Published in final edited form as:
Transl Res. 2013 July ; 162(1): 1–15. doi:10.1016/j.trsl.2013.03.001.

The Developmental Etiology and Pathogenesis of Hirschsprung disease

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The enteric nervous system is the part of the autonomic nervous system that directly controls the gastrointestinal tract. Derived from a multipotent, migratory cell population called the neural crest, a complete enteric nervous system is necessary for proper gut function. Disorders that arise as a consequence of defective neural crest cell development are termed neurocristopathies. One such disorder is Hirschsprung disease, also known as congenital megacolon or intestinal aganglionosis. Hirschsprung disease occurs in 1/5000 live births, and typically presents with the inability to pass meconium, along with abdominal distension and discomfort that usually requires surgical resection of the aganglionic bowel. This disorder is characterized by a congenital absence of neurons in a portion of the intestinal tract, usually the distal colon, due to a disruption of normal neural crest cell migration, proliferation, differentiation, survival and/or apoptosis. The inheritance of Hirschsprung disease is complex, often non-Mendelian, and characterized by variable penetrance. Extensive research has identified a number of key genes that regulate neural crest cell development in the pathogenesis of Hirschsprung disease including *RET*, *GDNF*, *GFRα1*, *NRTN*, *EDNRB*, *ET3*, *ZFHX1B*, *PHOX2b*, *SOX10*, and *SHH*. However, mutations in these genes account for only ~50% of the known cases of HSCR. Thus, other genetic mutations and combinations of genetic mutations and modifiers likely contribute to the etiology and pathogenesis of HSCR. The aims of this review are to summarize the HSCR phenotype, diagnosis and treatment options; to discuss the major genetic causes and the mechanisms by which they disrupt normal ENCC development; and to explore new pathways that may contribute to HSCR pathogenesis.

Gastrointestinal Tract, Enteric Nervous System and Hirschsprung Disease

The gastrointestinal (GI) tract is an endoderm derived organ system that begins at the mouth and terminates at the anus. The fetal GI tract is divided into three segments based on vascular supply. The foregut, supplied by the celiac artery, consists of the esophagus, stomach, part of duodenum, and biliary apparatus. The midgut, supplied by the superior mesenteric artery, comprises the rest of small and large bowel up to the splenic flexure. Lastly, the hindgut consists of the remainder of the large bowel to the superior part of anal

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The authors declare they have no conflicts of interest to disclose and have read the journal's policy on disclosure of potential conflict of interest.

canal, and is supplied by the inferior mesenteric artery. This organ system functions to digest and process foods and liquids taken in through the mouth via two types of digestion: mechanical, such as chewing and gut peristalsis, and chemical, such as enzymatic breakdown. The GI tract also plays a role as a major part of the immune system via the recognition of and response to introduced pathogens.

The ability of the GI tract to respond to the state of the lumen and gut wall by activating peristalsis, controlling blood flow and secretions and thus maintain proper physiological balance depends on the enteric nervous system (ENS) [1]. The ENS is the largest part of the peripheral nervous system and functions almost independently of the central nervous system [2] and is in direct control of the GI system [3]. ENS neurons and glia are organized into ganglia. The enteric ganglia are interconnected to form two plexi that extend along the length of the bowel: an outer myenteric (Auerbach) plexus running the full length of the gut, and an inner submucosal (Meissner) plexus, found only in the small and large intestine. The myenteric plexus develops first and is situated between the longitudinal and circular smooth muscle layers, and is involved in motility, while the submucosal plexus, which forms later in gestation, regulates motility, blood flow, and the transport of ions across the intestinal epithelium.

Gut motility is controlled by interdependent mechanisms including neural, such as the enteric ganglia, and nonneural, such as the interstitial cells of Cajal (ICC)[4, 5]. Interstitial cells of Cajal serve as pacemaker cells creating and propagating slow waves that lead to smooth muscle contraction in the gut [4]. Here, we focus on the necessity of the ENS to form a completely colonized gut that can maintain peristaltic activity of the gut wall and proper gut function. The absence of enteric ganglion cells of the myenteric and submucosal plexi along variable portions of the GI tract results in Hirschsprung disease (HSCR) [6], which is characterized by sustained contraction of the aganglionic bowel segment, leading to intestinal obstruction and distension of proximal segments (megacolon). The ENS is derived from a multipotent, migratory cell population called the neural crest, and the formation of a functional ENS requires coordination of the survival, migration, proliferation, and differentiation of these progenitor cells within the GI tract. Events that disrupt these processes can lead to HSCR. For example, a delay or arrest of NCC migration can result in the failure of ENCCs to reach their correct distal intestinal position [7]. Alternatively, NCCs can fail to survive, proliferate or differentiate after migration has occurred, due to abnormalities in the microenvironment [8–12].

Disorders such as HSCR that arise as a consequence of defective NCC development are termed neurocristopathies. HSCR is a particularly devastating neurocristopathy disorder that usually presents with the inability to pass meconium, together with abdominal distension and discomfort that usually necessitates surgical resection of the aganglionic bowel [13].

Clinical presentation

Histologically, aganglionosis is pathognomonic for HSCR. In 80–85% of HSCR cases, the aganglionotic region is limited to the rectum and sigmoid colon (short-segment disease). Long segment disease occurs in up to 20% of cases, and is characterized by aganglionosis extending proximally to the sigmoid colon. Total colonic aganglionononsis is more rare, occurring in 3–8% of patients with HSCR [14]. Another rare variant is ultra-short segment disease, affecting only the distal rectum (because this variant has such a short aganglionic zone, ganglion cells may be present in the biopsy)[15]. The portion of the bowel adjacent to the aganglionotic region with reduced enteric neuron density is termed the transition zone, and it is always cranial to the aganglionic region [16]. Hypoganglionosis and aganglionosis of the terminal gut can be caused by a reduction in enteric progenitor cells [17, 18]. The

zone of aganglionosis results in tonic contraction of the affected bowel, leading to obstructive symptoms. Most often, patients are diagnosed in the neonatal period [19], presenting with a distended abdomen, the delayed passage of meconium (>24 hours), and vomiting. When patients are diagnosed later in childhood, they often have short-segment aganglionosis and present with chronic constipation and distension, vomiting and failure to thrive [20]. In 10% of HSCR cases, patients can present with enterocolitis, fever, and even septicemia.

HSCR occurs in 1/5000 live births and has an overall 4:1 male predominance [21]. However, for short segment disease there is a 4.2–4.4 male: female predominance and for long-segment disease there is a 1.2–1.9 male: female predominance [22]. Furthermore, the risk of an HSCR sibling recurrence is 200 times higher than in the general population (4% versus 0.02%) [22, 23]. Up to 30% of patients with HSCR also exhibit other abnormalities [21, 24], such as velocardiofacial defects [25], congenital heart defects [26], gastrointestinal tract malformations, CNS abnormalities, genitourinary problems, craniofacial malformations, and spina bifida [22, 26–29]. In addition, 2–15% of HSCR cases are associated with Down's syndrome [27, 30].

Diagnosis

The diagnosis of HSCR can be made by a variety of methods. However, the preferred first diagnostic procedure is a contrast enema. This will define the transition zone between normal (dilated) bowel and the narrow aganglionic bowel. This transition zone is seen in 70–90% of cases [31, 32]. The rectosigmoid ratio is used to evaluate the transition zone. A rectosigmoid ratio greater than 1 is normal. A stool-filled proximal bowel will decrease the rectum to sigmoid ratio. Plain radiographs show dilated bowel loops, and anorectal manometry may also help with the diagnosis. When using anorectal manometry, clinicians note an absent rectoanal inhibitory reflex [33]. This absence of internal sphincter relaxation [34] is only a reliable test after neonatal day 12, when the rectoenteric reflex is present [35].

The gold standard for an HSCR diagnosis is a rectal biopsy. It is possible to obtain a submucosal rectal suction biopsy without anesthesia [36]. Analysis of the biopsy specimen is performed, to look for an absence of ganglion cells and hypertrophic nerve trunks. The clinician should be careful to biopsy proximal to the physiologically normal hypoganglionic zone at the pectinate (dentate) line, yet caudal enough to detect very short segment aganglionosis [37].

The biopsy specimen can be stained for an increase in acetylcholinesterase activity, which can contribute to the diagnosis [37, 38]. A full-thickness biopsy should be completed if the suction biopsy is unable to provide an accurate diagnosis. The length of aganglionosis is definitively determined at the time of surgical resection, confirming the absence of ganglionic cells in the myenteric and submucosal plexi.

Treatment

Currently, the only treatment for HSCR is surgery. Failure to surgically treat HSCR can be fatal due to malnutrition or sepsis following bowel perforation. Although surgery is the routine therapy for HSCR patients, surgical outcomes can vary widely, with a range of long term consequences, such as constipation, fecal incontinence and enterocolitis [39, 40]. The surgical treatments aim to remove the aganglionic bowel and anastomose the normal bowel to the anus while preserving sphincter function. The main techniques include the total transanal endorectal pull-through (TERPT) [41–43] and the laparoscopic assisted pull-through [44, 45] procedures.

As compared to traditional transabdominal open surgery, TERPT and laparoscopic assisted pull-through are associated with faster recoveries, shorter hospital stays, improved cosmetic appearance, and fewer perioperative complications [46, 47]. The TERPT is useful for aganglionosis confined to the rectosigmoid area [48], as it minimizes intraabdominal contamination, and adhesion formation, and the risk of damage to pelvic structures. The laparoscopic assisted pull-through has the benefit of collecting seromuscular biopsies for the identification of normal colon, better mobilization and dissection of the aganglionic colon, and minimized dilation of the anal canal [44, 46].

Although the TERPT and laparoscopic assisted pull-through procedures have better outcomes than traditional open surgery, there are risks and complications. Common post-surgical problems include long-term obstructive symptoms and soiling. Milder obstructive symptoms can be treated by dietary changes, laxatives, enemas or botulinum toxin injections every 3–4 months [49, 50]. Patients who are responsive to botulinum toxin injections but are unable to undergo repeated injections can have a myectomy procedure [51]. Although a repeat pull-through procedure can be done if there is residual or acquired aganglionosis, strictures, or dysfunctional, dilated proximal bowel, it is often very difficult due to scarring from the previous procedure [52, 53].

Pathogenesis

Proper neural crest cell migration, proliferation, differentiation, survival, and apoptosis all contribute to a functional ENS. Perturbation in any of these processes can lead to a Hirschsprung disease phenotype. Many genes which play a critical functional role in neural crest cell development have been implicated in HSCR, including the proto-oncogene *RET*, endothelin signaling genes, and transcription factors [28]. Although over a dozen genes have been identified that contribute to the etiology of HSCR, these pathways only account for about half of the known cases (Table 1).

Migration

The ENS is derived from migratory neural crest cells which originate at the vagal (somites 1–7) and sacral (caudal to somite 24) regions of the embryonic axis. These subsets contribute to different gut regions [54–57]. Vagal neural crest cells migrate in a rostral to caudal direction and sequentially contribute to the foregut, midgut, and hindgut [2, 55, 58]. In contrast, sacral NCCs are thought to contribute to the distal hindgut [59, 60]. Migration takes about six days in mice, with vagal NCC migration beginning around embryonic day (E) 8.5 and completing around E14.5 (Figure 1) [56, 61]. In humans, enteric neural crest cell (ENCC) migration takes about three weeks [62], beginning around week 4 and ending by week 7 [30, 63].

During migration, chains of interconnected neural crest cells at the leading edge of the population are referred to as the wavefront [63–65]. In these ENCC chains, there are two types of cell: multipolar and monopolar cells [66]. Previously, it had been proposed that the migration of ENCCs through the cecum (circumflex ENCCs: cfENCCs) was important for hindgut colonization [67–69], but recent photo-conversion real-time imaging experiments have shown that ENCCs destined for the hindgut traverse the mesentery (trans-mesenteric ENCSS: tmENCCs) as solitary cells when the midgut and hindgut are opposed in parallel (between E10.5–11.5), and these ENCCs are the major source of the ENS in the hindgut (Figure 2) [66]. Vagal NCC derived neuroblasts had been previously observed in the mesentery along the proximal colon prior to cecal colonization, but the significance of cells along this route were unclear [68]. Moreover cells in the mesentery near the distal hindgut between E11.5–13.5, were thought to be sacral neural crest cells [64].

Different populations of ENCCs exhibit different migration behaviors depending on their position along the migratory trail. Wavefront cells display significant caudal expansion, while trailing cells exhibit limited expansion. Therefore, it is the wavefront ENCCs that are responsible for the colonization of the hindgut [66]. This display of regional change in cell behavior is indicative of a positional microenvironment, suggesting the importance of cell-cell interactions during the process of NCC migration [65]. Since the midgut and hindgut are juxtaposed only during E10.5–11.5, and tmENCCs are the primary cell population contributing to the hindgut ENS, it was thought that these cells have only a limited period of time to reach the hindgut. Should these tmENCCs experience delayed migration through the mesentery, the result should be impaired colonization of the hindgut [66]. Furthermore a limited time window critical for complete ENS colonization of the terminal hindgut has also been proposed. In this scenario, a change in the microenvironment occurs at E14.5 when the gut is no longer permissive to migrating ENCCs, and high laminin levels may play a role in this non-permissive environment [70]. In contrast, other data shows that colonization can be completed after E14.5, suggesting that there may not be a strict permissive window of colonization [71].

GDNF/RET

Many genes contribute to normal enteric neural crest cell migration and the formation of a functional enteric nervous system, and mutations in any of these genes may cause an HSCR phenotype (Table 1). Two of the major contributing gene families responsible for HSCR cases are *Receptor tyrosine kinase* (*RET*) and *Glial cell line-derived neurotrophic factor* (*GDNF*). Mutations in the *RET* pathway account for 15–35% of patients with sporadic HSCR (HSCR in a single family member) and 50% of familial cases [72]. Patients who are heterozygous for mutations in *GDNF* have also been diagnosed with HSCR [73]. GDNF is a secreted protein that forms a complex with glycosylphosphatidylinositol-linked receptor (GFRα1), which binds and activates the transmembrane receptor tyrosine kinase RET. RET is then autophosphorylated and activates downstream pathways [74] that influence ENCC proliferation and survival, apoptosis, migration, and differentiation [75].

Prior to the entry of NCCs into the gut, GDNF is expressed in the mesoderm environment [76]. During their entrance into the gut, RET and GFRα1 are expressed in ENCCs [77]. When the NCC wave front reaches the esophagus, GDNF is expressed in the stomach, and is again elevated in the cecum as the NCC approach the distal portion of the small intestine, suggesting GDNF acts to attract RET and GFRα1 expressing ENCCs to the proper location [76, 78]. Following a concentration gradient of GDNF, tmENCCs migrate through the mesentery between the midgut and hindgut. Without GFRα1, tmENCCs are unable to leave the midgut [66]. Thus, without formation of the GDNF-RET-GFRα1 complex, aganglionosis is observed, irrespective of whether the mutation is in *RET*, *GDNF* or *Gfrα1* [66, 79–84].

Patients with long and short segment disease have been identified as having mutations in RET [85, 86]. However, penetrance is estimated at only between 50–70% [87]. Interestingly, the “gene dosage” necessary for normal ENS development differs between mice and humans. For example, a heterozygous mutation of *RET* in humans can lead to HSCR. In contrast, $Ret^{+/-}$ mice only exhibit hypoganglionosis [88]. Homozygous mutations in *Ret* are required for complete intestinal aganglionosis in mice [79]. To mimic the aganglionic phenotype of humans, mice need a loss of 60–70% of RET expression [89]. Due to alternative splicing, 2 RET isoforms can be formed: RET9 and RET51. These isoforms are conserved between human and mice [90]. When Ret51 is absent (*Ret9/9*), mice have a completely colonized distal colon, whereas when RET9 is absent (*Ret51/51*), colonization fails in the distal colon [91]. Other targeted RET signaling sites have been used to model

aganglionosis such as: Y1062 (*RetY1062F*), a multidocking site of RET9 (total intestinal aganglionosis)[92]; juxtamembrane serine 697 (*RetS697A*), a putative protein kinase phosphorylation site (loss of enteric neurons in distal colon) [93]; and a mutation in the extracellular cysteine residue 620 (*Ret620R*), where *Ret620R* null mice have total intestinal aganglionsis and heterozygotes have hypoganglionsis [88].

Interestingly, a new $Ret^{9/EGFP}$ hypomorphic mouse mutant ($Ret51^{C681F}$) has led to the proposition of a new mode of HSCR pathogenesis. These mutant mice display delayed and impaired transmesenteric migration of ENCCs from the midgut to the hindgut. This suggests that the tmENCCs have a limited time window to reach the hindgut, and if there is a delay, the result is intestinal aganglionosis [66]. However, delayed migration and reduced NCC progenitor numbers alone do not necessarily lead to HSCR. Rather, these deficiencies can be overcome through balancing neural crest cell proliferation and differentiation [71].

Noncoding mutations in *RET* can increase the susceptibility to other HSCR mutations [72, 94, 95]. These noncoding mutations may be involved with regulatory elements and cellular mechanisms, such as transcription, translation, location or level of gene expression, or may be associated with linked susceptible loci. For example, the 9q31 locus segregates with the HSCR phenotype in families with noncoding *RET* mutations, suggesting that a combination of these specific loci may cause a HSCR phenotype [72]. Additionally, a genome-wide association study of a Mennonite population (with a ten-fold increase in HSCR compared to the general population), identified a HSCR-associated, non-coding *RET* mutation in the transcriptional enhancer of intron 1, which associates with *EDNRB* mutations [96]. It was later found that this same *RET* Mennonite haplotype makes a 20-fold greater contribution to HSCR risk than the rarer protein-coding HSCR mutations [95]. However, not all *RET* variants increase susceptibility to HSCR. A common variant in the 3′ UTR of the *RET* gene has been shown to sl mRNA transcript decay [94]. The increasing number of identified non-coding susceptibility *RET* mutations is a reminder that although they may have low penetrance [95], they are capable of acting synergistically with other mutations to affect a disease phenotype.

Endothelin pathway

Endothelin signaling is also necessary for normal ENCC migration and may help maintain a permissive NCC environment. Endothelin 3 (EDN3 or ET3) is a secreted peptide expressed by gut mesenchyme [97] that binds to the G-protein coupled receptor ENDRB on migrating ENCC. Endothelin converting enzyme 1 (ECE1) post-translationally modifies the immature form of EDN3 into the active form [98, 99]. The EDN3-EDNRB signaling pathway is involved in regulating the normal migration of ENCCs, and maintains enteric progenitors in a proliferative state [69, 100]. Thus ENCC differentiation is inhibited by the presence of EDN3 [100–103].

Mouse models with mutations in *Edn3*, *Ednrb* or *Ece1* display various aganglionosis and pigmentation defects [98, 104, 105]. *Lethal spotted* mice have a mutation in the *Edn3* ligand, and *piebald lethal* have an *Ednrb* mutation. These mice lack enteric neurons in the distal bowel, have ENCC migration defects (delayed NCC arrival to the small intestine), and piebaldism [69, 98, 104, 106, 107].

Studies have shown that the incomplete neuronal colonization of gut [108] can be rescued by transgenic expression of the missing molecule [109, 110]. Additionally, conditional deletion studies of *Ednrb* show that the critical time window where EDN3-EDNRB signaling is necessary is between E10.5–12.5, as delayed migrating enteric neuroblasts were noted at E11.0 [111]. Additionally, beginning at E10.0, *Edn3* mRNA was detected ahead of migrating ENCCs [69]. Thus it has been suggested that because aganlionosis in *Ednrb*- and

Edn3-deficient mice is restricted to the distal colon, that the EDN3-EDNRB signaling pathway is required during later stages of gut colonization [112].

In *Ednrb*$^{flex3/flex3}$ mutant mice (null *Ednrb* allele with NCC labeled with YFP), ENCC advancement is delayed about 24 hours compared with wild-type littermates. By E14.5, wild-type guts are fully occupied by ENCCs, but mutant guts have altered trajectories and reduced speed. Grafting experiments suggested that the age of the recipient tissue, not genotype, restricts donor invasion. Furthermore, the time window when the gut microenvironment changes to become non-permissive to migration is E14.5, suggesting that if ENCCs have not finished migration by E14.5, an HSCR phenotype will result [70]. However, continued colonization has been observed between E14.5 to E18.5 in *Tcof1* haploinsufficient mice, even though reduced NCC numbers and delayed migration were observed between E10.5–E14.5 [71]. This evidence argues that delayed migration is not always predictive or sufficient for the pathogenesis of aganglionosis. Rather, a balance between NCC proliferation in concert with differentiation and extrinsic gut microenvironmental influences are required for complete ENS formation [71, 113].

Patients with heterozygous mutations in the *EDN3-EDNRB* pathway account for 5% of HSCR cases [27], including one case caused by a mutation in *ECE1* [114]. *EDN3* and *EDNRB* mutations are associated with both syndromic (Waardenburg Shah) and non-syndromic forms of Hirschsprung disease [27, 72]. Patients with Waardenburg Shah Syndrome have colonic aganglionosis, pigmentation defects and sensorineural deafness. The inheritance of this syndrome can be either recessive (*EDNRB* and *EDN3* cases) or dominant (*SOX10* cases) [27, 72]. Patients with *EDNRB* Hirschsprung disease are associated with a large Mennonite population [115]. Carriers of the Tryp276Cys mutation display incomplete penetrance, with heterozygous mutations leading to HSCR in 21% of the affected patients, and homozygous mutations leading to HSCR in 74% of the affected patients [116]. As with RET mutations, the human ENS is more sensitive to reduced ENDRB signaling than in mice [117].

Additional Genes Regulating Neural Crest Cell Migration

The zinc finger homeobox 1b/SMAL interacting protein (ZFHX1B/SIP1/SMADIP1), is expressed in premigratory and migratory vagal neural crest cells. This transcription factor is involved in neural specification and the epithelial to mesenchymal transition during early NCC development. *Zfxh1b*$^{-/-}$ mice exhibit complete absence of vagal NCC precursors and die around E9.5 due to cardiovascular and neural defects [118]. A human mutation in *ZFHX1B* is associated with Mowat-Wilson syndrome of which HSCR is a component [119].

Paired-like homeobox2b (PHOX2b) is a transcription factor expressed in migrating ENCCs, enteric neurons and glial cells [120]. This transcription factor is required for RET expression in ENCCs. Mouse and zebrafish models lacking *Phox2b* have total intestinal aganglionosis due to a failure of the ENCCs to colonize the gut properly [121, 122]. Consistent with this, mutations in *PHOX2b* have also recently been described in association with HSCR in humans, particularly in patients with congenital central hypoventilation syndrome (CCHS) and neuroblastoma (NB), referred to as the CCHS-HSCR-NB association [123–125]. Bone morphogenetic proteins (BMPs) also play a role in the regulation of NCC migration and ENS enteric ganglion formation [126, 127]. Targeted inhibition of intestinal BMP activity using noggin leads to delayed ENCC migration by interfering with the migratory effects of GDNF signals, suggesting that appropriate BMP and GDNF interactions are necessary for the proper formation of a complete ENS [126].

Sonic Hedgehog (SHH) is another key regulator of the migration of ENCCs of the gut [128] and plexus formation [129]. SHH and GDNF have opposing effects on ENCC migration.

NCCs migrate in the presence of GDNF, but addition of SHH reduces this migration by affecting the responsiveness of NCCs towards GDNF signaling or by regulating proliferation and differentiation of NCCs in the gut [128]. Thus, a balance between GDNF and SHH may be important for the proper positioning of the ENS plexus.

CXCR4 is a cell surface receptor for the CXC chemokine PBSF/SDF-1 (pre-B-cell growth-stimulating factor/stromal-cell derived factor). This factor is expressed in vascular endothelial cells. *Cxcr4* null mice display a failure of proper formation of the large vessels that supply the GI tract [130]. These mice also exhibit fewer tmENCCs at E11.5 and impaired hindgut colonization of ENCCs by E14.5 compared to wild type littermates [66]. This implies that tmENCC migration is partially dependent on vascular-derived signals, as CXCR4 indirectly supports trans-mesenteric migration by regulating vasculature development.

Proliferation, Survival and Differentiation

Proliferation rates in the developing GI system are equivalent throughout the ENS, with active ENCC proliferation at the wavefront necessary to colonize the gut, and behind the wavefront to fully populate the expanding intestine [131] and generate the millions of enteric neurons and glia present in the adult intestine [17]. The balance between proliferation and differentiation is necessary to maintain a sufficient progenitor pool of cells necessary to ensure complete ENS colonization [1, 18, 71, 113, 132].

Many of the genes discussed above also contribute to maintain ENCC and postmitotic enteric neuron survival in the gastrointestinal tract. *Ret*, *Gdnf*, and *Gfra1* are necessary for early ENCC survival [133]. *Gfra1* and *Ret* are also essential for survival of colonic enteric neurons. Furthermore, enteric neuron survival is sensitive to *Ret* dosage [89, 134]. Low *Ret* dosage does not result in increased apoptosis in ENCCs, but can lead to increased nonapoptotic cell death [89]. *Ret* hypomorphic mice have delayed migration and elevated nonapoptotic ENCC death. Consistent with this, when cell death is inhibited, a normal ENS is formed. This result led to the suggestion that cell death, not migratory delay of ENCCs, is a principle cause of isolated HSCR [135].

The EDN3-EDNRB signaling pathway is not only involved with regulating ENCC migration, but also plays a role in maintaining the enteric progenitors in a proliferative state [69, 100]. By keeping progenitors in a self-renewing state, the presence of Edn3 inhibits ENCC differentiation [100–103]. Gdnf and Ret promote proliferation of ENS progenitors and differentiation into neurons and glia [136, 137]. Gdnf also has a synergistic effect with Edn3 to enhance this proliferative effect, but inhibits neuronal differentiation to maintain the precursor cell population [101, 103].

Neurturin (NRTN or NTN) is a ligand in the GDNF family, which binds to GFRα2 to activate RET and promote the projection and branching of some enteric neuron axons. This trophic factor is important for the maintenance of neuronal projections, as neuturin-deficient mice have reduced nerve fiber density in the ENS, abnormal neurotransmitter release, and abnormal GI motility [17, 138]. Homozygous mice with null mutations in *Ntn* and *Gfra2* have reduced excitatory fibers present in the ENS and decreased gut motility along the entire GI tract [17, 138, 139]. Myenteric ganglion cells are smaller in $Ntn^{-/-}$ adult mice compared to wild-type mice as well, suggesting that Ntn plays a supportive role for neurons and cell size maintenance [138], and promotes the proliferation of ENS precursors and neuronal differentiation to increase the number of neurons and glia [136]. In addition, Ret+ cells from embryonic rat guts were cultured and failed to survive in the absence of GDNF or NTN [137]. Thus, it has been shown both *in vivo* and *in vitro* that Ntn promotes survival of enteric NCCs as well as the maintenance of enteric neurons and ganglia. Rare human cases

of HSCR have been documented with *NTN* mutations, and of those cases, occasional mutations have also been found in *RET* or another HSCR gene, suggesting a modifier role for *NTN* in the etiology and pathogenesis of HSCR [28, 73, 140–144].

SOX10

Sox10 (SRY-related high mobility group-box transcription factor) is expressed in vagal NCCs as they emigrate from the neural tube. Sox10 is used as a marker of ENS progenitors as it is expressed during NCC migration, and maintains the ENS progenitor state [100, 145]. This transcription factor regulates key genes required for ENS, melanocyte, and glial development [146], such as *Ednrb* and *Ret* in ENCCs [147–149]

Mouse and zebrafish *Sox10* homozygous mutants display abnormal ENS and melanocyte phenotypes. $Sox10^{Dom}$ mice have a single base pair insertion leading to a spontaneous dominant negative mutation. This truncates Sox10 downstream of its DNA binding domain [150]. These mice have a smaller pool of ENS progenitors as compared to wild-type mice [151, 152], and lack enteric neurons through the entire GI tract [150, 153]. The vagal NCC die prior to their entry into the gut, and these homozygous mutant mice die at birth [153]. Furthermore, up to 20% of the heterozygous mice have colonic aganglionosis and coat-color abnormalities, but the incidence is background-dependent [18, 150, 154, 155].

SOX10 mutations account for less than 5% of both syndromic and nonsyndromic forms of HSCR. In humans, *SOX10* mutations are associated with the dominant inheritance of HSCR in Waardenburg Syndrome [156], and some patients have additional neurologic phenotypes [157–159], due to dysmyelination of their CNS and PNS [157].

A model for CCHS-HSCR-NB, created by introducing a non polyalanine repeat expansion mutation (NPARM) *PHOX2B* in the *Phox2b* mouse locus, resulted in persistent *Sox10* expression in mutant embryos in enteric ganglion precursors, leading to reduced proliferation and biased glial differentiation [123]. This demonstrates that reciprocal inhibition between Sox10 and Phox2b is necessary to maintain appropriate differentiation of neurons and glia in the enteric ganglia.

Vitamin A Metabolism

Of all the human genes and mouse models identified, the RET pathway and its interacting components are the most commonly disrupted genes contributing to the HSCR phenotype. However, the mutations discussed above account for only about 50% of the documented cases of HSCR. Thus, there must be other pathways involved in the pathogenesis of Hirschsprung disease. Recently, a role for retinoid signaling in ENS development and the pathogenesis of colonic aganglionosis has been suggested [160, 161].

Vitamin A, an essential nutrient, plays a role in embryonic development in its active form, retinoic acid (RA). Insufficient or excess dietary vitamin A can result in congenital anomalies and even fetal death. Retinoic acid acts as a ligand for nuclear RA receptors (RARs) and retinoid X receptors (RXRs) to regulate transcriptional activity of target genes [162]. Vitamin A (retinol) is taken up by retinol binding protein 4 (RBP4), mediated by cell surface RBP receptor Stra6 (stimulated by retinoic acid gene 6). Once inside the cell, retinol is bound by cellular retinol binding proteins (CRBP) and undergoes two consecutive oxidation reactions to become retinoic acid: retinol is oxidized to all-trans-retinal by microsomal retinol dehydrogenase 10 (RDH10) or cytosolic alcohol dehydrogenases (ADH). Although studies initially indicated that members of the ADH family catalyzed this reaction ubiquitously throughout the embryo [163] a homozygous mouse mutation in *Rdh10*

[164] which displays a classic RA-deficiency phonotype, indicates RDH10 is the critical enzyme necessary for the regulation of this first oxidation step during embryogenesis [165].

All-trans retinal is then oxidized by retinaldehyde dehydrogenases RALDH1, RALDH2 and RALDH3 to retinoic acid. Retinoic acid can also be produced via an alternative route through beta carotene. Beta-carotene is cleaved by beta-carotene 15/15′-monooxygenase 1 (BCMO1/BCOX) to retinaldehyde which is then oxidized by RALDH1-3. RALDH expression patterns closely correlate with RA signaling, as shown through RA response element (RARE) reporter mice [166, 167]. *Raldh2*, the first retinaldehyde dehydrogenase to be expressed, is induced in the primitive streak and mesoderm during gastrulation, and is later restricted to the posterior embryonic region. This enzyme is responsible for almost all RA production during early embryogenesis. When necessary, to avoid the accumulation of excess RA, RA can be transformed to 4-hydoxy-RA for metabolism and elimination by cytochrome P450 26A1, B1, and C1 (Cyp26) enzymes. Collectively, the balance between retinoic acid synthesis and catabolism is critical to regulate the precise spatiotemporal domains of retinoid signaling during embryogenesis.

Retinoid Deficient mouse models

Currently, there are a two mouse models of depleted retinoid signaling that may phenotypically display some evidence for colonic aganglionosis, *Rbp4* $^{-/-}$ and *Raldh2*$^{-/-}$ [160, 161]. *Rbp4*$^{-/-}$ mice are unable to store retinol in their livers, and when subjected to an RA deficient diet during embryogenesis via maternal restriction from E7.5, these mice become fully depleted of Vitamin A and its active form, RA. Subsequently, these *Rbp4*$^{-/-}$ mice exhibit mild retinoid deficiency, with distal bowel aganglionosis and this phenotype is associated with increased phosphatase and tensin homolog (Pten) accumulation in migrating cells. Consistent with this, Pten overexpression leads to slowed ENS precursor migration. Thus RA has been proposed to maintain signals for migration and may do so through maintaining lamellipodia formation in migrating cells. This led to the suggestion that Vitamin A deficiency may be a non-genetic risk factor that increases HSCR penetrance and expressivity [161]. Consistent with this, the *Raldh2*$^{-/-}$ mouse model, which is also deficient in RA signaling, suggests that RA may play a role in NCC colonization of the gut. These mice typically die by E9.5, prior to development of the ENS. However, with RA maternal supplementation from E7.5, the animals live long enough to observe agenesis of the enteric ganglia as a result vagal NCC deficiency [160].

Although these studies suggest a role for retinoid signaling in the pathogenesis of colonic aganglionosis, the HSCR phenotype in the *Rbp4*$^{-/-}$ mice is only observed in response to a vitamin A deficient diet, creating a somewhat artificial situation. Similarly, the *Raldh2*$^{-/-}$ mutants die too early to examine a HSCR phenotype without providing RA rescue. These partially-rescued mice develop with a range of physical anomalies, so the precise role of RA remains to be characterized. Additional experiments are necessary to fully characterize the contribution of retinoic acid during gastrointestinal tract development, as the effects of RA on NCC formation, migration, proliferation and differentiation are not completely known. A mouse model without the need for dietary control or partial RA rescue would be ideal to properly investigate the role of RA in ENS development. This could be accomplished using conditional knockout mice. By genetically disrupting retinoic acid production, the precise temporal and tissue-specific requirement for RA during ENS development could be elucidated. Human studies are also needed to complement these animal models, and it would be ideal to conduct whole exome or whole genome sequencing to potentially identify new HSCR candidate genes and modifiers in the retinoid metabolism and/or signaling pathways. This would validate the clinical importance of the disrupted genes in many mouse models

currently available to study HSCR, while also providing new avenues for clinical research into treatments or prevention of individuals at-risk.

Discussion

Although more than half of the cases associated with Hirschsprung disease have associated genetic mutations, there is still much more to be learned about this disease. A continued effort is needed to identify responsible genes in experimental models, but also to identify relevant mutations in humans. Whole exome and whole genome sequencing of patient samples are an ideal way to identify these genes and mutations. With new genetic models of HSCR and new tools to examine them, new modes of pathogenesis may be proposed. For example, recent time lapse imaging using a photo-convertible mouse model showed that ENCCs reach the hindgut by migrating through the mesentery between the midgut and hindgut, as opposed to a sequential migration from midgut to cecum to hindgut [66]. Even this single deviation from the previous model of ENCC migration necessitates that mutants with migratory dysfunction be revisited to examine the possible changes in tmENCC and cfENCC behaviors [66], and the effects those changes may have on the progression of colonic aganglionosis. This new finding may also affect the way in which in vitro culture experiments are conducted to study the dymanics of NCC colonization and formation of the ENS. Instead of draping the midgut and hindgut across a well with a separated mesentery [168], experiments should now be conducted with the mesentery and blood vessels left intact, to allow for transmesenteric migration.

As discussed above, Hirschsprung disease is a complex, and often non-Mendelian disease, with high phenotypic variability and incomplete penetrance. Additionally, the same phenotype (HSCR) is associated with mutations of multiple distinct genes. These characteristics suggest the involvement of modifier genes or environmental influences [28, 87, 140, 143]. For example, rare human cases have been documented with *NTN* mutations, and many of these cases had a concomitant mutation in *RET* or another known HSCR gene, suggesting a modifier effect [144]. Although retinoid signaling has been implicated in the pathogenesis of HSCR, conditional knockout models are still required to fully elucidate the precise spatiotemporal role for RA during ENS development.

Great strides are being made to better understand HSCR, but much still remains unknown. The generation of new animal models, together with the continued identification of genes associated with HSCR in humans, and the development of new techniques to study the effects of these genetic disruptions are necessary. This may ultimately lead to better screening programs and prevention for at-risk individuals.

Acknowledgments

All experiments were approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research. We are grateful to Melissa Childers for excellent technical assistance and maintenance of our mouse lines. Research in the Trainor laboratory is supported by the Stowers Institute for Medical Research, the National Institute for Dental and Craniofacial Research (DE 016082) and the March of Dimes FY08-265.

Abbreviations

ADH	alcohol dehydrogenase
BCMO1/BCOX	beta-carotene 15/15′-monooxygenase 1
BMP	bone morphogenetic protein
cfENCC	circumflex enteric neural crest cell

CCHS	congenital central hypoventilation syndrome
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding proteins
CXCR4	cell surface receptor for the CXC chemokine PBSF/SDF-1
Cyp26	cytochrome P450 26
DAPI	4′,6-diamidino-2-phenylindole
E	embryonic day
ECE1	endothelin converting enzyme 1
EDNRB	endothelin receptor type B
ENCC	enteric neural crest cell
ENS	enteric nervous system
ET3/EDN3	endothelin 3
GDNF	glial cell line-derived neurotrophic factor
GFP	green flourescent protein
GFRα1	glycosylphosphatidylinositol-linked receptor alpha 1
GFRα2	glycosylphosphatidylinositol-linked receptor alpha 2
GI	gastrointestinal
HSCR	Hirschsprung Disease
ICC	Interstitial cells of Cajal
NB	neuroblastoma
NCC	neural crest cell
NPARM	non polyalanine repeat expansion mutation
NRTN/NTN	neurturin
PBSF/SDF-1	pre-B-cell growth-stimulating factor/stromal-cell derived factor
PHOX2B	paired-like homeobox2b
Pten	phosphatase and tensin homolog
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	response element
RBP4	retinol binding protein
RDH10	retinol dehydrogenase 10
RET	receptor tyrosine kinase
RXR	retinoid X receptor
SHH	sonic hedgehog
SOX10	SRY-related high mobility group-box transcription factor

Stra6	stimulated by retinoic acid gene 6
TCOF1	Treacher Collins-Franceschetti Syndrome 1
TERPT	transanal endorectal pull-through
tmENCC	trans-mesenteric enteric neural crest cell
ZFHX1B/SIP1/ SMADIP1	zinc finger homeobox 1b/SMAL interacting protein

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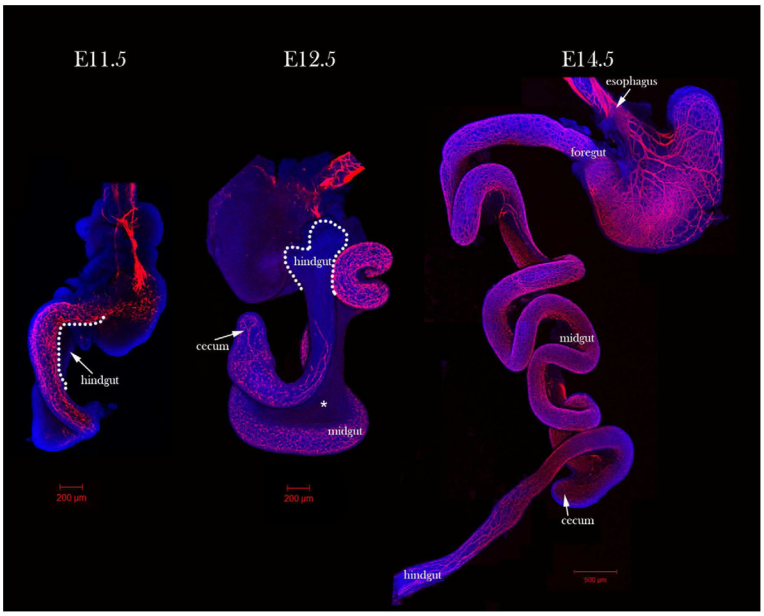

Figure 1. Enteric neuron immunostaining in wild-type embryonic guts

Tuj1 (red) immunostaining of E11.5 to E14.5 whole guts shows wavefront location. At E11.5, the embryonic gut is in a hairpin formation and neurons are present to the cecum. At E12.5 the gut has grown, and the neurons have reached the proximal hindgut. The E12.5 gut pictured is a back-side view to visualize the wavefront. At E14.5 neurons have reached the distal hindgut and grown significantly and the hairpin formation is no longer present. Asterisk (*) marks the mesentery at E12.5 through which tmENCCs migrate. Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue).

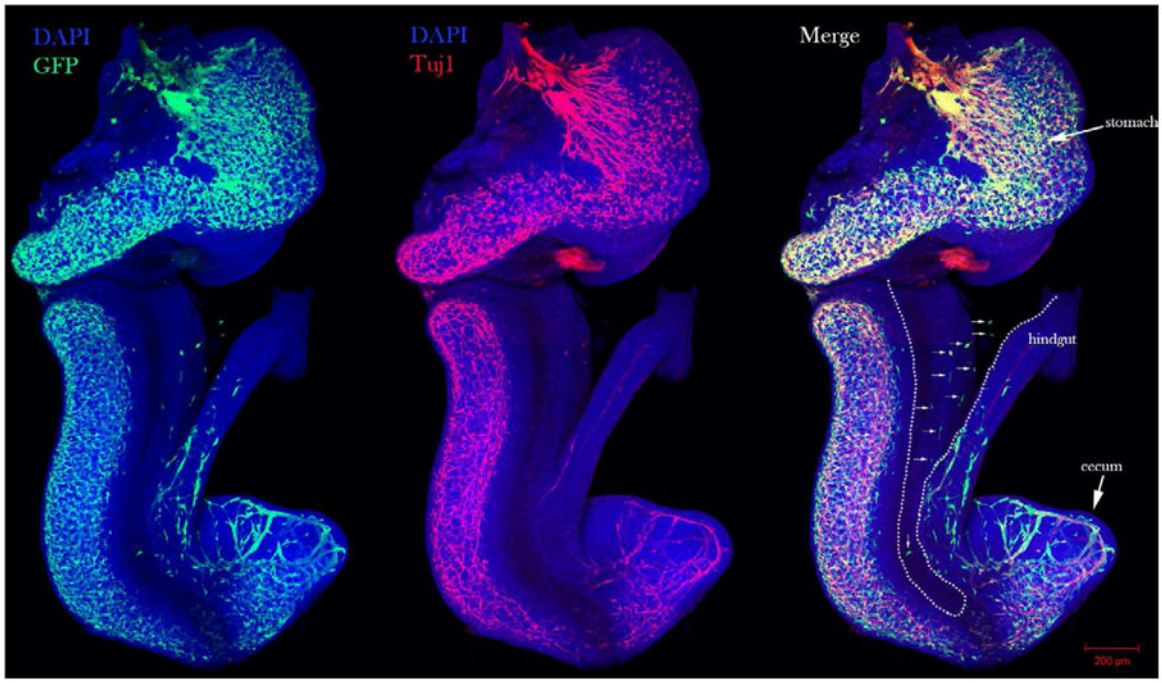

Figure 2. Trans-mesenteric enteric neural crest cells migrate through the mesentery of E11.75 guts

Whole-mount co-immunostain of GFP and Tuj1 staining of E11.75 gut from a *Rosa*^{eYFP}*; Wnt1Cre* embryo. White arrows depict tmENCCs in the mesentery between the midgut and hindgut. Mesentery is located between dotted lines. Neural crest cells are stained with GFP and mature neurons are stained with Tuj1 (red). Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue).

Table 1

Hirschsprung disease genes and contribution to neural crest cell migration, proliferation, differentiation and survival

Gene	Migration	Proliferation	Differentiation	Survival/Cell Death
RET	Attracted to GDNF	Promote proliferation	Promote neuronal differentiation	Promote ENCC and enteric neuron survival, Inhibit non-apoptotic cell death
GDNF	Gradient to attract RET+ and GFRα1+ ENCCs	Promote proliferation	Promote neuronal differentiation	Promote survival
GFRα-1	Attracted to GDNF, necessary to cross mesentery			Promote enteric neuron survival
EDNRB	Necessary for normal migration	Maintain proliferative state		
EDN3/ET3	Necessary for normal migration, may maintain permissive environment		Inhibit neuronal differentiation	
ZFHX1B/SIP1/SMADIP1	Neural specification, Epithelial-to-mesenchymal transition			
PHOX2b	Expressed in migrating ENCCs			
SHH	Oppose GDNF, reduce migration	Promote proliferation	Inhibit neuronal differentiation by affecting responsiveness to GDNF	
SOX10		Maintain ENS progenitor state		
NRTN/NTN		Promote proliferation	Promote neuronal differentiation	Promote survival

RET = receptor tyrosine kinase, *GDNF* = glial cell line-derived neurotrophic factor, *GFRα1* =glycosylphosphatidylinositol-linked receptor alpha 1, *EDNRB* = endothelin receptor type B, *ET3/EDN3* = endothelin 3, *ZFHX1B/SIP1/SMADIP1* = zinc finger homeobox 1b/SMAL interacting protein, *PHOX2b* = paired-like homeobox2b, *SHH* = sonic hedgehog, *SOX10* = SRY-related high mobility group-box transcription factor, *NRTN/NTN* = neurturin