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# Causes and consequences: development and pathophysiology of Hirschsprung disease

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## ABSTRACT

Hirschsprung disease (HSCR) is a congenital enteric neuropathy in which the enteric nervous system (ENS) fails to develop along variable lengths of the distal gastrointestinal (GI) tract. This aganglionosis results in a functional bowel obstruction and requires surgical resection of the aganglionic segment. Despite surgery, however, long-term bowel dysfunction affects many patients. Understanding the embryologic causes and pathophysiologic consequences of HSCR is critical to improving its diagnosis and treatment. During normal gut development, the ENS arises from neural crest cells (NCCs) that delaminate from the neural tube to populate the entire GI tract with enteric neurons and glia. This process requires NCCs to undergo proliferation, migration and differentiation to form the complex neuroglial network that regulates gut motility and other intestinal functions. This review discusses the cellular and molecular processes that control normal ENS formation and what goes awry to give rise to HSCR. The complex pathophysiologic consequences of aganglionosis are discussed, including recent observations that describe novel aspects of HSCR beyond the absence of ganglion cells. This review aims to expand the understanding of HSCR and to stimulate new ideas on how to improve current management of the disease.

## ENTERIC NERVOUS SYSTEM DEVELOPMENT

Hirschsprung disease (HSCR) is a congenital disorder that arises due to failure of enteric nervous system (ENS) formation in variable lengths of the distal gut during embryonic and fetal development, leading to life-threatening bowel obstruction in early life. The ENS is the largest and most complex division of the peripheral nervous system (PNS). It is located within the wall of the gastrointestinal (GI) tract, extends from the esophagus to the anus and controls coordinated smooth muscle contractile activity and many other gut functions. The human ENS contains approximately 400-600 million neurons that are grouped into ganglia located in two major plexuses; the myenteric (Auerbach's) plexus, located between the longitudinal and circular muscle layers, and the submucosal (Meissner's) plexus, located between the circular muscle and the mucosa. The ENS consists of

an intricate network of enteric glia and over 30 types of neurons, similar to those found in the central nervous system.<sup>1</sup> These include multiple functional subtypes such as intrinsic primary afferent neurons (IPANs), interneurons and motor neurons, which are further subdivided based on morphology, electrophysiology and neurotransmitter expression. The ENS is entirely derived from the neural crest,<sup>2</sup> a transient cell population often referred to as the fourth germ layer due to the diverse range of cell types and tissues to which it gives rise. Understanding the mechanisms underlying neural crest formation and development is important, not only for gaining insights into vertebrate evolution and development but also for increasing our knowledge of the pathophysiology of neural crest disorders, so-called neurocristopathies, that include Waardenburg syndrome, congenital central hypoventilation syndrome, CHARGE syndrome, DiGeorge syndrome, HSCR and more.

The study of the neural crest as an exemplar of developmental biology and of congenital disease has a rich history. In early development, neural crest cells (NCCs) delaminate from the neural tube and undergo epithelial-mesenchymal transition to become highly migratory cells that travel throughout the embryo along stereotypical pathways. These cells proliferate extensively and home to their target tissue, where they differentiate into a myriad of cell types, including melanocytes, craniofacial cartilage and bone, neurons and glia of the PNS and ENS and more. A complex gene regulatory network underlies the processes essential for neural crest development and includes signaling pathways, transcription factors and epigenetic modifiers that establish the migratory and multipotent characteristics of NCCs.<sup>3 4</sup> The use of quail-chick chimeras, devised by Nicole Le Douarin in the late 1960s, demonstrated that the vagal neural crest, adjacent to somite pairs 1-7, is the major contributor to the ENS



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along the entire length of the GI tract, with vagal neural crest-derived cells migrating in a proximal-to-distal direction along the length of the gut. A smaller contribution to the ENS of the distal intestine arises from the sacral level of the neural crest, which lies caudal to the 28<sup>th</sup> pair of somites.<sup>5,6</sup> These cells migrate in a distal-to-proximal direction. While it was accepted for decades that the majority of the ENS arises from vagal neural crest-derived cells that migrate along the gut mesenchyme,<sup>7</sup> more recent studies have identified additional complexity. For example, cell imaging in developing mouse gut showed that migrating cells take a 'shortcut' from the distal midgut to the proximal hindgut by traveling through the bowel mesentery to bypass the cecal region, and these 'trans-mesenteric' enteric NCCs form the majority of the distal colorectal ENS.<sup>8</sup> Whether similar trans-mesenteric migration occurs in humans is unknown. Furthermore, a new source of enteric NCCs was recently identified in mouse embryos, where Schwann cell precursors, which are neural crest-derived, enter the hindgut by migrating along extrinsic nerve fibers that extend into the gut, contributing about 20% of the enteric neurons in the colorectal ENS,<sup>9</sup> although again the relevance of this to human ENS development is unknown.

Classical animal models of developmental biology, including zebrafish, chick embryos and rodents have contributed to a detailed understanding of ENS development over the last 70 years. A description of the mechanisms underlying these processes, the genes and signaling pathways involved, the role of other non-neural crest cell types as well as the effect of the local environment on influencing ENS development is beyond the scope of this article and is covered by several excellent reviews.<sup>10-13</sup> Broadly speaking, formation of a normal ENS relies primarily on (1) craniocaudal migration of enteric NCCs from the foregut all the way to the distal hindgut, (2) NCC survival and proliferation to ensure enough cells to populate the entire GI tract and (3) appropriately timed cell differentiation such that some cells cease migration and settle down to become neurons and glia, while others continue on their journey. Coordination among these elements is a critical aspect of ENS development. In addition, patterning of neurons and glia into ganglionated plexuses, formation of a neuroglial network and proper communication with smooth muscle and other cell types, as well as interactions with the extracellular matrix (ECM) and local gut environment, are important components to a healthy ENS.

Two essential signaling pathways are of particular importance for ENS formation. Arguably the most important is the receptor tyrosine kinase, Rearranged during transfection (RET), present on enteric neural crest-derived cells, and its ligand, glial cell line-derived neurotrophic factor (GDNF), which is present in the embryonic gut mesenchyme. Mutations in *RET* account for approximately 50% of familial human HSCR cases,<sup>14</sup> and in mice null mutations in genes encoding RET, coreceptor GFR $\alpha$ 1, or ligand GDNF lead to total intestinal

aganglionosis.<sup>15</sup> Mice with monoallelic alleles of the *Ret* gene (*Ret*<sup>51/51</sup>) more accurately phenocopy human HSCR by displaying hindgut aganglionosis.<sup>16</sup> As RET-expressing NCCs migrate along the gut, they encounter GDNF in the surrounding environment, and the resulting activation of RET signaling regulates key ENS developmental processes, including NCC survival, proliferation and differentiation.<sup>13,17</sup> GDNF is also highly chemoattractive to Ret-expressing enteric NCCs. It is expressed in advance of the migrating NCC wavefront and in that way attracts the cells to continue their craniocaudal migration along the gut.<sup>18,19</sup>

The endothelin receptor B (EDNRB)-endothelin 3 (ET3) signaling pathway is the second key pathway in ENS development. Like RET, EDNRB is expressed on enteric NCCs while its ligand, ET3, is in the gut mesenchyme. Mutation of these genes in rodents leads to distal colorectal aganglionosis as well as melanocyte-related pigmentation defects.<sup>20,21</sup> EDNRB signaling normally delays the differentiation of enteric NCCs into neurons,<sup>22</sup> maintaining them in a progenitor state, which keeps them proliferative and migratory. Disrupted EDNRB-ET3 signaling leads to premature neural differentiation, thereby halting proliferation and migration, leaving the distal colon aganglionic.<sup>23</sup> EDNRB-ET3 signaling also acts synergistically with RET-GDNF, as activation of EDNRB enhances the proliferation-promoting effect of GDNF while inhibiting its chemoattractive role on these cells.<sup>24</sup> The coordinated activity of these two pathways is required for enteric NCCs to populate the entire length of the GI tract with mature neurons and glia.

The mouse models mentioned above have been powerful tools for elucidating the development of the ENS and have inferred an equivalence in the key steps involved in human ENS development based on conservation of genes, molecular mechanisms, signaling pathways and gut colonization patterns, the latter of which was described in human developing gut.<sup>25,26</sup> These studies showed that enteric NCC migration from foregut to distal hindgut in humans is completed by week 7 of gestation, suggesting that the distal aganglionosis of HSCR occurs very early in human development.

## WHAT GOES WRONG DURING ENS DEVELOPMENT TO CAUSE HIRSCHSPRUNG DISEASE?

During normal ENS development enteric neural crest-derived cells undergo a series of complex processes including cell migration, proliferation, differentiation and formation of ganglionated plexuses. To paraphrase the title of a review article,<sup>12</sup> what could possibly go wrong? Although there is no consensus on whether a single, primary defect underlies the pathogenesis of HSCR, there is a wealth of data implicating the processes above, which, when perturbed either individually or in combination, result in a failure of ENS development in the distal bowel. The primary contributing factors to the etiology of HSCR are outlined below.

### Cell number and proliferation

Critical numbers of NCCs are required for complete gut colonization. When the number of NCCs that migrate from the neural tube into the gut was experimentally reduced in chick embryos by ablating portions of the vagal-level neural tube (ie, the source of NCCs), the ENS was absent in the hindgut, phenocopying HSCR.<sup>27–28</sup> Proliferation at the NCC migratory wavefront is a key mechanism that ensures adequate cell numbers and drives neural crest invasion of the gut.<sup>29</sup> Experimental reduction in NCC number appears to disrupt the cell-cell interactions in the wavefront cells, which in turn may slow or halt their migration.<sup>27</sup> Although the effects of reducing NCC numbers on gut colonization can be tested experimentally in chick embryos, it is less clear how a genetic, or other insults during embryogenesis, reduces the number of NCCs available for gut colonization in humans. Analyses of mutant mice that phenocopy HSCR, as described above, highlight a reduction in NCC number and how this leads to slower migration along the gut, resulting in hindgut ENS deficiency.<sup>11</sup>

Active cell proliferation in the initially small population of NCCs that delaminate from the neural tube and enter the foregut is essential for ensuring there are sufficient numbers of cells to colonize the entire length of the gut during development, particularly since the gut is lengthening while the cells are migrating distally. RET-GDNF signaling has an essential role in this process as GDNF not only chemoattracts cells along the gut but also increases their proliferation.<sup>30</sup> In studies using heterozygous *Gdnf*<sup>+/-</sup> mice, which have enteric hypoganglionosis, this lower number of neurons was found to be caused by reduced enteric NCC proliferation, not to apoptosis of existing progenitors.<sup>31–32</sup> The authors suggested that the amount of GDNF available to enteric NCCs determines their proliferative capacity, and the extent of proliferation ultimately determines the number of enteric neurons in the postnatal gut.<sup>31</sup>

### Cell migration

The proliferation of NCCs is closely tied to their migration as proliferation drives both cell density and speed of migration. The latter aspect is an important factor for ENS development as the cells need to arrive in the gut while the microenvironment remains permissive to their arrival, expressing specific ECM components and secreting various components and niche factors that provide essential signaling cues to direct their migration.<sup>33</sup> Enteric NCCs at low-density migrate more slowly, which can result in those cells arriving to the distal gut late and being unable to colonize a microenvironment that has matured and is no longer permissive for their arrival.<sup>34</sup> Cell proliferation is the main driver of cell density and therefore migratory speed, inextricably linking proliferation and migration during ENS development.<sup>29–35</sup>

### Cell differentiation

While enteric NCC proliferation is essential to generate sufficient cells to populate the gut, this must be balanced with the need to stop proliferating and to undergo neuronal and glial differentiation. Just as insufficient cell proliferation will leave the distal gut aganglionic, so too will premature neuronal differentiation, since this stops cell proliferation and migration. Mutations in RET or EDNRB signaling, which work synergistically to coordinate enteric NCC proliferation, migration and differentiation, thus lead to failure of enteric NCCs to complete their migration, causing variable lengths of distal aganglionosis (reviewed in Lake and Heuckeroth and Sasselli *et al.*<sup>11–13</sup>).

### Genetics and associated syndromes

HSCR is regarded as a complex genetic disorder: it most commonly presents in sporadic form, which accounts for 80%–90% of cases and are mostly associated with short segment HSCR. The remaining 10%–20% of cases are familial, usually have autosomal dominant inheritance, and are often associated with long segment or total colonic HSCR.<sup>36–37</sup> There is a strong male bias, with a male-to-female ratio of 4:1, incomplete penetrance, and variable expression. HSCR is associated with a number of syndromes, congenital malformations and chromosomal abnormalities.<sup>36</sup> These include Shah-Waardenburg (ET3, EDNRB, SOX10), Haddad (PHOX2B), Mowat-Wilson (ZEB2), Goldberg-Shprintzen (KIAA1279), as well as Down syndrome, where patients have a >50-fold higher risk of developing HSCR than the general population, suggesting that overexpression of genes on chromosome 21 may contribute to HSCR etiology. However, the link between HSCR and Down syndrome remains to be fully elucidated.

Along with *EDNRB*, the *RET* gene has been shown to be the main gene associated with HSCR, with coding and splice site mutations in *RET* having been identified in up to 50% of familial cases and 15%–35% of sporadic cases.<sup>38</sup> In addition to *EDNRB* and *RET*, recent advances in genome-wide association analysis and next-generation sequencing have identified >30 HSCR candidate genes including genes in the RET-GDNF and EDNRB-ET3 signaling pathways, transcription factors such as SOX10, PHOX2B and ZEB2, and genes associated with a number of other signaling pathways involved in enteric neural development, such as the neuregulin, semaphorin, hedgehog and notch signaling pathways.<sup>37</sup> Adding to this complexity, a significant body of work has shown that the risk of HSCR appears to be a result of common non-coding variants, rare coding variants and copy-number variants affecting genes involved in ENS development that act individually or in concert to increase genetic susceptibility.<sup>37–39–40</sup> In addition, various epigenetic modifications such as histone modification, RNA methylation, DNA methylation and non-coding RNAs such as micro RNA (miRNA—small non-coding RNA) have been shown to modulate the expression of genes associated with

key ENS development processes such as cell migration, proliferation, differentiation and apoptosis that underly the pathogenesis of HSCR (reviewed in Diposarosa *et al.*, Sergi *et al.*, Torroglosa *et al.* and Yang *et al.*<sup>41–44</sup>). Although a substantial body of genetic information has been instrumental in helping to unravel the genetic underpinnings and the molecular mechanisms of the disease,<sup>37–40</sup> there are currently no evidence-based consensus guidelines for genetic testing of HSCR in the clinical setting. Thus a challenge for the HSCR field is how to translate the genetic research findings to better understand disease risk prediction, manifestation and penetrance, the risk of developing, for example, comorbid hereditary cancer syndromes associated with *RET*, and from a HSCR clinical standpoint, to determine if genotype is associated with postsurgical outcomes such as susceptibility to common complications including obstructive symptoms and enterocolitis.<sup>39</sup> Genetic advances could also be leveraged to stratify surgical approaches to help improve clinical outcomes for some subsets of patients, and even for the eventual development of novel tailored personal medicine approaches such as cell replacement or gene therapies.<sup>40</sup> Valuable research tools for the latter include human pluripotent stem cells (hPSCs) that can be used for disease modeling by introducing genetic alterations identified in HSCR patients, and/or by addition of hPSC-derived ENS cells in 2D culture assays or 3D engineered human gut functional studies.<sup>45</sup>

## PATHOPHYSIOLOGIC CONSEQUENCES OF INTESTINAL AGANGLIONOSIS

### Understanding intestinal motility in the context of HSCR

HSCR classically presents with failure of a newborn to pass meconium within the first 48 hours of life, a time by which nearly 100% of full-term neonates will have done so.<sup>46</sup> This failure to pass stool results from the functional intestinal obstruction caused by absence of enteric neurons, which leaves the aganglionic segment narrow and without coordinated motor activity. Since HSCR is due to failure of neural crest-derived cells to complete their craniocaudal migration along the entire GI tract, HSCR is always characterized by the absence of enteric neurons in the distal colon. In 85% of patients, the aganglionosis is confined to the rectosigmoid, and this is referred to as ‘short-segment’ HSCR. In the remaining patients, the aganglionosis extends for variable distances more proximally (long-segment HSCR) can involve the whole colon (total colonic aganglionosis) and rarely can involve the entire small and large intestine (total intestinal aganglionosis).

Given the important role of the ENS in motility, failure to pass meconium in these newborns is understandable. Interestingly, however, 37%–54% of infants with HSCR are able to pass meconium within the first 2 days of life.<sup>47–48</sup> This highlights the fact that while failure to pass stool should alert the clinician to the possibility of HSCR, the passage of meconium within the first 2 days of life

does not rule-out the disease. Some infants with HSCR, either with short-segment or long-segment disease, continue to pass stool during infancy and early childhood, accounting for the occasional later diagnosis of the disease. These observations suggest that the ENS is not absolutely required for colorectal motility. To understand the pathophysiology of HSCR, it is, therefore, helpful to review briefly the complex regulation of motor activity in the gut.<sup>49–51</sup>

Intestinal motility is under the control of two parallel mechanisms: myogenic and neurogenic. Myogenic activity refers to spontaneous smooth muscle activity and relies on three principal cell types: smooth muscle cells, interstitial cells of Cajal (ICCs) and fibroblast-like PDGFR $\alpha$ -expressing cells. These groups of cells are referred to collectively as the ‘SIP syncytium’ (Smooth muscle, ICC, PDGFR $\alpha$ ). Both ICCs and PDGFR $\alpha$ + cells are electrically coupled to smooth muscle cells via gap junctions. The muscularis propria of the gut thus functions as an electrical syncytium. ICCs possess spontaneous pacemaker activity that is characterized by the generation of rhythmic electrical slow waves, which are oscillations of membrane potential. The slow-wave currents generated by ICC depolarization propagate from cell to cell via gap junctions. Since ICCs are also electrically coupled with smooth muscle cells, the slow waves passively conduct to the muscle, leading to depolarization, excitation-contraction coupling and finally phasic contractions of the intestinal smooth muscle. The SIP syncytium is, therefore, responsible for ‘myogenic’ motor activity in the gut.

Parallel to this myogenic activity is the neuronally mediated activity controlled by enteric neurons, which modulates the baseline myogenic activity and allows for more complex and coordinated motor patterns. Enteric neurons are broadly classified into IPANs that sense luminal distension; ascending and descending interneurons that relay that sensory information orally and aborally, respectively; and excitatory and inhibitory motor neurons that contract and relax the muscle, respectively. The excitatory activity, primarily mediated via acetylcholine (ACh), leads to contraction of the gut. Inhibitory activity is mostly controlled by nitric oxide produced by a group of enteric neurons referred to as ‘non-adrenergic, non-cholinergic’ (NANC) neurons, which express neuronal nitric oxide synthase (nNOS). The ability of IPANs to sense the presence of intraluminal contents, combined with the coordinated activation of intestinal contraction and relaxation, leads to the propagation of luminal contents in an aboral direction. This neuronally mediated process is known as ‘peristalsis,’ or more specifically ‘content-dependent (i.e., distension-induced) peristalsis’. In addition to peristalsis, the neurogenic component of intestinal motor activity is also responsible for the migrating motor complex of the small intestine and the high-amplitude propagating contractions (HAPCs) of the colon.

Inhibition of neural activity in human and rodent colon explants leads to rhythmic, spontaneous, high-amplitude

contractions.<sup>52</sup> These result from the myogenic activity of the gut, which is unmasked by the absence of enteric neurons, especially the loss of inhibitory neurons. This myogenic activity underlies the mechanism whereby aganglionic bowel can be observed intraoperatively to have spontaneous muscle contractions. It also may account for why some children are able to pass stool despite having HSCR. Another possibility is that those with short-segment aganglionosis can pass stool due to the strength of upstream neurally-mediated contractions above the aganglionic segment, although it is difficult to envision these being capable of evacuating a longer segment of aganglionic bowel. It is likely that the amplitude and frequency of myogenic contractions vary among individuals and are further modulated by extrinsic sympathetic and parasympathetic innervation, contributing to variability in the timing and severity of presentation of the disease.

In addition to the functional obstruction created by aganglionosis, HSCR is also characterized by spasticity and narrowing of the aganglionic segment. The cause of this narrowing has been debated. Some have attributed it to the hypertrophic cholinergic nerve bundles that are a feature of the aganglionic segment and hypothesized that the absence of intrinsic innervation leaves the extrinsic cholinergic, and therefore excitatory, innervation to the gut unbalanced, causing spastic contraction of the aganglionic segment. Vizi *et al.*<sup>53</sup> attributed the spasticity to increased cholinergic activity in the aganglionic segment. They found that the narrow, aganglionic segment possesses higher levels of ACh than the dilated ganglionic bowel as well as increased ACh release both at rest and during transmural electrical stimulation. Another study, however, examined rectal muscle strips from mice with HSCR and showed that electrical stimulation of the muscle led to lower ACh release and no contractions in the aganglionic rectum, arguing that cholinergic innervation is not responsible for the distal spasticity.<sup>54</sup> An alternative theory argues that the net effect of the ENS is to relax the gut, an effect due to the action of the NANC inhibitory system. Therefore, in the absence of any intrinsic innervation, that relaxing effect is lost and the result is contraction and spastic narrowing.<sup>55</sup>

### HSCR IS NOT JUST AGANGLIONOSIS

If HSCR was simply due to distal aganglionosis, then removal of that segment should cure the disease. Unfortunately, that is not the case for many patients. At least 50% of adults who underwent pull-through surgery for HSCR early in life fail to achieve bowel function scores equivalent to healthy individuals. Up to 33% experience obstructive symptoms and up to 69% experience some degree of fecal soiling.<sup>56 57</sup> Xu *et al.*<sup>58</sup> outlined a number of explanations for poor post pull-through outcomes including mechanical obstruction, recurrent or acquired aganglionosis, disordered motility in the residual bowel, internal sphincter dysfunction or functional megacolon

caused by stool-holding behavior. One particular explanation may be that the remaining ENS is not normal and indeed ample evidence supports this. First, incomplete resection of the transition zone, which is the segment between aganglionic and normoganglionic bowel, can lead to obstructive symptoms. Using specific histopathologic features of the transition zone, which includes partial circumferential aganglionosis, severe myenteric hypoganglionosis, and hypertrophic submucosal nerves, Kapur and Kennedy found that the transition zone was typically less than 5 cm in length.<sup>59</sup> However, some transition zones extended higher and therefore may not be completely excised when the resection includes only 5 cm above the level of aganglionosis. Furthermore, the definition of 'hypoganglionosis' in the transition zone is highly subjective as no normative data exist for enteric ganglion density, leaving a significant burden on the pathologist to determine what is 'normal.' The functional consequences of transition zone pull-through can include obstructive symptoms, recurrent enterocolitis and fecal incontinence.<sup>60</sup> These authors note that the results of redo pull-through surgery in their experience were disappointing and recommended consideration of alternative approaches, including antegrade continence enema procedure. A recent retrospective study from Torre *et al.* suggested that the presence of a transition zone pull-through did not in fact increase the risk of constipation or enterocolitis, suggesting that redo pull-through surgery may not be necessary for all these patients.<sup>61</sup> The management of this problem, therefore, remains unclear and further clinical studies are warranted.

Recent studies on mouse and human HSCR tissue have highlighted additional abnormalities of the ENS proximal to the aganglionosis. Cheng *et al.* examined neurotransmitter expression in the ganglionic colon of both HSCR patients and Ednrb-deficient mice,<sup>62</sup> a well-established model of short-segment HSCR. They found a consistent overabundance of nNOS-expressing neurons, an observation that has been made by several other authors. Given the importance of maintaining a critical balance between excitatory and inhibitory innervation for normal motility, this excess of inhibitory innervation may contribute to postpull-through bowel dysfunction. Bhawe *et al.*<sup>63</sup> showed that the neuronal abnormalities in HSCR extend throughout the GI tract. In Ednrb-deficient mice, they documented neuronal defects involving the stomach, small intestine and colon. These included abnormalities in enteric ganglion number, ganglion size and neuronal fiber density. Interestingly, they quantitatively assessed motility and found accelerated gastric emptying, delayed small intestinal transit and a reduction in colonic motility in the HSCR mice. Pan-intestinal dysmotility has also been observed in children with HSCR, with abnormalities of esophageal, gastric, intestinal and colonic motility identified.<sup>64-66</sup> In a separate study, Bhawe *et al.* used single-cell RNA sequencing and found that the small



intestine of Ednrb-deficient mice is missing GABA-expressing neurons, although the impact of this is yet to be determined.<sup>67</sup> In addition to associated enteric neuropathies, abnormalities in the density of ICC cells have also been described in the aganglionic region, transition zone and ganglionated bowel, although these findings have been inconsistent,<sup>68</sup> a feature that is likely due to the cellular plasticity and transdifferentiation displayed by ICC and smooth muscle cells that is regulated by Kit signaling.<sup>69 70</sup> Whether similar neuronal or ICC changes are also present in human HSCR, and potentially explain the persistent dysmotility observed in so many patients after pull-through surgery, remains to be determined.

Another important consideration of HSCR pathophysiology that can contribute to postpull-through constipation is the absence of the recto-anal inhibitory reflex (RAIR), a diagnostic feature of the disease. The RAIR is characterized by transient relaxation of the internal anal sphincter in response to rectal distension. Its absence in HSCR is due to the absence of intrinsic innervation in the rectum, specifically the lack of nitrergic (i.e., NANC) neurons, which play an essential role in mediating the RAIR<sup>71</sup> by producing nitric oxide, an important inhibitory neurotransmitter for the internal anal sphincter.<sup>72</sup>

Finally, one needs to remember that pull-through surgery itself carries morbidity and can have long-term consequences on motor and sensory function in the pull-through segment. Inadvertent injury to the anal sphincters or the pelvic plexus, which provides sympathetic and parasympathetic innervation to the pelvic organs, can lead to anorectal dysfunction. Proctectomy, which is an integral part of any pull-through operation for HSCR, has potentially negative consequences given the important role of the rectum in storing stool prior to defecation. Also, without the rectum, HAPCs in the colon extend all the way to the anus, often with pressures that exceed anal sphincter pressure. Furthermore, some children have colonic hyperactivity after pull-through surgery, with a greater number of HAPCs in both fasting and postprandial states,<sup>73</sup> potentially contributing to fecal incontinence.

## SUMMARY

The developmental biology of the ENS and the embryologic mechanisms that give rise to HSCR are complex, but our understanding of the genetic, molecular and cellular causes have improved substantially over the past couple of decades. While we now have a much better understanding of why aganglionosis occurs, however, the pathophysiology of the disease remains complex and outcomes are still suboptimal. Ongoing research will not only further unravel the genetic and embryologic aspects of HSCR but will also help to develop novel treatments for the disease, such as cell replacement and other regenerative therapies<sup>74 75</sup> that

may improve outcomes and quality of life for affected individuals.

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