Functional Anatomy of the Enteric Nervous System

3

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3.1 Introduction

Congenital birth defects, of which Hirschsprung disease is an example, are among the most difficult of illnesses to study in the human patients who suffer from them. By the time the condition is identified in an affected individual, the process that brought it about is over and done with. It is thus impossible to study the ontogeny of birth defects, such as Hirschsprung disease, in the fetus in which the problems develop. An investigator seeking to uncover the pathogenesis of such a condition must search, like a detective, for clues left behind by the perpetrator who has fled the scene of a crime. Even the identification of genes that may have mutated, important an achievement as that is, does not, by itself, explain why the defect develops. Human life, moreover, is so precious that human subjects are terrible laboratory animals. As a result, more can often be learned about the origins of human illness by studying animal models than by investigating the patients themselves. Invasive research, which is only possible on animals, can be used to develop a conceptual framework to devise hypotheses that can subsequently be tested for applicability to human patients. Experiments, based on these hypotheses, can be targeted to what can be confirmed or denied by diagnostic tests or by analyzing the restricted materials available from human subjects. Studies of animal biology can thus make investigations of human biology possible.

The importance of animal models in learning why developmental defects occur and what can be done to prevent them cannot be emphasized too strongly. Recent research, facilitated by the use of rodents, avians, and zebrafish as model systems, has greatly advanced our understanding of the factors that govern the development of the enteric nervous system (ENS). Clearly, comprehension of the pathogenesis of the neuromuscular defects of the bowel, including Hirschsprung disease, requires a detailed understanding of the processes that govern normal enteric neuronal and glial ontogeny. This research has already provided enough insight to systematize current thinking about the origin of Hirschsprung disease. This review is concentrated on the important progress made in the developmental biology of the ENS (provided mainly by research on animals) that now provides a logical basis for explaining the origin of the human disease.

Hirschsprung disease is a well-defined clinical entity. It is a congenital absence of neurons in the terminal portion of the gut. The length of the aganglionic region varies, and short- and long-segment varieties have been distinguished, although these entities represent the extremes of a continuum. In fact, classical Hirschsprung disease, in which a segment of the bowel is totally aganglionic, is itself only one of a series of conditions that encompass a variety of allied disorders that include hypoganglionosis, neuronal intestinal dysplasias (hyperganglionosis), immaturity of ganglion cells, and dysganglionoses that have yet to be thoroughly classified. Most often, Hirschsprung disease is limited to the colon, although occasionally greater lengths of bowel may be involved. The gut is hypoganglionic in the region immediately rostral to the aganglionic segment and, in some patients, the junction between the abnormal hypoganglionic tissue and the normal bowel may not be obvious. The aganglionic segment is invariably narrowed in comparison to the bowel rostral to it, which often becomes massively dilated, so that another name for Hirschsprung disease is congenital megacolon. The aganglionic portion of the gut evidently functions as an obstruction causing the ganglionated bowel orad to the aganglionic segment to dilate.

Although various investigators have proposed a number of hypotheses to explain why the aganglionic tissue should be a functional obstruction, including denervation hypersensitivity of the smooth muscle and a selective deficiency of fibers able to relax the bowel [1, 2], a more general explanation is that the ENS is essential for normal propulsive intestinal motility [3]. Given the absence of the ENS from the aganglionic zone, a failure of propulsive reflexes and thus a functional obstruction are to be expected. Aside from propulsion, moreover, the net effect on intestinal muscle of the ENS is relaxant [4]; therefore, contraction and narrowing would be the predicted behavior of gut that lacks ganglia.

In thinking about the physiology of the colon in a patient with Hirschsprung disease, it is important to emphasize the difference between aganglionosis and denervation. Although the terminal bowel is aganglionic in Hirschsprung disease, it is not denervated [1, 2, 5–7]. Actually, many investigators have reported that the aganglionic gut may be hyperinnervated, especially by catecholaminergic and cholinergic nerve fibers [2, 8]. What is missing in the diseased bowel are the cell bodies of intrinsic enteric neurons, which are essential for the mediation of reflexes, not nerve fibers. Certain types of intrinsic axon are also selectively lost, including those which contain serotonin (5-HT) [9] or nitric oxide synthase (NOS) [10, 11]; however, the apparent selectivity of these deficiencies may be attributable to the fact that these are transmitters of intrinsic neurons. Given the lack of intrinsic neurons, one might expect that the transmitter of virtually any type of intrinsic neuron would be diminished. The confirmation that what is expected actually occurs is thus of limited value in understanding the pathogenesis of the disease, although a loss of relaxant fibers (such as those which contain NOS) is often invoked to explain the narrowing of the aganglionic segment as a contracted region. To understand why a loss of nerve cell bodies, despite an abundance of axons should be so devastating, it is important to consider the nature of the ENS.

3.2 The Normal Enteric Nervous System

The gastrointestinal tract is responsible for performing complex functions that are essential for host survival including (1) transport of food and waste; (2) digestion and absorption of nutrients; (3) secretion of water, electrolytes, mucus, signaling molecules, and antimicrobial substances; (4) preservation of intestinal barrier function;, (5) maintenance of healthy microbiota; and (6) protection from ingested pathogens, allergens, and toxins [12]. The regulation of these critical processes is under the control of the ENS. The mature ENS is absolutely unique and different from any other region of the peripheral nervous system (PNS). First, the ENS is independent and can function in the absence of input from the brain or spinal cord [13]. Second, in contrast to the remainder of the PNS, the ENS can mediate reflexes, even when it is isolated from the central nervous system (CNS). This ability of the ENS is often overlooked, even though it has long been known to be true. As the nineteenth century turned to the twentieth century, Bayliss and Starling reported that enteric reflexes could be mediated by "the local nervous mechanism" of the gut [14, 15]. These investigators described what they called the "law of the intestine" (now known as the peristaltic reflex) in extrinsically denervated loops of dog intestine. This is a reflex, evoked by increased intraluminal pressure, that consists of a wave of oral excitation and anal relaxation that descends in the bowel and is propulsive. Essentially, the same reflex can also be elicited in vitro in preparations of guinea pig intestine [16]. The fact that reflex activity can be manifested by segments of gut in vitro, which have clearly lost all connection to dorsal root or cranial nerve ganglia, the brain, and the spinal cord, indicates that every neural element of the peristaltic reflex arc (sensory receptors, primary interneurons, motor neurons, and effectors) must be intrinsic components of the wall of the gut.

These observations were known to J.N. Langley who first defined the autonomic nervous system [17]. Together with Langley's own idea that most enteric neurons receive no

direct input from the CNS, the independence of the ENS caused Langley to classify the ENS as a third component of the autonomic nervous system. The sympathetic division was defined as that with a thoracic and lumbar outflow of preganglionic axons from the CNS, while the parasympathetic was the division with a cranial and sacral outflow. The ENS, which mainly lacks either outflow, had to be classified as a separate division, since it met the criteria of neither of the other two. Anatomical observations have more recently confirmed the distinct nature of the enteric innervation. The internal ultrastructure of the ENS is more similar to that of the CNS than to any other region of the PNS [6–11, 18–23]. The ENS lacks internal collagen, and its neurons receive support from enteric glia; although enteric glia resemble astrocytes and Schwann cells, they are a distinct and unique cell type [24, 25]. Phenotypic diversity of peripheral neurons peaks in the ENS, and every class of neurotransmitter known to be present in the CNS is also represented in the ENS. Intrinsic neuronal reflexes evoke secretion as well as motility [26]; furthermore, most enteric neurons not only lack connection to the CNS, but some actually project centripetally, beyond the confines of the gut, to innervate extra-enteric targets [27]. These outside-the-bowel projections of enteric neurons make it possible for the ENS to affect directly the function of prevertebral sympathetic ganglia [28–30], the gallbladder [31], and the endocrine and exocrine pancreas [32, 33].

3.3 Organization of Enteric Neurons

The ENS of most adult mammals is comprised of two major interconnected ganglionated plexuses, the myenteric and the submucosal [3, 27, 34]. The myenteric plexus lies between the circular and longitudinal muscle layers of the gut. It manifests integrated neuronal activity and provides motor innervation to the circular and longitudinal muscle layers of the muscularis externa. The submucosal plexus is the smaller of the two major neural plexuses. The submucosal plexus is located between the mucosa and circular muscle of the gut within the dense irregularly arranged connective tissue of the submucosa, innervating secretory cells, endocrine cells, and blood vessels in the mucosa and submucosa. The two enteric plexuses also project to each other. In larger animals, including humans, the submucosal plexus can be divided into separate plexuses of Schabadasch (external) and Meissner (internal) [35]; however, these plexuses interconnect extensively and clear functional distinctions are not yet known. The submucosal plexus is thus usually treated as a single entity, although this practice will probably have to be changed in the future as new information accumulates that suggests a significant segregation of function to the subplexuses of Schabadasch and Meissner [36]. Submucosal neurons project to one another, to the mucosa, and to the myenteric plexus. The neurons that project to the mucosa include intrinsic sensory [37, 38] and secretomotor neurons [26, 39, 40]. Some submucosal neurons are bipolar or pseudounipolar in shape and also project to the myenteric plexus; these have been postulated to be primary afferent in function [37]. A subset of submucosal neurons, which evoke vasomotor responses when activated by mucosal stimuli, project both to the mucosa and to blood vessels [27, 41]. These cells may actually function as a unicellular reflex arc, which if true would be a structure that, in vertebrates, is unique to the bowel.

Both the submucosal and the myenteric plexuses contain many interneurons involved in interganglionic projections and the formation of complex microcircuits that are just beginning to be mapped. Motor neurons that excite or relax the muscularis externa are located exclusively in the myenteric plexus. The myenteric like the submucosal plexus [38] also contains intrinsic sensory neurons that project to the mucosa [27, 42–44]. Some myenteric neurons project centripetally out of the gut [27]. Projections that leave the bowel have been called "enterofugal" to distinguish them from the "centrifugal" extrinsic innervation, derived ultimately from the CNS, which enters the gut. Depending on the level of the bowel, targets of "enterofugal" neurons include prevertebral sympathetic ganglia (small and large intestine), the trachea (from esopha-

gus), pancreas (from duodenum and stomach), gall bladder (from stomach), brain stem (from stomach), and sacral cord (from colon). The extreme complexity of the ENS, the behaviors of the gut that it regulates, and the multitude of neurological disorders to which it contributes have only recently been appreciated [45]. Certainly, the ENS is not, as once was thought, a system of "relay ganglia" interposed between the brain and effectors in the bowel [46]. Because the ENS is so different from the other components of the PNS, it stands to reason that the factors and/or processes that dictate the development of the ENS are likely to be different from those of other peripheral ganglia [12, 34, 47].

3.4 The ENS Is Derived from the Neural Crest

The first clear demonstration that the ENS is derived from the neural crest was made by Yntema and Hammond who noted that enteric ganglia fail to appear when the "anterior" neural crest is deleted in chick embryos [48]. Their work was confirmed, and levels of the crest that contribute to the ENS were more precisely identified by Le Douarin and her colleagues [49]. These investigators took advantage of the distinctive nucleolar-associated heterochromatin of quail cells, which allows these cells to be readily identified following their transplantation into embryos of other species. Le Douarin and her co-workers replaced segments of the chick neural crest with those of quail (or the reverse) and traced the migration of crest-derived cells in the resulting interspecies chimeras by identifying cells of the donor (chick or quail, depending on the particular experiment). These studies suggested that the ENS is derived from both the vagal (somites 1–7) and the sacral (caudal to somite 28) crest. The vagal crest colonizes the entire bowel, while the sacral crest colonizes only the postumbilical gut. The conclusion that there are two sites of origin of enteric neuronal precursors was soon challenged, because other investigators could recognize only a single proximodistal progression of cells thought to be "neuroblasts" in the avian gut [50]. This progression was believed to imply that neuronal precursors in the bowel only descend, as would be expected of vagal progenitors. No ascent, of the kind predicted for precursors from the sacral crest, could be found. These observations led to the suggestion that the data derived from experiments with interspecies chimeras could have been obtained if crest-derived cells were to be more invasive in a foreign embryo than they are when they migrate in embryos of their own species. If so, then quail cells might reach ectopic destinations in a chick embryo and chick cells might behave in a similarly abnormal manner in a quail embryo. There are, however, reasons why only a single proximodistal progression of cells that can be recognized as belonging to a neuronal lineage can be detected, even though multiple levels of the crest contribute precursors to the bowel. Neuronal progenitors colonize various levels of the gut before they actually give rise to progeny that express recognizable neural properties [51]; thus, neurons develop in vitro in segments of gut that appear to be aneuronal at the time of explantation, thereby demonstrating that otherwise unrecognizable neural precursor cells were present in the explants. The delay, however short it might be, between the arrival of progenitors and their differentiation into neurons provides an opportunity for crest-derived precursors to interact with, and be influenced by, the enteric microenvironment. In fact, the enteric microenvironment plays a critical role in the development of enteric neurons and glia [34, 47, 52–54]. The observed proximodistal progression of perceived "neuroblasts," therefore, may be due to a proximodistal gradient in the maturation of the enteric microenvironment, rather than to the timing of the descent of the neuronal precursors. Subsequent studies, in which endogenous crest cells were traced by labeling them with a vital dye or a replication-deficient retrovirus, confirmed that both the avian and murine gut are each colonized by cells from both vagal and sacral levels of the neural crest [55, 56]. Most recently, studies involving lineage tracing in mice have confirmed that the ENS is derived from vagal and sacral levels of the crest [12] and, further, have shown the distal gut is

colonized by additional precursors that arrive in the bowel with the extrinsic innervation [57–59]. These latter precursors enter the gut as cells that appear to be in the Schwann lineage, but within the enteric microenvironment, they give rise to neurons. The avian and human bowel, like that of mice, thus appears to be colonized, not only by sacral and vagal crest cells but also by the Schwann cells of the extrinsic innervation [12, 60, 61]. In the mouse, studies with labeled crest-derived cells have also revealed that truncal crest contributes to the rostral-most foregut (esophagus and adjacent stomach) [62]. Retroviral and lineage tracing in avian embryos has suggested that vagal crest that contributes to the formation of the ENS is complex [12]. Crest-derived cells from somite levels 1–2 appear to give rise to neurons only in the esophagus, while those from levels 3–5 colonize the bowel from the stomach to the hindgut [12, 63]. Cells from levels 6–7 are more controversial and may go only to the hindgut [64], although labeling studies tracing cells in fetal mice with a lipophilic dye have suggested that crest-derived cells from somite levels 6–7 colonize the esophagus rather than the hindgut [62]. Crest-derived cells from somite level 3 seem to be the most invasive portion of the vagal crest and can compensate for the ablation of the remainder of the vagal crest [65].

The specificity of vagal and sacral regions as sources of enteric neuronal progenitors is well illustrated by back-transplantation experiments. Back-transplantation consists of grafting a developing organ or piece of tissue from an older to a younger host embryo. It is a technique that provides insight into whether cells in the older tissue retain and can manifest, in a suitably permissive environment, properties associated with earlier stages of development. Crest-derived cells that have colonized the bowel will leave segments of gut that are back-grafted into a younger embryo and re-migrate in their new host [66]. These cells will only reach the bowel of their host if the graft is situated so as to replace the host's vagal or sacral crest [67]. A subset of the vagal crest-derived cells that colonize the gut can be visually identified in transgenic mice directed to express *lacZ* by the promoter for dopamine β-hydroxylase (DBH) [68]. The *DBH-lacZ* transgene is permanently expressed in these mice by neurons that are not catecholaminergic in the adult gut. The colonization of the bowel by the transgenically labeled cells has been studied in detail in both normal mice and in murine models of Hirschsprung disease [69, 70]; however, it is important to note that the *DBH-lacZ* transgene probably demonstrates only a subset of vagal crest-derived cells and does not reveal those of sacral origin. Some enteric neurons develop from precursors that are transiently catecholaminergic (TC) [71–74]. DBH is one of the enzymes that participate in the formation of norepinephrine (NE), and thus its presence is a component of the catecholaminergic phenotype. Even in normal mice, and especially in rats, the genes encoding DBH are not completely repressed in the non-catecholaminergic neurons that develop from TC cell progenitors. Neurons derived from TC cells continue to express DBH, although they inactivate other elements of the catecholaminergic phenotype [72]. It is likely that the cells that are marked by the expression of the *DBH-lacZ* transgene are members of this lineage, that is, they are cells that originate from transiently catecholaminergic progenitors. Unfortunately, not every enteric neuron originates from a TC cell precursor. In fact, the subset of neurons that arises from progenitors that never exhibit catecholaminergic properties is larger than that which is TC cell-derived [74]. As a result, many enteric neuronal precursors are not subject to surveillance by the *DBH-lacZ* transgene-tracing technique. However cells are traced, it is now apparent that in both fetal mice and in avian embryos, the ENS arises from multiple regions of the neural crest, not just one [12]. Although the number of sources of enteric neurons in the neural crest is limited, it is necessary to take account of this multiplicity in attempting to explain the abnormal colonization of the gut that arises in Hirschsprung disease and other dysganglionoses.

Recent genetic studies have suggested that much of what has traditionally been considered vagal crest may not be [57]. Properties of the vagal crest are transitional between cranial and truncal [75]. Crest-derived cells next to somites 1–2 have been reported to give rise to Schwann cells that migrate to the esophagus and stomach along descending vagus nerve fibers and give rise

to the ENS in these regions [57]. These Schwann cell/neuronal precursors would literally have to be considered vagal-crest-derived, because they migrate as components of the vagus nerves. In contrast, crest-derived cells from somite levels 3–7 have been suggested to be analogous to the more distal truncal crest-derived cells and actually to be contributors to sympathetic chains; moreover, their ventral migration provides precursors, not only for sympathetic chain ganglia, but also for the ganglia of the entire GI tract. These data might account for the abnormalities that occur both in enteric and sympathetic ganglia when the gene encoding the receptor tyrosine kinase, ERBB3, is deleted [57, 76, 77]. The atrophy in half of the esophageal ganglia when the nerve-associated form of the ERBB3 ligand, neuregulin 1 (considered essential for parasympathetic neuronal development), is deleted may also be evidence that esophageal neurons are parasympathetic. As a result of these recent observations, the ENS has been postulated to have four origins: (i) crest-derived cells from levels 1–2 of the neuraxis, which reach the esophagus and stomach as Schwann cells in the descending vagus nerves; (ii) a "sympatho-enteric" component from levels 3–7 of the neuraxis, which colonize the entire remainder of the bowel; (iii) crest-derived cells from the sacral level of the neuraxis (caudal to somite 28) that contribute to the post-umbilical gut; (iv) Schwann cells that migrate into the bowel late with extrinsic nerves to the ileum and colon [59]. Further studies, perhaps including some carried out by means of live imaging of genetically labeled cell populations and new genetic tracers, are needed to verify whether this interesting new hypothesis is correct.

3.5 The Crest-Derived Cells that Colonize the Gut Are Originally Multipotent and Migrate to the Bowel Along Defined Pathways in the Embryo

The restriction of the levels of the premigratory crest that contribute precursors to the ENS raises the possibility that cells in the pre-migratory crest of these regions might be predetermined to migrate to the bowel and give rise to enteric neurons and/or glia. Such a predestination, however, is not supported by experimental evidence, which indicates instead that premigratory crest cells are multipotent. For example, when levels of the crest are interchanged so as to replace a region that normally colonizes the gut with one that does not, the heterotopic crest cells still migrate to the bowel and there give rise to neurons the phenotypes of which are ENS-appropriate, not level of origin-appropriate [78, 79]. An analogous process, moreover, is seen when the interchange of crest cells is reversed. Vagal and sacral crest cells give rise to non-enteric neurons in ectopic locations, such as sympathetic ganglia, when they are grafted so as to replace crest cells at other axial levels. Clones derived from single crest cells, furthermore, give rise, both in vitro [80–84] and in vivo [85–87], to progeny that may express many different phenotypes. A single cell that gives rise to a clone containing many phenotypes has to be multipotent.

The crest-derived cells that colonize the gut remain multipotent with respect to their ability to give rise to neurons and glia, even after they have completed their migration to the bowel. This potency is well demonstrated by back-transplantation experiments (similar to those described above). Again, back-transplantation involves the transplantation of tissues or organs into younger embryos. When already colonized segments of gut are back-transplanted into a neural crest migration pathway of a younger embryo at a truncal level, donor crest-derived cells leave the graft, but they do not migrate to the host's gut. Instead, they migrate to the host's sympathetic ganglia, adrenal gland, and peripheral nerves which are the classical targets of the truncal crest; moreover, despite their previous migration to and residence in the bowel, the donor crest cells now form catecholaminergic neurons in the ganglia, chromaffin cells in the adrenals, and Schwann cells in the nerves of the host embryos [66]. Analogous results have been obtained from in vitro studies of cells developing from cloned crest-derived cells of enteric origin. The progeny found in these clones express a variety of different phenotypes, including some that are not present in the normal

ENS [88]. Despite their multipotent nature, however, the developmental potential of enteric crest-derived cells in vivo [66] and in clonal culture is not as great as that of their progenitors in the premigratory crest [88, 89]. The pluripotency of the crest-derived cells that colonize the gut, revealed by studies of clones and the behavior of cells emigrating from back-transplants [67], indicates that the bowel does not become colonized by precursors from restricted regions of the neural crest because these regions are the only ones that contain crest cells endowed with homing information that programs them to migrate to the gut. Instead, these regions are the only levels of the crest from which there are defined migratory pathways that lead to the bowel.

The pathway from the vagal crest (taking the latest definition as "vagal" and including the critical axial level of the third somite) conveys the largest cohort of crest-derived emigres to the gut. In avian embryos, this migration pathway leads crest-derived cells to the entire bowel between the proventriculus and the cloaca. In mammals, the equivalent region would extend from the corpus of the stomach to the rectum. The cohort of crest-derived cells that follows the sacral pathway is much smaller and leads crest-derived emigres only into the portion of the bowel that is distal to the umbilicus. The cohort of crest-derived cells that follow the pathway that leads to the presumptive esophagus and the most rostral portion of the stomach is the smallest. The origin of this pathway is controversial; it may be rostral crest [57, 90] or truncal crest [62]. The possibility that crest-derived cells of different origins are intrinsically different has some experimental support. It is also conceivable that the crest-derived emigres from different levels interact with one another during the formation of the ENS.

The molecular nature of the migratory pathways and the nature of the mechanisms that guide progenitors to their correct destinations within the gut itself have yet to be fully understood. Chemoattractant or repellent molecules for growing axons have been identified in the vertebrate CNS [88]. These molecules include netrins [88, 91–93], semaphorins [94–96], and slit proteins [97]. The directional growth of migrating crest-derived cells is a property also shown by pathfinding axonal growth cones [98, 99]. Netrins are expressed in the developing bowel [91] and mice with a targeted mutation in netrin-1 die at birth with a bloated bowel and no milk in their stomach (Tessier-Lavigne, personal communication). Netrins play a role in the formation of the submucosal plexus [100]. There are radial secondary migrations of crest-derived cells, perpendicular to the longitudinal axis of the gut, that lead these cells out of the distal-directed stream of migration toward the enteric mucosa and the pancreas. These secondary migrations give rise, respectively, to submucosal and pancreatic ganglia. Netrins help to guide these secondary migrations. Netrin-1 and netrin-3 in mice and netrin-2 in chicks are expressed by the epithelia of fetal mucosa and pancreas. Crest-derived cells express the netrin receptors, deleted in colorectal cancer (DCC), neogenin, and adenosine A2b. Of these receptors, DCC, which is developmentally regulated, seems to be most important. Crest-derived cells migrate out of explants of gut in vitro toward co-cultured cells that express netrin-1. Enteric crest-derived cells also migrate in vitro toward co-cultured explants of pancreas and, in rings of cultured gut, inwardly toward the mucosa. Antibodies to DCC and inhibitors of protein kinase A, which interfere with DCC signaling, specifically block these migrations of crest-derived cells in the direction of the mucosa or pancreas. Mice that lack DCC also lack submucosal and pancreatic ganglia. Netrins, moreover, promote the survival/development of enteric crest-derived cells in addition to guiding their migration. These data strongly support the idea that netrins and DCC participate in the formation of submucosal and pancreatic ganglia. An important question, not answered by these data, is why crest-derived cells migrating toward the netrin that mucosal epithelial cells secrete stop migrating in the submucosa and fail to invade the mucosa itself. Part of the answer is that laminin, which is abundant in the fetal enteric mucosa, converts the netrin/DCC effect from attraction to repulsion [101]. Mucosal laminin repels advancing crest-derived cells. More recently, netrins have been found to be expressed within neurons

of the ENS and to attract vagal axons [102]. The sensory vagal innervation of the gut, moreover, appears to depend on the presence of intrinsic neurons within the bowel; sensory vagal axons grow away from explants of aganglionic ret-/- gut but toward explants of wild-type gut. It is thus possible that enteric neurons help to guide vagal sensory axons to the bowel. Such a role would help to explain the known termination of vagal sensory axons within the ganglia of the ENS.

New mouse genetic tools have formally confirmed that crest-derived precursors of neurons migrate from the myenteric to the submucosal plexus [103]. The *Confetti* transgene was used to map the fates in adult small intestines of individual crest-derived precursors that express Sox10 at E12.5 [103]. Most of the cells that were clonally related formed columns along the radial axis of the gut. Individual *Sox10*-expressing precursors differentiated into clones of neurons, glia, or both (called NG clones). Each type of clone participated in forming the myenteric plexus; however, but only glial and NG clones added to the submucosal plexus. Observations suggest that the cells that migrate from the myenteric to the submucosal plexus are bipotent. In the adult gut, moreover, neurons that were clonally related tended to display synchronous Ca^{2+} responses when stimulated with single electrical pulses, suggesting that developmental relationships are preserved in the functional wiring of the ENS [103]. Enteric neuronal subtypes are born in a reproducible phenotypic order [104], and subsets of neurons with similar birthdates may be more likely than cells with dissimilar birthdates to connect to one another. At least one additional factor is critical in the radial migration, at least of enteric glia. These cells respond to the presence of the enteric microbiota and are attracted toward the lumen as they migrate from their origin in the layer of the presumptive myenteric plexus [105, 106]. The extent to which lineage relationships and timing of phenotypic determination contribute to enteric circuit formation has not yet been explored.

Vagal crest-derived cells are clearly different from the sacral crest-derived cells that migrate to the bowel. Crest-derived cells from somites 8 and below express roundabout (ROBO) receptors; SLIT2, however, is expressed in the proximal gut [107, 108]. Slit proteins are repellent ligands for ROBO receptors. This distribution would allow vagal crest-derived cells, which fail to express ROBO to colonize the entire bowel (as they do) but would prevent the ascending stream of sacral crest-derived cells from entering the SLIT2-expressing rostral portion of the gut.

3.6 Differentiation of Crest-Derived Precursors Within the Enteric Microenvironment

No matter where in the crest enteric neuronal/glia precursors originate, after they colonize the bowel, the cells have to continue to proliferate to maintain an adequately sized precursor pool while at the same time giving rise to neurons. Because neurons are post-mitotic, their very formation necessarily diminishes the size of the proliferating precursor pool. The pool thus has to maintain itself even while engaged in an activity, neuronal differentiation, that threatens to deplete it. This paradoxical task, moreover, has to be solved as the crest-derived precursors move along the long routes of migration that lead from the rostral to the distal gut, into the pancreatic buds, and from the myenteric to the submucosal plexus. Proliferation at the colonizing wavefront is critical to keeping the mass of migrating cells sufficiently large to allow the population to keep on migrating [65, 109, 110]. If crest-derived precursors differentiate too quickly or prematurely into neurons, the distal bowel will not be adequately colonized [111–113].

Crest-derived cells encounter many molecules that influence their differentiation as they travel to their final destinations, and the cells must be endowed with receptors that enable them to respond to the molecules they encounter. Louis Pasteur said that “chance favours the prepared mind,” and in an analogous fashion, signaling molecules favor the receptor-bearing crest-derived cell. Profiles of transcripts that cells *Sox10* marks at two times during murine ENS ontogeny have identified over 150 signaling molecules and

receptors that developing enteric crest-derived precursors and the surrounding mesenchyme express [114]. Pathways that influence the development of the ENS are thus complex and have abundant molecular constituents; nevertheless, enteric crest-derived precursors express three regulatory molecules that are of central importance in preparing them to be enteric neurons or glia. These are the homeodomain paired-like homeobox 2B (PHOX2B) transcription factor, the Sry-related HMg-Box gene 10 (SOX10) transcription factor, and rearranged during transfection (RET) receptor tyrosine kinase.

When PHOX2B is deleted, vagal crest-derived precursors enter the foregut appropriately but fail to give rise to an ENS; expression of PHOX2B thus appears to be necessary for the subsequent expression of all other molecules critical for ENS development including RET and the transcription factors SOX10 and achaete-scute homologue 1 (ASCL1) [115, 116]. Loss-of-function mutations in human *PHOX2B* are not embryonically lethal but cause congenital central hypoventilation syndrome (CCHS), which in 16% of affected individuals is associated with Hirschsprung disease (HSCR) [117, 118]. PHOX2B is thus so important that even its haploinsufficiency can disturb ENS development. The risks for HSCR and neuroblastoma (a neural crest-derived tumor) are especially great in patients with CCHS who harbor a nonpolyalanine repeat expansion mutation in PHOX2B [116]. When a nonpolyalanine repeat expansion mutation of the PHOX2B was introduced into the mouse Phox2b locus, the clinical features of the association of CCHS with HSCR and neuroblastoma were recapitulated. Enteric and sympathetic ganglion precursors demonstrated sustained expression of Sox10. Proliferation of crest-derived precursors was impaired and their differentiation was biased against neurons and toward glia. Phox2B transactivation of target genes was affected in a dominant-negative fashion and the transcriptional effect of PHOX2B on a Sox10 enhancer was converted from repression to transactivation. The nonpolyalanine repeat expansion mutation of PHOX2B thus acts both as a dominant-negative and gain-of-function mutation. The ability of PHOX2B to regulate SOX10 is thus critical in development of the ENS and other autonomic ganglia.

SOX10, which is expressed by virtually every crest-derived cell as it delaminates from the neural tube, is nearly as fundamental to ENS development as PHOX2B. The initial expression of SOX10 is independent of PHOX2B. Once in the bowel, precursors need to express SOX10 in order to survive and maintain their multipotency. One of the first manifestations of the importance of SOX10 to the developing ENS to be realized came from the study of a naturally occurring autosomal dominant mutation in mice (*Dom*) that caused a pronounced hypoplasia of myenteric neurons, terminal bowel aganglionosis, and congenital megacolon [119]. *Dom* was subsequently found to be located in the *Sox10* locus [120, 121]. Even the haploinsufficiency of *Sox10* is enough to lead, in mice, to a hypoganglionic colon and megacolon [122]. *SOX10* haploinsufficiency in humans is associated with the HSCR-like aganglionosis of Waardenburg-Shah syndrome [123].

SOX10-expressing cells colonize the bowel and must maintain SOX10 expression. That allows them to survive and remain multipotent as they initiate their expression of PHOX2B and another gene important to a subset of enteric neurons, ASCL1 [124, 125]. Interestingly, as crest-derived precursors lose potency and differentiate, SOX10 expression is retained as the precursors generate glia; SOX10 expression thus becomes a glial marker in the adult ENS [122, 124]. Downregulation of SOX10 expression appears to be important in enteric neurogenesis [125]. Genetic lineage tracing has recently been employed to investigate enteric crest-derived cells in *Sox10*$^{Dom/+}$ mice [126]. Varying lengths of distal aganglionosis was found in the colons of *Sox10*$^{Dom/+}$ mice; nevertheless, the ENS of the small intestines of the *Sox10*$^{Dom/+}$ animals contained normal numbers of neurons and glia [126]. There were, however, abnormalities in the types of neurons generated in *Sox10*$^{Dom/+}$ mice and associated deficits in gastrointestinal motility [126]. It is possible that the extreme sensitivity of the terminal colon to SOX10 haplo-

insufficiency is due to the role that SOX10 plays in maintaining survival and multipotency of the colonizing stem cells. Premature depletion of the stem population (due to precocious differentiation) in the absence of adequate SOX10 leads to a failure to colonize the last (most distal) portion of the bowel. Surprisingly, however, SOX10 evidently also plays a role in specifying small intestinal neuronal subtypes. It is interesting to speculate that defects in the types of cells that populate enteric ganglia may occur as a result of diminished activity of SOX10 or developmentally active factors and give rise to an unsuspected dysmotility of the bowel. Alternatively, these defects of the proximal ganglionated bowel might be the cause of residual dysmotility of the gut that is frequently seen after the surgical correction of HSCR.

The *Ret* protooncogene is a gene upon which most enteric neurons are critically dependent for survival [62, 127, 128]. This gene encodes the third member of the trio of crucial regulators of ENS development, RET, a receptor tyrosine kinase, for which glial cell line-derived growth factor (GDNF) is a functional ligand [129–131]. GDNF was first identified as a factor, produced by a glial cell line (B49) that promotes the survival of midbrain dopaminergic neurons [132]. GDNF was later observed to enhance the survival of spinal motor neurons [133]. GDNF is a distant relative of transforming growth factor-β (TGF-β). It is a homodimer, consisting of two peptide chains of 134 amino acids linked by a disulfide bridge. A larger precursor of 211 amino acids is synthesized first. This big molecule is proteolytically cleaved intracellularly to produce mature GDNF, which is secreted. During development, GDNF is not restricted to the brain, but rather is very highly expressed in the gut and other peripheral organs [133, 134]. In keeping with its peripheral distribution, GDNF is not just a survival factor for central CNS neurons [130], but also enhances the in vitro survival of peripheral sensory and sympathetic neurons, and also promotes their extension of neurites [133]. The observation that GDNF affects sympathetic neurons suggests that it should also affect at least some neurons of the ENS. In fact, both enteric and sympathetic neurons express *Ret*, at least transiently [127, 135]. When *Ret* is knocked out in transgenic mice, the ENS totally fails to develop in the entire bowel, with the exception of the rostral foregut [62, 128]. Since Ret is a functional receptor for GDNF, the fact that a similar lesion occurs in the bowel of knockout mice lacking GDNF [136–138] is not surprising. Neither is the observation surprising that, in contrast to the trophic effects that GDNF exerts on autonomic neuroblasts from control mice, GDNF fails to exert trophic effects on analogous cells from *Ret*−/− animals [131]. Activation of the Ret receptor by GDNF is thus a critical event in the formation of the ENS. Actually, GDNF does not bind directly to the Ret receptor itself. Instead, GDNF binds to a glycosylphosphatidylinositol-linked cell surface protein called GFRα-1, which then complexes with Ret to trigger the autophosphorylation and other actions of Ret [129, 139]. Other growth factors, which are members of the GDNF family of ligands, can also associate with their preferred GFRα proteins to activate Ret [140, 141]. These growth factors include neurturin (GFRα-2), artemin (GFRα-3), and persephin (GFRα-3). Of these, only the combinations of GDNF/GFRα-1 and neurturin/GFRα-2 appear to be important in ENS development [142–144]. Because of its role in GDNF signaling through Ret, GFRα-1 is as essential for ENS (and kidney) development as Ret and GDNF [145].

Despite the fact that most of the bowel is aganglionic in *Ret*−/− mice [128], there are neurons in the portions of the gut that develop from the rostral foregut of these animals [62]. Although the superior cervical ganglion is missing in *Ret*−/− mice, most other sympathetic ganglia do develop. The crest-derived cells that colonize the rostral foregut and the superior cervical ganglion have been traced by injecting a fluorescent dye (DiI) that intercalates into the lipid of the plasma membrane. The DiI-labeled cells that colonize the presumptive esophagus and rostral stomach originate from the same pool of truncal crest cells that gives rise to the sympathetic chain ganglia below the superior cervical ganglion. In contrast, the post-otic vagal crest cells that colonize the entire bowel distal to the

rostral foregut also contribute the crest-derived cells that form the superior cervical ganglion. There thus appears to be not one but two common sympathoadrenal-enteric lineages. One of these is *Ret*- and GDNF-dependent, while the other is *Ret*- and GDNF-independent. The bulk of the ENS is constructed of cells in the *Ret/*GDNF-dependent sympathoadrenal-enteric lineage, which evidently also gives rise to the superior cervical ganglion. The *Ret/*GDNF-independent lineage forms the ENS of the rostral foregut and the entire sympathetic chain, except for the superior cervical ganglion. The *Ascl1*-dependent and *Ret*-dependent lineages seem superficially to be opposite sides of a single coin [62]. For example, the ENS of the esophagus, which is totally *Ascl-1*-dependent, happens to be the region of the gut that is *Ret*-independent. In contrast, the ENS of the bowel below the proximal stomach is totally *Ret*-dependent; yet it contains neurons in *Ascl-1* knockout mice. Still, as noted above, there is no region of the ENS that is completely *Ascl-1*-independent. Although there are neurons in the intestines of *Ascl-1* knockout mice, TC cells and all the neurons derived from TC cells are missing. Still to be explained as well is why the presumably *Ret*-independent crest-derived cells of the rostral foregut do not migrate distally in the bowel of *Ret*−/− mice (or mice lacking GDNF). Possibly, the evident inability of the *Ret*-independent cells of the rostral foregut to expand their territory in *Ret*−/− mice is due to an inhibition of their migration. Consistent with this possibility, GDNF signaling has been shown to attract and guide RET-expressing enteric crest-derived cells as they migrate proximo-distally down the developing bowel [146]. GDNF is expressed at high levels early in the mesenchyme of the primordial stomach. As development proceeds, the locus of highest GDNF development moves anally ahead of the front of migrating crest-derived cells until the caecum is reached. Distal progression of GDNF then ceases and the caecum becomes a stable site of high mesenchymal GDNF expression. These observations are consistent with the idea that GDNF acts as a guidance molecule for RET/GFRα-1-expressing cells and is at least partly responsible for the descent of emigres from the vagal crest. A difficulty with this concept is that the accumulation of GDNF in the caecum would trap the migrating crest-derived cells in this segment of the gut. An additional factor has to be postulated that enables enteric crest-derived cells to free themselves from the attraction to GDNF and migrate out of the caecum. Endothelin 3 (EDN3) signaling via the endothelin-B receptor (EDNRB) has been postulated to play this role [147, 148]. Alternatively, all enteric neurons may be GDNF/Ret-dependent but able to survive in the rostral foregut, despite the absence of GDNF or Ret, because a compensatory factor (currently unknown) is expressed only in this region of the bowel.

During development of the ENS, GDNF plays an essential role in survival, proliferation, migration, and neuronal differentiation. Recently, McKeown et al [149] showed that GDNF enhances the ability of enteric neural progenitors to grow as enteric neurospheres and to migrate and generate an ENS. Exposure to GDNF, furthermore, resulted in a 14-fold increases in neurosphere volume and a 12-fold increase in cell number. The GDNF/RET interaction thus shows promise for the future as approaches are sought to replace an ENS when that becomes a clinical necessity.

3.7 The Development of the ENS Is Influenced by a Neurotrophin

For a long time, neurotrophins were thought to play little or no role in the development of the ENS. Unlike developing sensory and sympathetic ganglia, explanted enteric neurons can be cultured without nerve growth factor (NGF) or even in the presence of neutralizing antibodies to NGF [150, 151]. Neuritic outgrowth from organotypic cultures of gut, moreover, is not stimulated by NGF. Autoantibodies to NGF produce severe sensory and sympathetic defects in the progeny of immunized animals [152, 153]; nevertheless, the same autoantibodies to NGF do not induce ENS lesions. These observations, however, suggest only that the development of the ENS is

independent of NGF, not that the ENS does not require the action of any neurotrophin. NGF was the first neurotrophin to be discovered and the studies outlined above were carried out before the existence of other neurotrophins became known. NGF, together with brain-derived neurotrophic factor (BDNF), neurotrophin- 3 (NT-3), NT-4/5 [115, 154, 155], and NT-6 [156] are members of a family of small, very basic proteins. Each of these neurotrophins is able to interact independently with a common receptor, p75NTR, and with a specific Trk receptor tyrosine kinase, TrkA for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. At higher concentrations, the neurotrophins become somewhat promiscuous and activate Trks other than their primary receptor. NT-3, for example, activates TrkA and TrkB, but it binds to those receptors with an affinity that is lower than its affinity for its natural ligand, TrkC, or that of NGF or BDNF for TrkA or TrkB, respectively.

The common neurotrophin receptor, p75NTR, regulates many cellular functions [157]. These include an enhancement of the affinity of Trks for their neurotrophins, increasing the rate at which NGF binds to TrkA, and improving the specificity of Trk receptors by decreasing their receptivity to activation by the wrong neurotrophin. p75NTR also affects apoptosis, axonal growth and degeneration, cell proliferation, myelination, and synaptic plasticity [157]. The kaleidoscopic multiplicity of cellular functions that p75NTR governs stems from the equivalent multiplicity of the ligands and co-receptors that associate with p75NTR and modulate its signaling. Survival is promoted through the interactions of p75NTR with Trk receptors; inhibition of axonal regeneration through interactions with the Nogo (Nogo-R) and Lingo-1 receptors, apoptosis is promoted through interactions of p75NTR with sortilin. Signals downstream of the interactions of p75NTR and its various partners are additionally modulated by regulated intramembrane proteolysis of p75NTR and by interactions of the receptors with a wide variety of cytosolic partners.

The first observation to suggest that one or more neurotrophins probably are important in the formation of the ENS was the discovery that the common neurotrophin receptor, p75NTR, is expressed by the crest-derived cells that colonize the fetal mouse and rat gut [71, 72]. The cells that express p75NTR give rise to neurons and glia in vitro [73]. Antibodies to p75NTR specifically immunoselect crest-derived cells from the fetal bowel [158, 159]; moreover, almost no cells able to give rise to neurons or glia remain in dissociated cell populations after p75NTR-expressing cells have been removed by immunoselection. These observations suggest (but do not prove) that all, and not just some, of the crest-derived cells that colonize the gut express p75NTR. No marker has yet been found that reveals a greater number of enteric crest-derived neural precursors than p75NTR. Although p75NTR may not be required for stimulation of cells by a neurotrophin, which can activate a specific Trk, p75NTR is commonly expressed by cells that are neurotrophin-responsive. The fact that enteric neuronal precursors express p75NTR, however, is not the only reason to believe that a neurotrophin plays an important role in the development of enteric neurons and/or glia. The concept that at least one lineage of enteric neurons arises from a common sympathoadrenal-enteric progenitor [57, 90, 160] suggests that at least the enteric neurons of this lineage should share the neurotrophin dependence of their sympathoadrenal equivalents. Sympathetic neural precursors are not at first NGF-dependent [161–164]. Instead, they are supported by NT-3 before they respond to, and become dependent on, NGF [161, 162]. This change in neurotrophin responsivity and dependence is matched in sympathetic neural precursors by a change from TrkC to TrkA expression [162, 163, 165]. This switch in receptor expression may occur spontaneously [165], or it may require the exposure of cells to NT-3 [161]. NT-3 thus promotes the development of sympathoadrenal precursors [161, 163]; moreover, both the knockout of NT-3 in transgenic mice [166, 167] and the administration of neutralizing antibodies to NT-3 impair the normal development of sympathetic neurons [168]. Excessive apoptosis of sympathoadrenal neuroblasts occurs when NT-3 is absent during development [169]. If the enteric neurons that arise from a common sympathoad-

renal-enteric progenitor were to diverge from the common lineage before TrkA and NGF dependence are acquired, then the evident NGF independence of virtually all enteric neurons could be explained. In this model, the acquisition of NGF dependence would be considered, for sympathetic neurons the time when their progenitors diverge from the common lineage. Acquisition of NGF dependence would also be an event that does not occur in the enteric microenvironment, where the successors of TC cells lose their catecholaminergic properties and acquire other, gut-specific, phenotypes. Since NT-3 plays such an important role in the early development of sympathoadrenal cells, NT-3 might be expected to play a similar role in the development of those enteric neurons that are derived from the common sympathoadrenal-enteric lineage. NT-3 would be predicted to affect the enteric neuronal progenitors during the predivergent phase, when they share properties with sympathetic neural precursors. Clearly, the logic of this argument suggests that NT-3 would support the development of the subset of enteric neurons that is derived from the *Ascl-1*-dependent TC cells (the common sympathoadrenal-enteric progenitor). What the argument does not suggest is that NT-3 or any other neurotrophin is likely to exert a global effect similar to that of GDNF. GDNF stimulation of the Ret receptor appears to be critical at a very early stage of development, so that the loss of precursor cells that are GDNF/Ret-dependent results in the total failure of both neurons and glia to arise in the affected region of the bowel. The idea that NT-3 is the critical neurotrophin in enteric neuronal development is supported by the observations that TrkC is expressed by enteric neurons, where both full-length and truncated forms of the receptor can be detected in newborn mice [170] and fetal rats [159, 171]. Transcripts encoding TrkC have been shown by in situ hybridization to be located in the developing and mature ENS [171, 172]. mRNA encoding the full-length TrkC (containing a kinase domain) is enriched in purified populations of crest-derived neural and glial precursor cells immunoselected from the fetal rat bowel [159]. NT-3 binding to both full-length and truncated forms of TrkC has been detected in the E13.5 chick gut [173], although affinity labeling has not revealed the presence of significant amounts of NT-3 binding to TrkC in the bowel of newborn mice [173]. NT-3, as well as TrkC, is expressed in the developing gut [174]. The expression of *lacZ* driven by the NT-3 promoter in transgenic mice has enabled cells that express NT-3 to be located and identified in the fetal bowel [174]. The cells that express NT-3 are located in the outer gut mesenchyme of fetal mice. The outer gut mesenchyme is the layer of the bowel within which myenteric ganglia arise, suggesting that NT-3 is secreted in situ, where it can reach and affect TrkC expressed by developing enteric neuronal precursors and/or neurons. NT-3 expression has not been detected in the submucosa. The development of submucosal neurons follows that of myenteric neurons [175, 176] and all submucosal neurons are born late [177]. The neurons of the submucosal plexus, therefore, probably are not derived from the *Ascl-1*-dependent TC cell lineage, which gives rise only to neurons, such as serotonergic cells (of which there are none in the submucosal plexus) that are born early [74]. These considerations are consistent with the idea that a subset of enteric neurons, most likely the *Ascl-1*-dependent TC cell lineage, are affected by NT-3.

3.8 NT-3 and Bone Morphogenetic Proteins Promote the Development of Enteric Neurons

A major breakthrough, which has enabled the effects of growth factors on the development of enteric neurons or glia to be studied in vitro, has been the development of a means of isolating crest-derived cells from within the wall of the fetal bowel. If crest-derived cells are not so isolated, then the direct actions of growth factors on crest-derived neural and/or glial precursors cannot be distinguished from indirect effects of these molecules on other cells of the enteric mesenchyme. The isolation of enteric crest-derived cells takes advantage of the phenomenon that these cells express cell-surface differentiation antigens or

markers that are not expressed by non-neuronal cells of the gut wall. Antibodies to these cell surface antigens are utilized for immunoselection of the crest-derived cells. The first differentiation antigen used for the immunoselection of crest-derived cells from the fetal gut of chicks and rats was a protein recognized by HNK-1 monoclonal antibodies [158, 159]. Since then, p75NTR [140] and Ret [89] have each been employed with good effect. In general, the fetal gut is dissociated and the separated cells are incubated with primary antibodies, which selectively decorate the surfaces of the crest-derived cells. The antibody-labeled cells can then be immunoselected with secondary antibodies coupled to magnetic beads, and eventually isolated with a magnet [158, 159]. Alternatively, the primary antibody-labeled cells can be identified with fluorescent secondary antibodies and isolated with a cell sorter [89] or by manual selection [88, 178–180]. The non-immunoselected cells proliferate much more than do the immunoselected crest-derived cells. The crest-derived precursors that colonize the gut are still dividing when they arrive in the bowel [71, 110, 177, 181]; however, crest-derived cells withdraw from the cell cycle when they give rise to neurons. In contrast, the non-neuronal cells of the residual population do not give rise to cells that become postmitotic and thus continue to divide in vitro. The ability of isolated populations of crest-derived cells, immunoselected from the fetal rat gut, to differentiate into neurons and glia is promoted by NT-3 [159, 182]. In contrast to the immunoselected cells, NT-3 has no effect on crest-depleted populations of cells that remain after the crest-derived cells have been removed by immunoselection. In these experiments, it is necessary to identify cells as neurons or glia by demonstrating chemical markers, because the morphological appearance of the cells in culture can be misleading. Neurons can be identified by the expression of the immunoreactivity of specific marker proteins, such as HuC/D [183–186]. Glia can be identified by the expression of the immunoreactivity of markers such as glial fibrillary acidic protein (GFAP), S100, and proteolipid protein 1 (PLP1), which, in contrast to GFAP, is expressed by all enteric glial cells [24, 25]. The ability of NT-3 to promote neuronal and glial development is concentration-dependent and is maximal at 40 ng/ml. In addition to promoting the development of enteric neurons and glia, NT-3 enhances neurite outgrowth, but it is not mitogenic. Similarly, NT-3 does not induce dorsal root ganglion cell precursors to proliferate; on the contrary, when administered early in ontogeny, NT-3 causes sensory neurons to differentiate prematurely, thereby reducing their ultimate numbers [187]. NT-3 thus exerts an effect on the postmigratory crest-derived cells that colonize the bowel and dorsal root ganglia that is different from its action on premigratory crest cells, which are stimulated to proliferate by NT-3 [188, 189]. The action of NT-3 on immunoselected cells, in common with the effects of most growth factors, is associated with the transient induction of the *c-fos* protooncogene in responding cells [159]. Other neurotrophins, such as NGF, BDNF, and NT4/5 affect neither the in vitro development of neurons and glia in populations of immunoselected cells, nor the in vitro proliferation or differentiation of the non-immunoselected cells. NT-3 thus specifically promotes the in vitro differentiation of crest-derived cells as enteric neurons and glia, and may be the only neurotrophin that can do so. Although a physiological role for NT-3 in the normal development of the ENS has not yet been identified, NT-3 has been shown to be able to affect the development of enteric ganglia in vivo. The DBH promoter has been used to direct the overexpression of NT-3 in the developing ENS. When this is done, the myenteric plexus of the small and large intestines of the DBH/NT-3 transgenic animals becomes hyperplastic. There are significant increases in the number of neurons/ganglion, the number of neurons per unit length of gut, the packing density of neurons within ganglia, the proportion area of ganglia, and the size (maximal diameter and volume) of individual neurons. In contrast, none of these parameters are changed in the submucosal plexus and there is no change in the numbers of CGRP-containing neurons (the majority of which are submucosal). CGRP-containing neurons are the latest-born of enteric neurons and are derived from cells in the *Ascl-1*-independent lineage [74, 177]. In fact, the entire set of submucosal neurons tends to be born late. These findings suggest that

the late developing *Ascl-1*-independent lineage of enteric neurons is probably not affected by the DBH/NT-3 transgene. Both the myenteric hyperplasia and the increase in neuronal size induced by the overexpression of NT-3 in transgenic mice are thus probably due to a response of the *Ascl-1*-dependent precursor lineage.

An ENS is present in mice that lack NT-3 or TrkC despite the ability of NT-3 to promote enteric neuronal development in vitro [190]. The mere presence of an ENS and the survival of an animal do not, however, demonstrate that the ENS is either normal or complete. Further analysis of mice lacking NT-3 or TrkC demonstrated abnormalities of subsets of enteric neurons. After isolated enteric neurons are exposed to NT-3, the TrkC-expressing subset becomes NT-3 dependent and undergoes apoptosis upon NT-3 withdrawal. Function blocking antibodies to NT-3 inhibit neuronal development in mixed cultures of crest- and non-neural crest-derived cells but do not do so in cultures containing only crest-derived cells isolated from the fetal bowel; therefore, the endogenous source of NT-3 for the support of enteric neuronal development is probably the non-crest-derived mesenchymal cells of the gut wall. Retrograde transport of (125)I-NT-3 reveals the locations and projections of NT-3-responsive neurons in the adult bowel. The submucosal plexus contains NT-3-responsive neurons that project to the mucosa, while the myenteric plexus contains NT-3-responsive interneurons and neurons that innervate distant ganglia, the tertiary plexus and smooth muscle. The numbers of neurons in both plexuses in mice lacking NT-3 or TrkC are less than those of wild-type animals. The neuropoietic cytokine (CNTF) enhances the effect of NT-3 in vitro and can prevent apoptosis of neurons upon NT-3 withdrawal. NT-3 is thus required for the development of a normal ENS although its affects are not as global as those of GDNF.

Another set of regulatory molecules that are important in ENS development is the set of bone morphogenetic proteins (BMPs) [191]. These molecules play a major role early in the morphogenesis of the primordial bowel and are critical in the regulation of mucosal stem cells [192]. Expression of BMP-2 and BMP-4, BMPR-IA (BMP receptor subunit), BMPR-IB, and BMPR-II, and the BMP antagonists, noggin, gremlin, chordin, and follistatin are all expressed in the fetal gut when neurons can first be detected. When applied to crest-derived cells immunopurified from the fetal mouse intestine at E12, moreover, BMP-2 and BMP-4 induce translocation of phosphorylated Smad-1 from cytosol to nucleus, suggesting that these cells are responsive to BMP signaling. Low concentrations of the same BMPs promote neurogenesis from isolated enteric crest-derived cells in vitro, while high concentrations impede neurogenesis because they drive many cells to apoptosis. The BMPs also cause the precocious expression of TrkC in neurons as well as their dependence on TrkC for survival. BMPs synergize with GDNF in enhancing neuronal development; however, the promotion of neuronal development depletes the pool of proliferating precursor cells. Interestingly, when the actions of BMPs 2 and 4 are inhibited by overexpressing noggin in the developing ENS of transgenic mice under the control of the neuron-specific enolase promoter, neuronal numbers in both enteric plexuses are increased throughout the postnatal gut. In contrast to the overall increase in total numbers of neurons, the specific set of neurons that express TrkC are decreased. BMP-2 and/or BMP-4 thus limit the size of the ENS but enhance the development of specific subsets of enteric neurons, such as those that express TrkC. The BMPs thus appear to be differentiating factors that enhance phenotypic expression at the expense of precursor proliferation. These observations of BMP signaling during murine ENS development have been confirmed and extended to the ENS of the avian hindgut [193]. Again, BMP-2, BMP-4, and BMPR-II are strongly expressed in the ENS during hindgut development. The phosphorylated Smad1/5/8 proteins are present in the enteric ganglia, suggesting ongoing BMP signaling. Inhibition of BMP within the developing gut inhibits the normal migration of crest-derived precursor cells and also causes hypoganglionosis and failure of clustering of neurons into ganglionic aggregates.

Evidence that BMPs affect not only neurogenesis and gliogenesis but also migration of crest-

derived precursors has come from studies of the neural cell adhesion molecule (Ncam1) [194]. BMP signaling has been found to restrict murine ENS precursors to the outer bowel wall during migration; moreover, inhibition of BMP signaling accelerates colonization of the murine colon but diminishes the formation of ganglionic aggregates and neurite fasciculation. The migration of crest-derived cells through the bowel and the fasciculation of neurites may be related to a BMP-enhanced addition of polysialic acid to Ncam1. Enzymatic removal of polysialic acid from Ncam1 blocks the effects of BMPs on migration of enteric crest-derived cells and the fasciculation of neurites. Additional insight supports a role for BMP regulation of sialyltransferases during ENS development [195]. Transcripts encoding the sialyltransferases, ST8Sia IV (PST) and ST8Sia II (STX), which polysialylate Ncam, have been detected in fetal rat gut by E12 and are downregulated postnatally. Numbers of neurons that express Ncam1with polysialic acid, but not those that express just Ncam, are similarly developmentally regulated. Circular smooth muscle transiently (E16-20) expresses Ncam with polysialic acid at the time when it is traversed by migrating crest-derived cells migrating from the myenteric to the submucosal plexus, which they do under the guidance of netrins [100]. Neurons developing in vitro from crest-derived cells immunoselected at E12 express both Ncam and Ncam polysialic acid [195]. BMP-4 promotes the addition of polysialic acid to neuronal Ncam and the clustering of neurons into ganglionic aggregates. N-butanoylmannosamine, which antagonizes addition of polysialic acid to Ncam, but not N-propanoylmannosamine, which does not, blocks BMP-4-induced formation of ganglionic aggregates. BMP signaling is thus critical in the addition of polysialic acid to Ncam. This process evidently allows crest-derived precursors to migrate and form ganglionic aggregates during ENS development.

Although the migration, differentiation, and survival of enteric neurons during development are reliant on a variety of trophic factors, relatively little is known about the mechanisms that regulate the maturation of enteric neurons in postnatal life. Still, it is now clear that enteric neurons continue to arise in the adult gut [196–198]. One molecule that the maturation of enteric neurons and glia require is a transcriptional cofactor, homeodomain interacting protein kinase 2 (HIPK2) [199]. Deletion of HIPK2 causes distention of the colon and slowing of GI transit. Curiously, loss of HIPK2 does not affect enteric neurons in prenatal life; however, in Hipk2-/- mice, there is a progressive postnatal loss of enteric neurons. Enteric dopaminergic neurons are lost preferentially. The action of HIPK2 in postnatal ENS development is intertwined with the response of enteric neuronal BMPs. The proportion of enteric neurons in Hipk2-/- mutants that have a high level of phosphorylated Smad1/5/8 is greater than that in wild-type animals. Smad protein 1/5/8 are pivotal in BMP signaling. Gliogenesis is also increased in the ENS of Hipk2-/- mutants, suggesting that a diminution of neurogenesis in the adult ENS in Hipk2-/- mutants is compensated by an increase in gliogenesis. Autophagy is increased in enteric neurons in Hipk2-/- mutants and synaptic maturation is impaired. HIPK2 is thus an important transcriptional cofactor in the regulation of BMP signaling during enteric neuronal and glial maintenance. The development of the ENS thus does not cease at birth.

3.9 The Development of the ENS Is Influenced by Neuropoietic Cytokines

Ciliary neurotrophic factor (CNTF) was first identified as a factor in the eye that promotes the survival of chick ciliary ganglion neurons [200]. CNTF has since been purified, cloned, and found to affect many different neurons, both developing and mature [201]. CNTF does not resemble any of the neurotrophins and is a member of the cytokine family, which includes distantly related molecules, such as leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin-1, neuropoietin (formerly known as cardiotrophin 2), and cardiotrophin-like factor (CLCF1) [201–204]. CNTF primarily acts on neurons and skeletal muscle [205], while the actions of the other cyto-

kines are exerted on many other types of cell. The active CNTF receptor (CNTFR) is an assembly of three molecular components, only one of which, CNTFRα, actually binds CNTF [206]. There are two β receptor subunits, gp130 and LIFRβ. These are signal-transducing molecules and also serve as components of receptors for cytokine relatives of CNTF, such as LIF and IL-6 [203, 205, 207–209]. The three molecular components are not initially associated with one another on cell surfaces but are recruited to form a complex when stimulated by CNTF. CNTF binds first to CNTFRα and the β components then join to form the tripartite complex [209]. The signal transduction process thus begins with formation of the CNTFRα/LIFRβ/gp130 complex, involves the dimerization of LIFRβ with gp130, and proceeds by activating Jak tyrosine kinases, which are constitutively associated with the cytosolic tails of each of the β components [210, 211]. CNTF neither binds to, nor activates the β components in the absence of CNTFRα. CNTFRα, moreover, is restricted to the nervous system and skeletal muscle, which thus explains the relative neural specificity of the actions of CNTF [205]. Other cytokines have different specificity determinants, which are expressed extraneuronally. Levels of CNTF in embryonic and fetal animals are very low [201], although expression of mRNA encoding CNTF can be detected in the developing bowel by using reverse transcriptase and the polymerase chain reaction. In contrast to CNTF, CNTFRα is expressed by many cells of the developing nervous system, including the ENS [212]. The natural or targeted knockout of genes encoding CNTF does not cause notable developmental defects in mice [213] or humans (about 2.5% of the Japanese population) [214]. CNTF, furthermore, unlike the majority of secreted proteins, lacks a signal sequence and thus is probably cytosolic. In the absence of cell death, such a protein requires a chaperone to be secreted [204]. CNTF has thus been proposed to be an emergency factor, which is released only in response to injury; moreover, mice that lack CNTF appear normal at birth but lose motor neurons excessively as they age, further supporting the idea that CNTF is helpful in protecting neurons from injury [205].

In situ, all myenteric ganglia of the fetal stomach and intestine express the immunoreactivity of CNTFRα by E16-E18 [215]. In vitro, CNTF and LIF cause nuclear translocation of signal transducer and activator of transcription 3 (STAT3), a concentration-dependent increase in neurons and glia, and a decrease in expression of the precursor marker, nestin. CNTF and LIF were additive with NT-3 in the ability to enhance neuronal but not glial development. Specifically, the development of NOS-expressing neurons (a marker of inhibitory motor neurons) was promoted by CNTF and NT-3. These observations suggest that a ligand for the tripartite CNTF receptor complex plays a role in ENS development; however, the identity of that ligand is not yet clear.

In contrast to the relatively normal development of CNTF knockout mice, there are profound motor and other defects at birth in animals with targeted deletions of CNTFRα [202]. Mice lacking CNTFRα fail to feed and die with a massive dilation of the bowel during the perinatal period. Neurons immunoreactive for substance P (SP) and NOS are markedly reduced in the enteric plexuses of these animals. Almost no SP- or NOS-immunoreactive axons are found in the circular muscle of CNTFRα knockout mice. Motor neurons that excite smooth muscle contain SP [216] and motor neurons that relax smooth muscle contain NOS [217–221]. An identical defect is seen in the gut of mice in which the expression of *LIFRβ* has been knocked out. These observations suggest that the tripartite CNTFR, and particularly its α component, plays a vital role in the development of enteric motor neurons. Although enteric ganglia are present in mice that lack CNTFRα or LIFRβ, the ENS cannot function in the absence of motor neurons. Since a similar effect is not seen in mice lacking CNTF, there may be another endogenous ligand in the fetal gut that can bind to CNTFRα. This yet-to-be-identified ligand is essential for the development of enteric motor neurons. Many relatives of CNTF, LIF, OSM, IL-6, IL-11, and cardiotrophin-1, do not require CNTFRα and thus are unlikely to be the unknown CNTFRα ligand [202]. In contrast, neuropoietin (formerly known as cardiotrophin

2) and cardiotrophin-like factor (CLCF1) bind to CNTFRα and activate the tripartite CNTF receptor [204]. A role for these agents in ENS development may thus be important and should be investigated. Mice that lack CLCF1 die perinatally due to a suckling defect. The state of the ENS in these animals has not yet been described but may well be deficient.

3.10 An Aganglionosis Similar to That in Hirschsprung Disease Occurs in *ls/ls* and *sl/sl* Mice

The terminal colon of lethal spotted (*ls/ls*) and piebald lethal mutant mice (*sl/sl*) becomes aganglionic [222]. These murine aganglionoses, inherited as autosomal recessives, provide the best-known spontaneous animal models of Hirschsprung disease [223]. Related models in other species have now been discovered. These include megacolon inherited as a recessive trait in the spotting lethal rat (*sl/sl*) [10, 224–228], white patterned foals [229], and the homozygous spotted rabbit (en/en) [230]. What all of these models have in common, whether they are inherited as a recessive or a dominant trait, is that the terminal region of the gut is aganglionic, megacolon develops, and the animals exhibit a spotted coat. The megacolon can be attributed to a loss of the reflexes normally mediated by the ENS. The presence of nerve fibers thus does not compensate for the aganglionosis which contain the neural circuitry responsible for enteric reflexes. The spotted coat color reflects an abnormality of melanocytes, which like enteric neurons are derivatives of the neural crest. The defects in the animal models, however, like that which occurs in patients with Hirschsprung disease, do not extend to all derivatives of the neural crest, or even to all crest-derived neurons. The constant association of enteric neuronal and melanocytic deficiencies thus suggests that there is a common factor or requirement for normal differentiation that the ENS of the terminal gut shares with melanocytes; this common factor is the signaling of endothelin 3 and its receptor endothelin B.

3.11 Genetic Abnormalities in Genes Encoding Endothelin-3 and Its Receptor, Endothelin-B, Are Associated with Spotted Coats and Aganglionosis

The genes that are abnormal in lethal spotted (*ls/ls*) and piebald lethal (*sl/sl*) mice, as well as the spotting lethal rat, have recently been identified. The loci that are involved in these models are also abnormal in a subset of patients with Hirschsprung disease. Aganglionosis in *ls/ls* mice is associated with a mutation in the gene encoding the peptide hormone, endothelin-3 (EDN3) [231], while the somewhat more severe aganglionosis that occurs in *sl/sl* mice [232], spotting lethal rats [225, 227, 228], white patterned foals [233], and some patients with Hirschsprung disease [234] is linked to abnormalities of genes encoding the endothelin-B receptor (EDNRB). This is the receptor normally activated by EDN3. The discovery that EDN3 and EDNRB are important in the development of the ENS (at least in the colon) was made as a result of analyses of the effects of knockouts of the genes that encode these molecules in mice. Endothelins 1–3 represent a family of peptides, each with a chain length of 21 amino acids that activate one or both of two serpentine (G-protein coupled) receptors, endothelin-A (EDNRA) and/or EDNRB [235, 236]. Each of the endothelins has an equivalent potency for stimulating EDNRB, but that for activating EDNRA is EDN1 > EDN2 >> EDN3 [237]. EDN1 was discovered as a product of vascular endothelial cells that is a strong vasoconstrictor [238]. Since their initial discovery, however, the endothelins and the EDNRs have been found to be widely distributed [235]. The endothelins are initially synthesized with a signal sequence (a preproendothelin) that is responsible for translocation of the proteins across the membranes of the rough endoplasmic reticulum into the cisternal space. This translocation enables the proteins to be packaged for secretion. The signal sequence is removed cotranslationally to yield an inactive precursor, called a big endothelin, which is secreted. Big endothelins, in turn, are again cleaved by a spe-

cific membrane-bound metalloprotease, the endothelin-converting enzyme-1 (ECE-1), to produce the smaller active peptides [239]. Craniofacial defects arise in transgenic knockout mice that fail to produce EDN1 due to the abnormal development of first branchial arch derivatives [240]. Missense mutations in *ednrb* occur in *sl/sl* mice [232]. Similar mutations can be found in *EDNRB*, which is the analogous human locus, in patients with Hirschsprung disease [234]. When *ednrb* is knocked out by homologous recombination, an aganglionosis of the colon develops that is identical to that seen in *sl/sl* mice [232]. More recently, lethal spotting in rats has also been demonstrated to arise as a result of an interstitial deletion in an exon of the *ednrb* gene that prevents expression of the rat EDNRB [225, 227]. The *edn3* gene is mutated in *ls/ls* mice (*EDN3$^{ls/ls}$*) so that an arginine is replaced with a tryptophan residue in the C-terminus of big EDN3 [231]. This defect prevents the conversion of big EDN3 to the active EDN3 by ECE-1. In an analogous fashion, the knockout of *edn3* also causes the terminal colon to become aganglionic. It is thus clear that the receptor, EDNRB; the ligand, EDN3; and the converting enzyme, ECE-1, play critical roles in the development of the ENS. The nature of these roles, however, remains to be fully explained. The genetic loss of EDN3 stimulation could, in theory, lead to aganglionosis by affecting the crest-derived precursors of enteric neurons themselves. Alternatively, the effect of EDN3 could be mediated indirectly, through an action on another cell type that interacts with crest-derived cells in a manner that is essential for neuronal and/or glial development. Why the absence of EDN3 interferes with the development of neurons only in the colon is also an issue that must be resolved. The inability of EDN1 or EDN2 to compensate for the loss of active EDN3 in *EDN3$^{ls/ls}$* or *edn3* knockout mice [231] is also hard to understand, in view of the fact that all endothelins are equally good as ligands for the EDNRB [237]. The effects of EDN3, therefore, must be quite local and the circulating concentrations of EDN1 and EDN2 must be too low to be effective at those EDNRBs that are critical for development of the ENS of the terminal bowel.

3.12 An Action of EDN3 on Crest-Derived Precursors Does Not, by Itself, Account for the Pathogenesis of Aganglionosis

Several hypotheses have been advanced to explain the critical role played by EDN3 on the development of enteric neurons. One idea is that EDN3 is an autocrine growth factor [231]. This proposal considers (1) that EDN3 is essential for the development of migrating crest-derived cells as enteric neurons or melanocytes, and (2) that the crest-derived cells themselves are both the source and target of EDN3. The nice feature of this hypothesis is that it explains why the coats of all of the animal models of Hirschsprung disease are spotted or white. The lack of EDN3 deprives both the precursors of melanocytes and enteric neurons of a necessary growth factor. The hypothesis postulates that the migrating crest-derived cells that colonize the bowel synthesize big EDN3, convert it to active EDN3, and express EDNRBs. A problem for this autocrine hypothesis is that it fails to explain why the development of enteric neurons in mice lacking EDN3 [231] and in both piebald mice [232, 241] and spotting lethal rats [225, 227, 228] that lack EDNRBs only becomes abnormal in the colon. If no factors other than the crest-derived cells themselves were to be involved, then there is no obvious reason why enteric neuronal development should be independent of EDN3 in the esophagus, stomach, and small intestine but EDN3-dependent in the terminal colon. An idea that has been advanced to account for this problem is to assume that the ability of the vagal population of crest-derived cells to migrate as far as the terminal colon requires that the starting population be large and proliferating [109, 242]. This hypothesis postulates that EDN3 is a mitogen that is required to provoke vagal crest cells to multiply sufficiently to generate a population that is large enough to colonize the entire bowel. EDN3, in fact, has been demonstrated to be a mitogen for cells cultured from the premigratory neural crest [243]. Addition of EDN3 causes these cells to proliferate massively; however, following their multiplication,

the cultured crest cells go on to develop primarily as melanocytes. The neural crest cells, therefore, do not respond to EDN3 exactly as predicted by the hypothesis that EDN3 is required to generate adequate numbers of neural precursors to colonize the entire gut. EDN3 promotes the formation of melanocytes, not neurons, suggesting that, at least in culture, the precursors that proliferate in response to EDN3 are not neurogenic but melanogenic. The data are even consistent with the possibility that EDN3 shifts the originally pluripotent neural crest population toward the melanocytic lineage. Unless EDN3, therefore, were to exert a different effect in vivo, this outcome would not enhance the formation of neurons in the colon. The proliferative action of EDN3 on premigratory crest cells in vitro thus is consistent with the idea that its mitogenic properties are needed to enlarge the number of melanogenic precursors enough to colonize the skin, but the data do not support the concept that the mitogenic properties of EDN3 are needed for the formation of the ENS. To apply the hypothesis to the ENS, it is necessary to assume that the effects of EDN3 on crest-derived cells that have colonized the gut are different from those which EDN3 exerts on cells isolated from the neural crest itself. There are no longer any cells with a melanogenic potential in the crest-derived cell population that colonizes the bowel; moreover, the cohort of crest-derived cells that colonizes the gut is still proliferating [71, 72, 181].

To identify enteric cells that express EDN3 and EDNRB, the fetal mouse gut was dissociated at E11-13, and positive and negative immunoselection with antibodies to p75NTR were used to isolate neural crest- and non-crest-derived cells [111] Transcripts encoding EDNRB were detected in both crest-and non-crest-derived cells; however, transcripts encoding preproendothelin-3 were exclusively found in the non-crest-derived population. These data suggest that both crest- and non-crest-derived cells express EDNRB, but that EDN3 is produced by non-neuronal cells of the gut wall and is thus a factor of the enteric microenvironment. Neurons and glia develop in cultures of crest-derived cells isolated from the bowel in the absence of EDN3 and even in the presence of the EDN3 antagonist, BQ 788 [111]. EDN3 is thus not an autocrine factor that is required for the development of enteric neurons. In fact, the addition of EDN3 or the EDNRB agonist, IRL 1620, actually inhibits neuronal development, an effect that BQ 788 antagonizes. The ability of EDN signaling to inhibit enteric neurogenesis has been confirmed [112, 147, 244]. Neurons do not develop in explants of the terminal bowel of E12 $EDN3^{ls/ls}$ mice, but addition of extrinsic EDN3 makes them do so if a source of crest-derived cells is present in the bowel proximal to the aganglionic region. These observations are compatible with the suggestion that EDN3/ECNRB signaling prevents premature differentiation of crest-derived precursors [111, 147, 244]. The underlying assumption is that precursors migrate while post-mitotic neurons do not. The inhibitory effect of EDN3/ECNRB signaling thus enables the precursor population to persist long enough to finish colonizing the bowel. In fact, when EDNRB is selectively deleted only in crest-derived cells, the bowel is still aganglionic; thus, although both crest- and non-crest-derived cells express EDNRB, the expression by the crest-derived cells is necessary for the terminal bowel to become ganglionated [245].

EDN3/EDNRB signaling plays additional roles in the developing gut. EDN3 opposes the attraction of Ret-expressing crest-derived cells to sources of GDNF [147]. This allows crest-derived cells to avoid becoming trapped in the cecum, where GDNF expression peaks. EDN3 also opposes the accumulation of semaphorin 3A in the colon [246]. When EDN3 is deleted, semaphorin 3A accumulates in the presumptive aganglionic bowel. Given the ability of semaphorin 3A to repel migrating crest-derived cells, it is likely that the excess of semaphorin 3A in the terminal gut of mice lacking EDNRB contributes to the development of the aganglionosis. Such an effect is compatible with the in vitro observation that the presumptive aganglionic bowel of $EDN3^{ls/ls}$ mice can be colonized in vitro if extrinsic EDN3 is provided [111]. Presumably, the EDN can rescue the gut by suppressing the overabundance of semaphorin 3A.

3.13 The Pathogenesis of Aganglionosis Is Not Explained by an Abnormality Limited to Crest-Derived Neural Precursors

The enteric microenvironment may become inhospitable for colonization by crest-derived cells if EDN3 is deficient or if the EDNRB is lacking [68, 70, 241, 247]. Such an effect could be the result of an action of EDN3 on EDNRBs expressed by non-neuronal cells of the bowel wall. Alternatively, the crest-derived cells themselves may respond to EDN3 by secreting a factor that stimulates their non-neuronal neighbors to make the enteric microenvironment tractable for invasion by crest-derived emigres. As noted above, the advancing front of crest-derived cells in the developing gut cannot be recognized by the expression of neural or glial markers, but can be detected indirectly by explanting and culturing the bowel [51, 248, 249]. Neurons develop in cultures of the normal murine terminal colon explanted after stage 33, but not before [250]. In contrast, neurons never arise in the terminal 2 mm of an EDN3-deficient (*EDN3*$^{ls/ls}$) gut, no matter what the stage of the fetus at the time of explantation [248, 249]. These observations establish that the final segment of the *EDN3*$^{ls/ls}$ bowel is the presumptive aganglionic region and they suggest that viable crest-derived cells do not enter this zone. In co-culture experiments, crest-derived cells from a variety of sources, including the ganglionated proximal gut of *EDN3*$^{ls/ls}$ mice, have been shown to enter explants of the terminal bowel from control mice and give rise to neurons; however, no source of crest-derived cells migrates into an *EDN3*$^{ls/ls}$ terminal colon [250]. In contrast to the normal colon, moreover, the *EDN3*$^{ls/ls}$ colon also fails to promote the acquisition of gut-appropriate phenotypes when it is co-cultured with sources of crest cells [53]. These observations suggest that there may be non-neuronal EDNRB-expressing targets of EDN3 in the colon. The EDNRB that non-neuronal cells express as well as that expressed by crest-derived cells may contribute to making it possible for crest-derived cells to complete their colonization of the bowel. The possibility that EDN3 acts on non-neuronal cells of the gut wall has received strong experimental support. Aganglionosis does not occur in *EDN3*$^{ls/ls}$ × C3H aggregation chimeric mice, as long as >5% of enteric cells are of C3H origin; moreover, *EDN3*$^{ls/ls}$ neurons, identified with an endogenous marker (β-glucuronidase activity), are found even in the most distal enteric ganglia [247]. Similarly, ganglia containing mutant neurons (marked by the expression of a transgene, *lacZ* driven by the DBH promoter) develop in the terminal colon of aggregation chimeras constructed between wild-type and either *EDN3*$^{ls/ls}$ [69, 70] or *sl/sl* embryos [241]. It has been postulated that intercellular signals "downstream" from the EDNRB mediate colonization of the terminal gut by crest-derived cells [241]. An alternative hypothesis is that there is an additional, non-neuronal cell in the wall of the colon that expresses the EDNRB and must be stimulated by EDN3 in order to open the colon to colonization by crest-derived neuronal precursors. This latter idea is supported by the observation that the migration of vagal crest-derived cells, visualized by their expression of the *DBH/lacZ* transgene, is entirely normal in *EDN3*$^{ls/ls}$ mice until the cells reach the colon; however, the migration of vagal crest-derived cells becomes abnormal within the colon, which is not fully colonized [68, 70]. These observations imply that the ability of crest-derived cells to migrate within the colon is influenced by the enteric microenvironment, which is abnormal in EDN3-deficient *EDN3*$^{ls/ls}$ mice. This suggestion has been confirmed by back-transplantation experiments [247]. When segments of wild-type or *EDN3*$^{ls/ls}$ colon are placed in a neural crest migration pathway of a quail embryo, the avian crest-derived cells enter wild-type, but not *EDN3*$^{ls/ls}$ grafts. There is no reason to suppose that the quail crest-derived cells in these experiments fail to express either EDN3 or the EDNRB. Their inability to enter the *EDN3*$^{ls/ls}$ colon, therefore, cannot be explained by an autocrine hypothesis; furthermore, the back-transplantation experiment demonstrates that the absence of active EDN3 in the aganglionic *EDN3*$^{ls/ls}$ colon has pro-

duced an environment that crest-derived cells will not enter even if they are themselves normal. In sum, the accumulated evidence suggests that crest-derived cells are capable of colonizing the gut and forming enteric neurons whether or not they produce EDN3 but that the enteric microenvironment becomes abnormal in the absence of EDN3/EDNRB stimulation, so that the colon becomes resistant to colonization by crest-derived cells. In fact, extracellular matrix abnormalities have been described, both in the colon of *EDN3*$^{ls/ls}$ mice and in human patients with Hirschsprung disease.

3.14 The Extracellular Matrix Is Abnormal in the Presumptive Aganglionic Bowel of *EDN3*$^{ls/ls}$ Mice

A variety of defects involving components of the extracellular matrix have been found in mice with deficient EDNRB signaling [113, 251–254] and in human patients with Hirschsprung disease [255, 256]. A common feature that unites these abnormalities is that they all involve an over-abundance and/or maldistribution of constituents of basal laminae. Molecules that have been noted to be over-abundant include laminin, collagen type IV, nidogen non-sulfated glycosaminoglycans, and proteoglycans. In the developing colon of fetal *EDN3*$^{ls/ls}$ mice, the abnormal molecules are diffusely distributed throughout the mesenchyme of the colon and the surrounding pelvis and are not, for the most part, aggregated in formed basal laminae [251–253]. The mucosal basal lamina of the terminal and distal colon, however, is also thickened relative to that of a wild-type fetus of the same age. The location of the accumulated molecules of the extracellular matrix is in the paths both of vagal crest-derived cells migrating down the bowel [257] and of sacral crest-derived cells approaching the gut [258]. Double-label electron microscopic immunocytochemistry, moreover, has revealed that crest-derived cells, identified by their expression of HNK-1 immunoreactivity, migrate through the enteric mesenchyme of the developing bowel in contact with what appears to be diffuse tufts of electron-opaque material that is laminin-immunoreactive [259].

The over-abundance of laminin and type IV collagen can be detected in the colon of *EDN3*$^{ls/ls}$ mice at an earlier age [251] than that when crest-derived cells colonize the terminal colon in wild-type mice [250]. This timing and the fact that the extracellular matrix molecules accumulate in the path of incoming crest-derived cells are consistent with the possibility that the abnormal extracellular matrix in *EDN3*$^{ls/ls}$ mice (and by analogy, in patients with Hirschsprung disease) contributes to the pathogenesis of aganglionosis. This suggestion, however, presumes that the accumulation of laminin and other constituents of the extracellular matrix is a primary event rather than a secondary response to the absence of neurons and/or their precursors.

Studies with *EDN3*$^{ls/ls}$ mice have indicated that, at least in that model, the accumulation of molecules of the extracellular matrix in the fetal bowel is probably due to an increase in their biosynthesis [253]. Transcripts encoding the β1 and γ1 subunits of laminin, as well as the α1 and α2 chains of collagens type IV, were found to be overly abundant in the colons of *EDN3*$^{ls/ls}$ mice. Transcripts encoding laminin α1 were also found to be increased; however, the abundance of transcripts encoding the α1 chain was so much less than that of the β1 and γ1 subunits that the α1 protein had to be evaluated quantitatively with reverse transcription and the competitive polymerase chain reaction (RT-cPCR). The abundance of mRNA encoding laminin α1 was developmentally regulated and declined as a function of age after E11; nevertheless, at all ages, the abundance of mRNA encoding laminin α1 was higher in the *EDN3*$^{ls/ls}$ colon than in an age-matched wild-type colon or in the small intestine of the same *EDN3*$^{ls/ls}$ animals. The location of the cells responsible for the bulk of the biosynthesis of laminin α 1 and β1 and the α2 chain of collagen type IV was found by in situ hybridization (with 35S-labeled antisense riboprobes) to change as a function of developmental age. In the fetal colon, transcripts encoding these molecules are first concentrated in the endodermal epithelium; however, by day E15, the transcripts are more abundant in mesen-

chymal cells of the outer gut wall than in the epithelium. More mRNA was found in the colonic mesenchyme of the *EDN3^ls/ls^* colon than in the wild-type colon at an equivalent age.

To determine whether the increase in transcripts encoding subunits of laminin is a primary or secondary event, the expression of laminin-111 in E15 and newborn *Ret* knockout mice were compared with that in age-matched *EDN3^ls/ls^* and wild-type animals. The assumption behind this comparison was that the aganglionosis that occurs in both *EDN3^ls/ls^* and *Ret* knockout mice does so for different genetic reasons. In *Ret* knockout mice, the entire bowel distal to the rostral foregut becomes aganglionic because early crest-derived precursors lack functional Ret receptors and thus cannot respond to GDNF [128–131, 136]. In the *EDN3^ls/ls^* mice, the animals lack EDN3 and the aganglionic region is restricted to the colon [231]. If the increase in transcripts of laminin and the associated accumulation of laminin and other molecules of the extracellular matrix in the colon of *EDN3^ls/ls^* mice were to be a secondary response to the absence of neural precursors, then one would expect to see the same increase in the aganglionic bowel of *Ret* knockout mice. In contrast, the increase in mRNA encoding laminin subunits should not occur in the aganglionic bowel of *Ret* knockout mice if the change is *EDN3^ls/ls^* -specific and related to an effect of the absence of EDN3 on the colonic mesenchyme. No difference from controls either at E15 or in newborn mice was detected by RT-cPCR in the abundance of mRNA encoding laminin α1 in the *Ret* knockout colon [253]; furthermore, the overabundance of immunocytochemically visualizable laminin characteristic of the *EDN3^ls/ls^* colon was not seen in *Ret* knockout mice.

The results of these experiments suggest that the increase in abundance of transcripts encoding components of the extracellular matrix occurs in *EDN3^ls/ls^* mice as a primary effect of the genetic defect in EDN3, and is not a consequence of the aganglionosis. The observations also suggest that at least one isoform of laminin that is present in excess in the *EDN3^ls/ls^* mouse is laminin-111 (α1-β1-γ1). It should be noted that the accumulation of laminin-111 and other molecules of the extracellular matrix is not limited to the colon, although it occurs there. The excess of these molecules is also found in the pelvic mesenchyme that surrounds the terminal bowel. As a result, the abnormal extracellular matrix is located in the paths both of the vagal crest-derived cells that descend within the gut and of the sacral crest-derived cells that approach the bowel within the pelvis. The location, as well as the *EDN3^ls/ls^* specificity of the abnormal matrix, therefore, are compatible with the possibility that it contributes to the pathogenesis of aganglionosis. Suggestive evidence has been found showing that the effect of laminin-111, which normally enhances migration of crest-derived cells, changes to inhibition in the terminal bowel of EDNRB-deficient mice [113, 254]. Whether the extracellular matrix defects are actually contributory to the condition, however, remains to be confirmed.

Although molecules of the extracellular matrix have been demonstrated to inhibit the migration of crest cells in a number of locations, including the dorsolateral path between the ectoderm and the somites [260, 261], the posterior sclerotome [262, 263], and the perinotochordal mesenchyme [264], in none of these regions have the inhibitory effects been linked to accumulations of components of basal laminae [260, 263, 265]. In fact, the extracellular matrix in these regions behaves rather differently from that of either the aganglionic *EDN3^ls/ls^* [175] or Hirschsprung bowel [7, 266]. The aganglionic bowel in each of these conditions is heavily innervated both by axons of neurons from the more rostral hypoganglionic gut and from extrinsic ganglia [175]. The defect in the colon of *EDN3^ls/ls^* mice and patients with Hirschsprung disease thus impedes its colonization by crest-derived cells, but it does not antagonize the ingrowth of axons.

In contrast, the other regions that normally exclude crest-derived cells also inhibit the outgrowth of axons [265]. It seems paradoxical that laminin-111 should be one of the molecules that is overly abundant in a zone where crest-derived cells fail to migrate. Laminin is a favorable substrate for the adherence of crest-derived cells [98, 267]; moreover, laminin-111 also stimulates the migration of cells away from the neural crest itself [99, 268]. Antibodies to integ-

rins that block attachment of crest-derived cells to laminin [269, 270], as well as antibodies that bind to a laminin-proteoglycan complex [271] inhibit cranial crest cell migration in vivo. The abundance of laminin in the aganglionic *EDN3^ls/ls^* colon, therefore, might be expected to promote rather than inhibit the colonization of this region of the bowel by cells from the neural crest. On the other hand, the abundance of laminin-111 in the aganglionic colon of *EDN3^ls/ls^* mice and human patients with Hirschsprung disease could explain why this region of the gut is so well innervated by extrinsic axons; laminin promotes neurite extension and axonal growth [272–277].

3.15 Laminin-111 Promotes the Development of Neurons from Enteric Cells of Neural Crest Origin

Molecules of the extracellular matrix have been demonstrated to be biologically active and able to alter the fate of stem cells from the neural crest in vitro [278]. Extracellular matrix molecules, therefore, can provide more than just an adhesive substrate for crest-derived cells; they are also able to provide signaling information and are, at least potentially, capable of influencing the differentiation of crest-derived cells. Specifically, with respect to crest-derived cells that colonize the bowel, a substrate that includes laminin-111 has been found to increase the in vitro development of neurons relative to that which occurs on substrates of tissue culture plastic or type I collagen [158]. Neurons in these studies were defined as cells that express markers (such as peripherin, neurofilament proteins, neuron-specific enolase, or PGP9.5) that were visualized by immunocytochemistry. The ability of laminin-111 to promote the development of enteric neurons was initially observed in cultures of crest-derived cells immunoselected from the developing avian or rat gut with HNK-1 monoclonal antibodies. An even more pronounced effect of laminin-111 is seen in cultures of cells immunoselected from the mouse gut with antibodies to a cell-surface laminin-binding protein, known as LBP110 [273, 279, 280].

3.16 The Effect of Laminin-111 on Enteric Neuronal Development Depends on the Binding of Its α1 Chain to LBP110

LBP110 is not an integrin, but is similar to β-amyloid precursor protein [281]. Given the relationship of LBP110 to β-amyloid precursor protein, it is interesting that a recent whole genome mapping study has implicated β-amyloid precursor protein in susceptibility to Hirschsprung disease [282]. Specifically, alterations in β-secretase (BACE1) signaling via β -amyloid precursor protein and BACE2 have been proposed as contributors to the pathogenesis of Hirschsprung disease.

The domain of laminin that binds to LBP110 contains an isoleucine-lysine-valine-alanine-valine (IKVAV) sequence and is located on the laminin α1 chain, near its globular C-terminal end [283–285]. The IKVAV peptide also binds to integrins [286] and nucleolin [287]. Expression of LBP110 by PC12 cells is downregulated by transfection of the cells with an antisense amyloid precursor protein cDNA [281, 283]. The ability of NGF to induce neurite extension on a laminin-111 substrate is reduced in such antisense-treated PC12 cells. Kleinman and colleagues have concluded that LBP110 is a laminin-111 receptor that mediates the effects of laminin-111 on neurite outgrowth and also is responsible for controlling a variety of behaviors in non-neuronal cells [281, 283, 288–293]. The only cells in the bowel that express LBP110 are those of neural crest origin; therefore, LBP110 immunoreactivity co-localizes in the gut with crest markers [259] and cells immunoselected from the fetal mouse gut with antibodies to LBP110 preferentially differentiate as neurons or glia [158]. The ability of laminin-111 to promote the development of crest-derived cells as neurons or glia is specifically blocked by a synthetic peptide that contains the IKVAV sequence (IKVAV peptide). A variety of control peptides exert no effect on neuronal differentiation, including a nonsense peptide, a peptide with the same amino acids in a different sequence, or a peptide with a sequence found elsewhere in the laminin-1 molecule. The

IKVAV peptide, moreover, does not affect the development of neurons and glia when similar populations of anti-LBP110-immunoselected crest-derived cells are cultured on poly-d-lysine or fibronectin. The IKVAV peptide, therefore, does not exert a generally inhibitory action on the development of enteric neurons, but only blocks the increment in neuronal development that is a response to laminin-111. Since the addition of an IKVAV peptide does not reduce the total number of cells in culture, the IKVAV peptide appears not to antagonize the adhesion of cells to laminin-111. Adhesion is probably integrin-dependent [269, 270] and independent of LBP110 [283].

Further evidence that the IKVAV peptide does not interfere selectively with the attachment of a small neurogenic subset of crest-derived cells (which could be too small to affect the total number of cells counted in the cultures) has come from the observation that laminin-111 is just as effective when added in soluble form to already adherent cells as it is when it is used as the substrate upon which cells are plated. Soluble laminin is also equally efficacious when applied to cells immunoselected from the fetal mouse gut with antibodies to p75NTR as when it is applied to cells immunoselected with antibodies to LBP110. The effectiveness of soluble laminin-111 does not necessarily indicate that laminin-111, in a soluble form, is able to activate the receptors responsible for its effect on enteric neuronal development. Even when added as a soluble molecule, laminin-111 might bind to the substrate and then, after becoming bound, activate the receptors on cell surfaces that mediate its effects; nevertheless, the observation that laminin-111 retains its efficacy many hours after cells have adhered to poly-d-lysine, indicates that the ability of laminin-111 to increase the numbers of neurons developing in vitro is not due to the selective adherence of neurogenic crest-derived cells to laminin-111 at the time of plating.

As is true of the responses of cells to the addition of a growth factor, the response of immunoselected crest-derived cells to laminin-111 is associated with a rapid, but transient induction of the expression of the *c-fos* protooncogene. The effect of laminin-111 on *c-fos* expression is evident within one hour of adding laminin-111 and is no longer detectable by 24 hours. The *c-fos* response to laminin-1, like the promotion by laminin-111 of neuronal development, is abolished by the IKVAV peptide, but not by control peptides. The specific antagonism by the IKVAV peptide of both the laminin-111-induced development of neurons and the expression of *c-fos* suggests that both of these responses are mediated by LBP110, which is the cellular binding site for the IKVAV domain of laminin-1. Since the IKVAV peptide is an antagonist, and not an agonist, the observations also imply that activation of the putative receptor function of LBP110 requires more than simply its binding to the IKVAV domain of laminin α1. It is likely that the binding of the IKVAV domain to laminin-111 is necessary but not sufficient to stimulate the LBP110 receptor. Other sequences of laminin-111 and/or the whole laminin-111 molecule must be required for agonist activity. Although the IKVAV peptide does not stimulate LBP110, however, its presence in excess in the medium indicates that it probably occupies IKVAV binding sites on LBP110 and competitively antagonizes the binding of laminin-111.

These ideas have recently been supported by additional experiments that have shown that an antipeptide neutralizing antibody directed against the IKVAV domain of the α1 chain of laminin-111 mimics the effect of the IKVAV peptide and blocks the promotion of the development of enteric neurons in vitro by laminin-111 [294]. In contrast, precipitating antibodies to the β1 chain of laminin-111, applied in the same manner, fail to interfere with the in vitro differentiation of enteric neurons. Neither the antibodies to the α1 chain, nor those to the β1 chain, cause cells to detach from a laminin-111-containing substrate. Laminin-111 promotes the extension of neurites, as well as the development of neurons. This action is also specifically antagonized by an IKVAV peptide and by antibodies to the IKVAV domain of laminin α1.

3.17 The Effects of Laminin-111 on Crest-Derived Cells Immunoselected from the Fetal Bowel Are Different from Those of Laminin-111 on Cells Isolated from the Crest Itself

In contrast to its action on crest-derived cells immunoselected from the fetal gut, laminin-111 does not induce neural crest stem cells to differentiate as neurons [278, 295]. The ability of crest-derived neuronal precursors to respond to laminin-111 must thus be a characteristic the cells acquire, either while migrating to the bowel, or after they enter it. The difference in responsivity to laminin-111 between neural crest stem cells and their crest-derived successors, could be accounted for by the timing of LBP110 expression. Although premigratory and early-migrating crest cells express integrins, and thus are able to bind to laminin [98, 267, 268, 270, 271], which is abundant in the embryonic mesenchyme and basal laminae [258, 259, 296, 297], premigratory and early-migrating crest cells do not express LBP110 [259]. LBP110 is expressed only in target organs; moreover, the crest-derived emigres that colonize the bowel express LBP110 for the first time within the gut itself. If the induction of neuronal development by laminin-111 depends on the interaction of LBP110 with the IKVAV domain of the α1 chain of laminin-111, as suggested by the in vitro studies outlined above, then enteric neuronal precursors could adhere to laminin-111 while migrating to the bowel without being induced to prematurely differentiate into neurons. The premature differentiation of crest-derived cells into neurons prior to their arrival in the gut would prevent them from colonizing the bowel. Neurons are not notably migratory; thus, for ganglia to develop within a given region of the bowel, that region must first be colonized by crest-derived neural precursors. Crest-derived cells, within the gut, acquire LBP110 asynchronously. Some of the vagal crest-derived emigres express LBP110 as soon they enter the proximal bowel. Others, however, acquire LBP110 later and by the time they express LBP110, they have moved distally [259]. This asynchronous delay in the timing of LBP110 expression may enable the late-responding crest-derived cells to make their way distally into the caudal bowel before they differentiate and cease migrating.

3.18 Premature Neuronal Differentiation May Result When Inadequately Resistant Progenitors Encounter an Excessively Permissive Extracellular Matrix

The expression of LBP110 and the evidently related ability of laminin-111 to promote enteric neuronal development from crest-derived precursors may explain the seemingly paradoxical association of an excess of laminin-111 with aganglionosis in the terminal colon of EDN3-deficient *EDN3*$^{ls/ls}$ mice and human patients with Hirschsprung disease. As has been noted previously, it is possible that the deficiency of EDN3 removes an inhibitory influence on neuronal differentiation [111, 113, 254]. By simultaneously leading to an excess laminin-111 in the colonic mesenchyme, the lack of EDN3 also causes crest-derived cells to become exposed to an overabundance of laminin-111, a signal that promotes neuronal development. On the one hand, a brake (EDN3) to neuronal differentiation is absent, while on the other hand, a drive to differentiate (laminin-111) is enhanced. The consequence of the combined effect may be the premature differentiation of crest-derived emigres as neurons. Premature differentiation in turn causes the cells to cease migrating before colonization of the gut is complete. The genetic deficiency of EDN3 may thus exert both direct and indirect effects, which combine synergistically to prevent the formation of ganglia in the terminal bowel. Effects of EDN3 deficiency include allowing crest-derived cells to become trapped by their attraction to GDNF in the caecum, allowing accumulation of semaphorin A, enhancing synthesis of laminin-111, allowing exhaustion of the supply of proliferat-

ing precursor cells to occur, enabling premature neuronal differentiation. These ideas predict that vagal crest-derived cells of *EDN3*$^{ls/ls}$ mice (or the subset of patients with Hirschsprung disease with defects in EDN3 or the EDNRB) would encounter an abnormally strong inducement to differentiate (the over-abundance of laminin-111) when they enter the proximal colon. Consistent with this prediction is the observation that the progression of crest-derived cells, visualized in *EDN3*$^{ls/ls}$ mice by their expression of the *DBH-lacZ* transgene, is comparable to that in wild-type animals until the cells cross the ileocecal threshold, but becomes abnormal immediately thereafter [70].

Since laminin-111 is present in excess in the pelvic mesenchyme that surrounds the bowel, the hypothesis also predicts that sacral crest-derived precursors will not even enter the gut [251]. This prediction too has been confirmed, in that unique ectopic ganglia are present outside the terminal bowel in *EDN3*$^{ls/ls}$ mice [175, 248]. It is likely that these extra-enteric ganglia are formed by migrating sacral crest-derived cells that prematurely differentiate and stop before entering the gut. In the hypoganglionic region of the *EDN3*$^{ls/ls}$ colon, the aberrant ganglia actually pierce the longitudinal muscle and fuse with ganglia of the myenteric plexus. This peculiar configuration of ganglia, partly in and partly out of the gut, provides strong support for the idea that sacral crest-derived cells cease migrating short of their destination in the *EDN3*$^{ls/ls}$ bowel. This concept, that the aganglionosis of EDN3 deficiency (or absence of EDNRB) has a dual origin in an abnormal extracellular matrix driving an inadequately resistant crest-derived progenitor, would account for the observations that the failure of neurogenesis in the terminal bowel in these conditions is not neural crest-autonomous.

3.19 Both Crest-Derived and Non-neuronal Cells of the Colon Probably Respond to EDN3

There is evidence that EDN3 affects both crest-derived and non-crest-derived cells in the colon. Clearly, the excess of laminin-111, which occurs independently of crest-derived cells in the *EDN3*$^{ls/ls}$ bowel, is most easily explained by the postulate that EDN3 normally acts on one or more of the cells of the fetal enteric mesenchyme to downregulate their secretion of laminin-111. This postulate assumes that the EDNRB must be expressed, not only by crest-derived cells, but also by other cells of the fetal mesenchyme. Smooth muscle precursors and cells that form interstitial cells of Cajal (ICCs) are each candidates. In the mature gut, EDNRBs have been demonstrated to be expressed by the smooth muscle cells of the muscularis externa of both the large intestine [298] and small intestine [299]; moreover, intestinal smooth muscle responds directly to EDN3. When, during development, smooth muscle cells acquire EDNRBs is unknown. Transcripts encoding EDN3 and those encoding EDNRB are each found in the totally aganglionic bowel of *Ret* knockout, confirming (albeit indirectly) that enteric neuronal and glial precursors are not the only cells in the bowel wall that synthesize these molecules.

Direct evidence that non-neuronal cells contain mRNA encoding the EDNRB has been provided by in situ hybridization carried out in mice in which the crest-derived cells are marked by their expression of the *DBHlacZ* transgene (Kapur R and Yanagisawa M, reported at the 1996 Meeting of the American Motility Society). Both the *lacZ*-expressing crest-derived cells in primordial myenteric ganglia and non-*lacZ*-expressing cells that surround the ganglia were found to express the EDNRB. The location of the *lacZ*-negative cells that contain mRNA encoding the EDNRB is compatible with the idea that these cells are ICCs. That possibility must still be confirmed; however, ICCs have been found to be abnormal in patients with Hirschsprung disease [300, 301].

3.20 Interstitial Cells of Cajal Are Present, but Abnormal, in the Aganglionic Bowel of Hirschsprung Disease

The nature of the ICC has long been the subject of debate [302, 303]. An old idea that ICCs might be fibroblasts [304] has now been discarded [304]. A more recent suggestion is that

ICCs are modified or primitive smooth muscle cells [305, 306]. Whether or not they are related to smooth muscle, ICCs can be identified as a distinct cell type by their expression of, and dependence on, the *ckit* protooncogene [307–309]. *c-kit* encodes a receptor tyrosine kinase (Kit) and is allelic with *White Spotting* (*W*) [310]. Kit ligand (KL; also known as *Steel* factor or stem cell factor) is allelic with *Steel* (*Sl*). Activation of Kit by KL is probably critical for the development and/or maintenance of ICCs, because *W* [307, 311] and *Sl* [308] mutations interfere with the appearance of ICCs, the injection of neutralizing antibodies to Kit causes ICCs to disappear [309, 312], and the development of Kit-expressing ICCs in vitro is dependent on KL in the culture medium [260]. ICCs appear to be the pacemakers for myogenic intestinal slow waves because these waves are impaired when the network of ICCs is lost or fails to develop [307–309, 311, 312]. Once the ICC network is disrupted and slow waves are lost, intestinal motility becomes abnormal and the bowel dilates in a manner that is not dissimilar to that seen in aganglionosis.

During fetal development and, in some regions (the longitudinal muscle) extending into postnatal life, ICCs express markers in common with smooth muscle cells [302]. These markers include the intermediate filament protein, desmin, and smooth muscle isoforms of actin and myosin. ICCs never express Ret, which can serve as a marker for crest-derived cells in the wall of the gut [127, 128, 135]. These observations suggest that ICCs are not crest-derived cells, but that instead, they share a common precursor with smooth muscle. A similar conclusion has been reached from studies of stably marked crest-derived cells in avian interspecies chimeras [313]. Interestingly, Kit-immunoreactive ICCs assume a variety of shapes in different locations in the intestinal wall and may be divided by the timing of their divergence from the common smooth muscle/ICC precursor into subtypes of ICC [302]. It has been proposed that those ICCs that surround myenteric ganglia and those that are found within the deep muscle plexus, circular, and longitudinal muscle layers constitute functionally distinct cell classes [314].

Since ICCs are not crest-derived cells, it follows that their abnormality in the affected region of the bowel of patients with Hirschsprung disease [300, 301, 314, 315] demonstrates that the genetic lesion in these patients affects more cells than just neurons and their precursors. ICCs, however, are reduced in number and disrupted in pattern, but they are not totally absent from the aganglionic region of the colon in Hirschsprung disease. ICCs are also found in the terminal colon of *ls/ls* mice and in the aganglionic bowel of *Ret* knockout mice, although again, their numbers are reduced in comparison to those of wild-type mice, and the distribution pattern of ICCs is abnormal. These observations indicate that ICCs can develop in the absence of EDN3 and even in the absence of neurons. Conceivably, the abnormal numbers and distribution of ICCs in the aganglionic bowel of patients with Hirschsprung disease and *ls/ls* mice are secondary effects, resulting from the aganglionosis. Supporting this possibility, in situ hybridization has indicated that enteric neurons do contain transcripts encoding KL [302]. Enteric neurons thus are likely to be a source of KL; moreover, the physiologically active form of KL is not the secreted protein, but a membrane-bound ligand [316, 317]. To be stimulated by neuronal KL, therefore, neurons probably must come into contact with target cells so that the Kit receptors of the targets can be activated by the KL bound to neuronal surfaces. The requirement that cell-to-cell contact must occur for the KL/Kit interaction to take place could explain the close spatial relationship of a subset of ICCs to myenteric ganglia. It could also explain the paucity of ICC of the myenteric type in the aganglionic region of the Hirschsprung bowel [314, 315].

The aganglionosis of Hirschsprung disease and that of *EDN3*$^{ls/ls}$ mice, therefore, might each be expected to be associated with ICC abnormalities; the KL-dependent ICCs would be deprived of neuronal KL in the aganglionic bowel in these conditions. Neurons, however, are probably not the only source of KL in the bowel. First, if they were, then ICCs would be expected to be totally absent from the aganglionic zone of the Hirschsprung and *EDN3*$^{ls/ls}$ colon, but they are not. Second, ICCs develop in the *Ret* knockout

gut, which contains no neurons at all; moreover, mRNA encoding KL (as well as that encoding Kit) can be detected in this tissue. It is possible that ICCs do not require EDN3 for their development, but they might still express the EDNRB and be EDN3-responsive. The abnormalities noted in the numbers and distribution of ICCs in the aganglionic regions of the Hirschsprung and *EDN3*$^{ls/ls}$ colons are consistent with this idea. Certainly, the location of the non-neuronal cells of the colon of *DBH-lacZ* mice found by in situ hybridization to contain mRNA encoding the EDNRB conforms to the known location of Kit-immunoreactive ICCs in the bowel. One might speculate that EDN3 speeds the development of ICCs or smooth muscle. In its absence, the respective precursors might remain secretory for a longer period of time than normal and secrete more laminin-111. As the cells mature as smooth muscle and/or ICCs, laminin-111 secretion diminishes. This hypothesis is consistent with the observed developmental regulation of laminin-111 and the slower than normal rate of decline found in its expression in the *EDN3*$^{ls/ls}$ colon [253].

3.21 Serotonin (5-HT) and Cross-talk Between Progenitors in the Developing ENS

Crest-derived precursors of enteric neurons interact, not only with the microenvironment they encounter as they migrate to and within the bowel, but they also interact with one another. Serotonergic neurons, which are among the first neurons to arise during ontogeny [104], synapse on dividing precursors in the developing guinea pig ENS [318]; moreover, in mice that lack tryptophan hydroxylase 2, which is essential for neuronal biosynthesis of 5-HT, the ENS is profoundly hypoplastic [319]. The same defect, a hypoplastic ENS, also occurs in mice with a gain-of-function mutation (G56A) in the serotonin transporter (SERT; *Slc6a4*). SERT takes up 5-HT, which has the effect of removing it from its receptors. The overly active G56A isoform of SERT removes 5-HT so fast that it does not have the opportunity to activate its receptors adequately [320]. In contrast, a hyperplastic ENS develops in mice that lack SERT or which have been treated during gestation with a selective serotonin-reuptake inhibitor (SSRI) [320]. ENS hyper- and hypoplasia each cause gastrointestinal motility to be abnormal.

Many subtypes of 5-HT receptor are expressed in the bowel during ontogeny and in adult life. Of these, the 5-HT$_4$ receptor has been most extensively studied with respect to mediation of the developmental effects of 5-HT. Both 5-HT and 5-HT$_4$ receptor agonists, such as tegaserod and prucalopride, enhance enteric neurogenesis when they are applied to isolated enteric crest-derived cells in vitro; moreover, the neurogenic effects of each are blocked by 5-HT$_4$ receptor antagonists [196, 319]. Similarly, 5-HT and 5-HT$_4$ agonists promote enteric neurogenesis, even in adult bowel *in vivo*, and again, 5-HT$_4$-promoted neurogenesis is 5-blocked by HT$_4$ antagonists [196, 321, 322]. These observations imply that 5-HT from enteric serotonergic neurons is necessary for adequate neurogenesis in the developing and mature ENS. Importantly a 5-HT$_4$ receptor agonist, such as prucalopride, rescues ENS development and prevents abnormal gastrointestinal motility in mice that carry the overly active G56A isoform of SERT [320]. Early-born enteric serotonergic neurons may thus unwittingly be affected by drugs used during pregnancy. Depression is commonly treated with tricyclic antidepressants or SSRIs and drugs of abuse (cocaine, for example) may be taken by pregnant or lactating women. These compounds all alter the function of serotonergic neurons and thus, by changing the development of the ENS, the drugs may lead to unanticipated and long-lasting effects on the ENS of offspring [320, 323].

SERT is transiently expressed during CNS development in glutamatergic neurons [324–327]. Although these neurons do not synthesize 5-HT, their ability to take it up enables them to utilize 5-HT, which they do to help pattern the innervation of the sensory cortex. It is likely that SERT expression in the developing ENS is also not limited to serotonergic neurons. The role of SERT-mediated 5-HT uptake in ENS develop-

ment appears to be important but has not yet been fully explored. SERT is expressed in the gut, not only in the ENS, but also in the enterocytes of the fetal and adult gastrointestinal mucosa; moreover, SERT is functional in the early fetal endoderm and ENS even before 5-HT itself can be detected [328]. Epithelial SERT probably helps to compartmentalize the bowel wall and prevent the ENS from becoming swamped by the very large amount of 5-HT that mucosal enterochromaffin cells constitutively secrete. If it were to reach the ENS, mucosal 5-HT would make it impossible for the much smaller quantity of enteric neuronal 5-HT to function properly [329]. 5-HT is a multifunctional molecule. It is not only a neurotransmitter, a paracrine factor, and a hormone [329], but also a growth factor that is critical for ENS ontogeny and its clinical effects, though currently unknown, may be highly significant.

3.22 Deficiency of Platelet-Derived Growth Factor Receptor-α-Expressing Cells in Hirschsprung Disease Colon

Gastrointestinal smooth muscle contraction is controlled by coordinated interaction of three main cell types: enteric nerve cells, intestinal cells of Cajal (ICCs), and smooth muscle cells (SMCs). In recent years, a fourth cell type has been described as forming part of this complex network, namely platelet-derived growth factor receptor alpha-positive cells (PDGFRα$^+$-cells). These PDGFRα$^+$-cells were, for many years, known as “fibroblast-like cells” or “ICC-like” cells, as they resembled ICCs morphologically, but were c-kit negative [330]. More recently, enhanced green fluorescent protein (eGFP) labeling of these cells, as well as commercial availability of antibodies directed against PDGFRα, has enabled specific and reliable identification of this cell type [331]. PDGFRα$^+$-cells form discrete networks in the region of the myenteric plexus and within the circular and longitudinal muscle layers [332]. PDGFRα$^+$-cells express the small-conductance Ca^{2+}-activated K^+ channel (SK3), which is an important mediator of purinergic neurotransmission in gastrointestinal smooth muscle [332, 333].

Many studies have investigated the expression of PDGFRα$^+$-cells in the gastrointestinal tract of various animals in recent years. Iino et al. [330] who were the first authors to examine PDGFRα$^+$-cells found that they form a cellular network with their ramified processes and encompass myenteric ganglia. Kurahashi et al. [334] were the first group to confirm a functional role for PDGFRα$^+$-cells in gastrointestinal smooth muscle using transgenic mice with constitutive expression of enhanced green fluorescent protein (eGFP) in PDGFRα$^+$-cells. The eGFP label allowed the authors to isolate and study the function of PDGFRα$^+$-cells. They found that PDGFRα$^+$-cells expressed appropriate receptors and effectors to receive and transduce purinergic neural signals [334]. O’Donnell et al. [335] have verified that the mucosal PDGFRα$^+$-cells in the human colon, like that of the mouse, also express TLR4, TLR5 and P2RY1, suggesting a role for these cells in the immune response and in purinergic neurotransmission. The results of their study have revealed that there are many fewer PDGFRα$^+$-cells in both ganglionic and aganglionic regions of the bowel in patients with Hirschsprung disease than in control colon [335]. The same authors have also shown that the expression of both ICCs and small conductance Ca^{2+}-activated K^+ (SK3) channels is much less than normal in Hirschsprung disease colon [315, 336]. The reduced expression of PDGFRα$^+$-cells is consistent with the existence of a deficiency in inhibitory neurotransmission in Hirschsprung disease bowel and may contribute to the state of tonic contraction of aganglionic segments of gut [335].

3.23 Hirschsprung Disease Is Associated with Many Different Genetic Abnormalities: Conclusion from Animal Models

Congenital neuromuscular disorders of the gut are commonly encountered during the neonatal period. These conditions include, in addition to

Hirschsprung disease (long- and short-segment varieties), the allied disorders, hypoganglionosis, neuronal intestinal dysplasia (hyperganglionosis), ganglion cell immaturity, and dysganglionoses. There are also additional defects such as hypertrophic pyloric stenosis, volvulus, and intussusception that may also involve abnormalities of the development of the ENS. HSCR is quite common and occurs in up to 1 in 5000 live births [337]. The major susceptibility gene is the *RET* protooncogene [47, 337–345]. Hirschsprung disease has been shown to be associated with loss-of-function mutations in the coding sequence of *RET* mutations [337–339] or with non-coding regions that reduce expression of RET protein [47, 342–345]. Both long- and short-segment Hirschsprung disease can occur in patients with identical *RET* abnormalities and patients may also exhibit other problems, including multiple endocrine neoplasia type A (more commonly associated with gain-of-function mutations in *RET*), maternal deafness, talipes, and malrotation of the gut. Identical mutations in *RET* may thus give rise to distinctly different phenotypes in affected individuals. Unfortunately, there is no obvious relationship between the *RET* genotype and the Hirschsprung phenotype; moreover, the frequency of coding mutations of *RET* in Hirschsprung disease is sufficiently low that other genetic and/ or environmental conditions must be invoked to explain susceptibility to Hirschsprung disease in the majority of patients. Another genetic defect that has been associated with Hirschsprung disease involves mutations in *EDNRB,* which accounts for many fewer instances of the disorder than *RET* (<5% of isolated cases) [234, 346]. Again, many patients with Hirschsprung disease do not exhibit mutations of *EDNRB* or *RET* and there are individuals who carry these mutations (and also those of *RET*) who do not express the Hirschsprung disease phenotype [234]. As might be expected, not only are some cases of Hirschsprung disease linked to mutations in *EDNRB*, but mutations of genes encoding the ligand, EDN3, are also associated with Hirschsprung disease, albeit rarely [346]. In the case of the *EDN3* mutations, the phenotype is reminiscent of that which is seen in $EDN3^{ls/ls}$ mice. Hirschsprung disease occurs together with pigmentary abnormalities and is combined with a Waardenburg type 4 or 2 phenotype (Shah-Waardenburg syndrome) [346–348]. Hirschsprung disease is thus a multigene abnormality and a wide variety of mutations (many of which are still to be identified) predispose toward it [47, 234, 282, 337, 342–345]. The environmental background within which these mutations operate also influences the phenotypic outcome. A major whole-genome sequencing study has recently expanded the known genes that increase the risk for Hirschsprung disease [282]. This study identified 4 susceptibility loci, including one in the phospholipase D1 gene. The patients in the new whole genome analysis had a significant excess of rare protein-altering variants in genes that were already known to be associated with Hirschsprung disease, but an excess was also found in the gene encoding BACE2. Many common and distinct pathways were identified that enhanced risk of Hirschsprung disease when they were associated with variants in RET. The study linked BACE-1, β-amyloid precursor protein and BACE-2 to Hirschsprung disease.

3.24 Conclusion

The ENS is a complex and independent nervous system that is formed by precursors that migrate to the bowel from vagal, truncal, and sacral regions of the neural crest. Very recently, a claim has been made that endodermal pancreatic duodenal homeobox-1-expressing cells also contribute to the murine ENS [349]. The crest-derived enteric neuronal progenitors are initially multipotent; however, their developmental potential decreases as a function of time and place during ontogeny [350]. The crest-derived emigres that arrive in the bowel have lost the potential to give rise to some derivatives, such as ectomesenchyme and melanocytes, but the emigres retain a high degree of multipotency and their ultimate fate is influenced by the enteric microenvironment. The effects of the microenvironment are played out on cells that vary in their receptivity according to the lineages and sublineages into

which they have been sorted. One set of neurons is born early, is transiently catecholaminergic, is dependent on expression of the *Ascl-1* gene, and gives rise to serotonergic neurons. The other, from which CGRP-containing neurons are derived, is born late, is never catecholaminergic, and is *Ascl-1*-independent. A variety of signals have been identified that influence the differentiation and/or survival of enteric neurons. An early-acting factor is GDNF, which activates the Ret receptor. Other factors, such as EDN3, the neurotrophin, NT-3, neuropoietic cytokines, and laminin-111 act later.

Natural or targeted mutations in genes that encode factors required by crest-derived precursors early in development affect cells that are still relatively multipotent; therefore, the resulting defects tend to be large, such as those associated with the deletion of GDNF or Ret. Later-acting factors give rise to many fewer global abnormalities, although even a small loss of a critical neuron may be lethal. Knockout of CNTFRα, which results in an apparent loss of motor fibers to smooth muscle, is an example. A still more localized abnormality occurs in mice lacking EDN3 or EDNRB. The terminal colon of these animals becomes aganglionic. This defect may result from an effect of the mutation both on the crest-derived precursors of enteric neurons and on the non-neuronal cells of the bowel wall that produce the matrix through which crest-derived cells must migrate to colonize the gut. There is an excess of laminin-111 in the colon of EDNRB signaling-deficient animals and humans that may combine with the loss of the effect of EDN3 on crest-derived cells to cause premature differentiation of precursors as neurons. Since neurons do not migrate, the consequence of premature differentiation is an early cessation of migration leading to a distal aganglionosis. Many mutations have been associated with Hirschsprung disease, although the most important contributing gene is *RET* [282].

Hirschsprung disease is a multigene abnormality that cannot be completely accounted for by known mutations or easily identified by genetic testing [282, 342, 343]. Each of the many factors that are critical for the formation of the normal ENS [114] are potential targets of mutations that might cause Hirschsprung disease or other birth defects in humans. Future research should begin to reveal genes that, when abnormal, cause not just an aganglionosis, but hypoganglionosis, neuronal intestinal dysplasia, and intestinal dysganglionoses, as well as additional contributors to Hirschsprung disease. Hopefully, progress made in understanding the pathogenesis of Hirschsprung disease and allied disorders will provide better means of treating these conditions and, better yet, preventing them.

During the last decade, there has been much progress in the understanding of ENS development including how neural crest-derived progenitors migrate and colonize the bowel, the formation of ganglionated plexuses, and the molecular mechanisms of enteric neural and glial diversification [12, 47, 114, 282, 351]. Recent advances have highlighted the potential of enteric neural stem cell transplantation as a possible treatment option for enteric neuropathies including Hirschsprung disease [352]. Much research has already resulted in the development of robust and reproducible methodologies to facilitate the harvesting and propagation of neural stem cells, their potential and safety in murine models. Recent studies have transplanted human enteric neural progenitors into the mouse colon and shown engraftment [352, 353]. The identification and isolation of neuronal progenitor cells from the human postnatal intestine raise the possibility of transplanting these cells to replace missing neurons in patients with Hirschsprung disease.

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