to enter the gut, retaining them within the gut, and may also play a role in their caudally directed migration along the gut. Although GDNF/Ret-GFRα1 signaling plays multiple roles in ENS development, the circumstances under which Ret activation induces different processes (survival, proliferation, differentiation or migration) are unknown.

Other GDNF Family Members

In addition to GDNF, there are three other known members of the GDNF family—neurturin, artemin and persephin.[73] All GDNF family members signal through Ret, but bind to specific GFRα coreceptors. Persephin is not expressed outside of the CNS. Although artemin is expressed by the esophagus and stomach of embryonic rats,[89] its preferred coreceptor, GFRα3, is not expressed by enteric neurons,[90] and the sizes and numbers of enteric neurons in the small and large intestine of mice lacking artemin are no different from wildtype mice.[91] Neurturin is expressed by the mesenchyme of the developing gut, but at later developmental stages than GDNF, and its preferred coreceptor, GFRα2 is expressed by neural crest-derived cells within the gut.[90,92,93] In vitro studies have shown that neurturin promotes the survival, proliferation, differentiation, neurite outgrowth and migration of neural crest cells in the developing gut.[85,86,90] Mice lacking neurturin or GFRα2 are viable and fertile and have only minor differences in enteric neuron numbers from wild-type mice.[55,94,95] Thus, neurturin does not appear to be essential for the survival, proliferation or differentiation of enteric neural crest cells. However mice lacking neurturin or GFRα2 have smaller enteric neurons, reduced numbers of nerve fibres in the gut wall and some motility defects,[93] suggesting that neurturin may provide trophic support to enteric neurons.

Endothelin-3 (Et-3)/Endothelin Receptor B (Ednrb)

Et-3 is a secreted peptide that signals through a G-protein-coupled 7 trans-membrane spanning receptor, Ednrb. *Lethal spotted* (*ls*) and *piebald lethal* (s^l) are naturally occurring mutants for Et-3 and Ednrb respectively, and have been shown to lack enteric neurons in the distal bowel[96-98] (Fig. 3). Mutations in *ET-3* and *EDNRB* in humans can also result in Hirschsprung's disease.[80] Et-3 is expressed by the mesenchyme of the developing gut, with highest expression in the caecum.[69,99] Ednrb is expressed by both migrating neural crest cells and some mesenchymal cells.[69,100] Although enteric neurons are absent only from the distal colon of *Et-3* and *Ednrb*-null mice and rats, the migration of neural crest cells through the small intestine is also delayed.[58,69,100-102]

It is still unclear why perturbations in Et-3/Ednrb signaling result in delayed neural crest cell migration, and their failure to colonize the distal regions of the gastrointestinal tract. In vivo and in vitro studies have shown that Et-3 signaling does not effect the survival of enteric neural crest cells.[58,100,103] Different studies have reported variable effects of Et-3 on enteric crest cell proliferation,[58,69,84,87,103] and it appears that if Et-3 does influence proliferation, the effect is slight. Some in vitro studies have found that Et-3 inhibits neuronal differentiation induced by GDNF; it was therefore proposed that Ednrb signaling normally prevents the premature differentiation of crest cells into neurons, and thereby maintains sufficient numbers of undifferentiated migratory cells to colonize the entire gastrointestinal tract.[84,87] However, a recent study of Ednrb null embryonic rats has shown that the percentage of crest cells expressing neuronal markers was lower in the mutant rats than in wild-type animals.[58]

Other Secreted Factors

Neurotrophin 3 (NT-3) acting via TrkC receptors appears to be the only neurotrophin involved in the development of the ENS.[104] In vitro, NT-3 promotes the neuronal differentiation of crest-derived cells, and mice with null mutations in *Nt-3* or *Trk3* have reduced numbers of enteric neurons.[105] Interestingly, crest-derived cells respond to NT-3 at later developmental stages than they respond to GDNF.[76]

A number of studies have shown that BMP-2 and BMP-4 influence the differentiation of crest-derived cells in vitro, and mice that overexpress noggin, a BMP antagonist, have more enteric neurons.[58,106-108] Thus, BMPs seem to contribute to the regulation of enteric neuron number and differentiation. BMPs could also play a role in the concentric patterning of enteric ganglia within the gut wall and to ganglion size.[109,110]

Both Indian hedgehog (Ihh) and sonic hedgehog (Shh) have been shown to play a role in the development of the ENS.[109,111,112] Embryonic *Ihh*$^{-/-}$ mice are missing enteric neurons from some regions of the small intestine (Fig. 3), but the cellular and molecular mechanisms underlying the phenotype have not been elucidated.[111] In embryonic *Shh*$^{-/-}$ mice, some neurons are ectopically located under the epithelium instead of being restricted to myenteric and submucosal ganglia.[111] In vitro, Shh promotes the proliferation of enteric crest-derived cells directly, and inhibits GDNF-induced neuronal differentiation and migration.[112] Thus, the ectopic location of enteric neurons observed in the null mutants could be due to direct effects of Shh on the migratory behaviour of crest cells, or to influences of Shh on the mesenchyme and the concentric patterning of the gut wall.[109,111,112]

Transcription Factors

Gene knockout studies have revealed some of the transcription factors necessary for the development of the ENS. Mice lacking Phox2b or Sox10 lack enteric neurons throughout the entire gastrointestinal tract[113-115] (Fig. 3). Mice lacking Pax3 lack enteric neurons from the small and large intestine,[43] while mice lacking Mash1 lack enteric neurons in the esophagus[116] (Fig. 3). These null mutant mice all die during late embryogenesis or shortly after birth.

Phox2b is required for the expression of *Ret*.[115] Sox10 regulates the expression of a number of different genes; it can activate Ret directly by interacting with Pax3,[43,117] or indirectly by inducing Phox2b.[118] Sox10 also binds to the *Ednrb* gene and regulates the temporal and spatial expression of *Ednrb* in the gut.[72] Mash1 can also indirectly activate *Ret* by regulating Phox2a, a closely related protein to Phox2b.[119,120] Thus, Phox2b, Sox10, Pax3 and Mash1 seem to be required for normal ENS development because they regulate the expression of *Ret* and/or *Ednrb*.

In Sox10 null mutants, crest-derived cells die early.[113] Most Sox10 heterozygous mutant mice are viable, although some exhibit aganglionosis of the terminal colon[113,121] (Fig. 3). In *Sox10*$^{-/-}$ mice, the migration of crest cells through the gut is delayed and there is a reduction in the numbers of progenitor cells and an increase in the proportion of cells expressing neuronal markers.[66,121] These data show that Sox10 is required for the survival and correct cell fate of neural crest cells, in particular for the development of glial cells.[66] Sox10 is probably required for the early survival of enteric crest cells because it is necessary for *Ret* expression, and its role in glial development is probably due to interactions with other genes.[122] Sox8 is a transcription factor that is closely related to Sox10. Sox8 is expressed by enteric neural crest cells, but *Sox8*$^{-/-}$ mice are viable and fertile and show no ENS phenotype.[57] However, double heterozygous mice (*Sox8*$^{+/-}$, *Sox10*$^{+/-}$ mice) have a more severe ENS phenotype than *Sox10*$^{+/-}$ mice alone, suggesting that Sox8 functions as a modifier gene.[57]

SIP1 (smad-interacting protein-1), encoded by the *ZFHX1B* gene, is a transcription factor that appears to repress expression of target genes. Mutations in *ZFHX1B* have been implicated in HSCR-mental retardation syndrome, which is HSCR associated with multiple congenital anomalies.[80,123] In mice lacking *Zfhx1b*, the neural tube does not close and vagal neural crest cells fail to form.[124] Hence Sip1 appears to be required for the formation of the ENS because it is necessary for the formation of vagal neural crest cells in the neural tube.

Hox11L1 is expressed by enteric smooth muscle cells and by developing enteric neurons.[125] Mice with a null mutation in the Hox11L1 gene exhibit pseudo-obstruction (mega-colon),[125] and have been reported to have ENS hyperplasia in the colon.[126,127] However, a more recent study reported no detectable differences in the numbers of enteric neurons in the colon of mutant mice compared to wild-type mice.[125] Thus, it seems likely that the pseudo-obstruction phenotype observed in the Hox11L1 null mutant mice is due to myogenic, rather than ENS, defects.[128]

Other Molecules

Retinoic acids (RA) act at nuclear retinoic acid receptors to regulate the transcriptional activity of target genes. The main synthetic enzyme for retinoic acids during development is retinaldehyde dehydrogenase 2 (RALD2). *Raldh2*$^{-/-}$ mice die around E10 because of severe

cardiovascular defects, but maternal retinoic acid supplementation can prolong the survival of *Raldh2*$^{-/-}$ embryos until late gestational stages.[129] RA-rescued *Raldh2*$^{-/-}$ embryos lack an ENS, probably due to defects in the posterior pharyngeal arches and vagal level hindbrain, and consequent defects in vagal level crest cell migration.[129]

Conclusions

The development of the ENS from the neural crest encapsulates nearly all the major events of development, such as induction, cell migration, proliferation, stem cell replication and lineage restriction, cell associations, differentiation, neurite elaboration and connectivity. ENS development from the neural crest shows many desirable attributes both intrinsically and as a model of general developmental processes. This system appears to be highly conserved in vertebrates,[130-133] and many of these processes are of greater scale or duration, better defined and timetabled, and more accessible to experimental manipulation, compared to other early developmental systems. This is allied to a large number of genes implicated in ENS development, several serious clinical problems stemming from ENS dysgenesis (many already related to gene defects), and an array of informative animal mutant models (mostly mouse) (see above). In addition mathematical simulations of early ENS development are also being formulated.[134] Combined, these have placed the ENS at the forefront of research into clinically relevant, genetically complex developmental events.

ENS research is sufficiently detailed now to give an idea of what is not known; it drives home how complex development really is, and highlights the common difficulty in bridging the knowledge gap between genotype and phenotype. For the future, the discovery and isolation of ENS stem cells[107,135-138] and the recent use of neural stem cells in replenishing ENS neural deficits in animal models,[58,139] and even in providing some functional recovery[140] provides hope for clinical applications. But to reap these rewards requires even more exact basic research on ENS genes, molecules and cell biology.

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CHAPTER 11

Neural Crest and the Development of the Enteric Nervous System

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Abstract

The formation of the enteric nervous system (ENS) is a particularly interesting example of the migratory ability of the neural crest and of the complexity of structures to which neural crest cells contribute. The distance that neural crest cells migrate to colonize the entire length of the gastrointestinal tract exceeds that of any other neural crest cell population. Furthermore, this migration takes a long time—over 25% of the gestation period for mice and around 3 weeks in humans. After colonizing the gut, neural crest-derived cells within the gut wall then differentiate into glial cells plus many different types of neurons, and generate the most complex part of the peripheral nervous system.

The Enteric Nervous System

The ENS contains more neurons than the spinal cord.[1,2] There are many different classes of enteric neurons that differ in their neurotransmitters, electrophysiological properties, targets (circular muscle, longitudinal muscle, other neurons, blood vessels, epithelium etc), inputs and the direction along the gut in which their axons project.[3,4] The neurons are grouped into ganglia, and each ganglion contains many different types of neurons. Myenteric ganglia are located between the circular and longitudinal muscle layers, and submucosal ganglia are located internal to the circular muscle layer. The ENS plays a critical role in mediating motility reflexes, as well as regulating blood flow within the gut wall and water and electrolyte transport across the mucosal epithelium. The fact that some regions of the gastrointestinal tract can function autonomously (without CNS input),[5] and because of its high degree of complexity, the ENS has been termed "the second brain".[2]

Origins of the ENS

Although neural crest cells arise along the entire length of the body axis, studies by Yntema and Hammond (1954) and later by Le Douarin and Teillet (1973) showed that the ENS is derived from crest cells that originate from two specific regions of the neuraxis—the vagal (defined as post-otic hindbrain adjacent to somites 1-7) and sacral (caudal to somite 28 in chick embryos and caudal to somite 24 in embryonic mice and humans) levels[6] (Fig. 1). The vagal region includes the transition zone between the head and neck.[7]

By ablating neural crest cells from the chick post-otic hindbrain, Yntema and Hammond (1954) showed that enteric ganglia were absent from the esophagus, stomach and small and large intestine. In a series of elegant chick-quail transplantation studies, Le Douarin and Teillet (1973) later showed that vagal neural crest cells adjacent to somites1-7 are the major source of

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