REVIEW ARTICLES

The Developing Nervous System: A Series of Review Articles

The following article is the third in our series on the developmental biology of the nervous system and its relation to diseases and disorders that are found in newborn infants and children. In this article, Cheryl Gariepy describes the development of the enteric nervous system and the relation between genetic abnormalities of that system and enteric disease.

Alvin Zipursky
Editor-in-Chief

Intestinal Motility Disorders and Development of the Enteric Nervous System

CHERYL E. GARIEPY

*Department of Pediatrics, Pediatric Gastroenterology and Nutrition, University of Texas
Southwestern Medical Center, Dallas, Texas 75390-9063, U.S.A.*

The enteric nervous system (ENS) is an independent nervous system. It contains as many neurons as the spinal cord, every class of neurotransmitter found in the CNS, and reflex arcs capable of functioning independently of CNS input. Whereas the majority of enteric neurons are not directly innervated by the brain or spinal cord, the ENS is in two-way communication with the CNS via parasympathetic and sympathetic neurons. The ENS processes information regarding the state of the intestinal lumen and gut wall and modulates intestinal contractility, secretion, vascular supply, and inflammatory responses (1, 2).

Complaints related to the ENS represent a large percentage of pediatric office visits. Common clinical complaints of gastrointestinal motility and sensation include gastroesophageal reflux, dyspeptic syndromes, constipation, chronic recurrent abdominal pain, and irritable bowel syndrome (3). Less common but life-threatening disorders of the ENS include Hirschsprung disease and neuropathic chronic intestinal pseudoobstruction. Our understanding of the pathophysiologic processes underlying these complaints is being aided by the study of ENS formation and structure. Ultimately, these studies will improve our diagnosis and treatment of these disorders.

As our understanding of the ENS improves, it becomes very clear that it is no longer sufficient to simply determine whether

enteric ganglion cells are present. We are learning the importance of determining whether the correct number and types of ganglion cells are present. This task is complicated by the fact that the morphology of the myenteric plexus varies with location as well as age (4, 5). Only through improved understanding of the normal development of the ENS can we hope to accurately diagnose relatively subtle abnormalities in ENS development (6, 7). Further, with continued basic research, we may be able to isolate an ENS stem cell population from postnatal individuals. The identification and study of multipotential stem cells may allow for their eventual use in transplantation to correct intestinal neuronal deficiencies (8).

Recent studies have identified six genes causing Hirschsprung disease, as well as genes that may be related to other disorders of the ENS. This review focuses on studies of ENS development in rodents that have substantially improved our understanding of the genetic basis of human disorders of the ENS.

DISCUSSION

Origin of the ENS

The ENS is composed of autonomic ganglia in the myenteric and submucosal plexuses and associated connecting neural structures in the bowel wall. The ENS, like all other branches of the peripheral nervous system, is derived from the NC.

Vagal, truncal, and sacral neural crest (NC) cells colonize the gut (Fig. 1). Vagal- and truncal-derived ENS precursors enter the foregut mesenchyme and colonize the developing gut in a rostral-to-caudal progression. Enteric ganglia throughout

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Correspondence and reprint requests: Cheryl E. Gariepy, M.D., Department of Pediatrics, UT Southwestern Medical Center, Dallas, Texas 75390-9063, U.S.A.; e-mail: Gariepy@utsw.swmed.edu

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Figure 1. Schematic presentation of the colonization of the developing gut by NC-derived cells. Vagal NC-derived cells (shown in *red*) migrate ventrally to populate the entire gut. Truncal NC-derived cells (shown in *blue*) populate the foregut. The foregut is, therefore, populated by both vagal and truncal NC-derived cells (shown in *purple*). Sacral NC-derived cells (shown in *green*) colonize the hindgut before the arrival of the vagal NC-derived cells.

the entire length of the gut originate in the vagal NC (somites 1–5 in the mouse, red in Fig. 1). The foregut (esophagus and proximal stomach) is also populated by cells derived from the

truncal NC (somites 6 and 7 in the mouse, blue in Fig. 1) (9). Sacral-derived NC cells enter the hindgut mesenchyme and colonize the hindgut in a caudal-to-rostral progression (green

in Fig. 1) before the arrival of the vagal-derived ENS precursors to the hindgut (10). Studies in the avian system provide strong evidence for the contribution of the sacral NC to the hindgut ENS. The final contribution of these cells to the ENS in mammals is an area of active research. Failure of the vagal-derived NC cells to colonize the hindgut results in failure of hindgut ENS development, suggesting that interaction between sacral and vagal enteric NC cells may be necessary for sacral NC contribution to the ENS.

There are several markers of NC cells as they colonize the gut. The extent to which these markers identify all or just subsets of ENS precursors is generally controversial. Expression of specific markers may identify specific lineages of NC-derived cells restricted in their ultimate developmental potential. Among the first recognized markers of ENS precursors were two enzymes specifically involved in catecholamine biosynthesis, tyrosine hydroxylase (TH) and dopamine- β -hydroxylase (D β H). At least a subset of ENS precursors is transiently catecholaminergic during the colonization process.

Molecules Implicated in Control of ENS Development

Aided in large measure by the advent of knockout and transgenic technology to manipulate the mouse embryo, many molecules and several signaling pathways are identified as important in the control of mammalian ENS development (see Table 1). Seven of the resulting strains are mouse models of Hirschsprung disease. Mutation of six of the genes implicated in distal intestinal aganglionosis in mice is confirmed to cause Hirschsprung disease in humans (11). In addition, we now have several rodent models of abnormal ENS development other than distal intestinal aganglionosis. Rodent models of abnormal ENS development are discussed in approximate order of severity of their ENS abnormality.

Sox10. Sox10 is a member of the sry-related family of transcription factors (12). *Sox10* is expressed by ENS precursors before and throughout colonization of the gut mesenchyme (13, 14).

The role of sox10 in ENS development was identified by positional cloning of the *Dominant megacolon (Dom)* locus of mice (15, 16). *Sox10*^{Dom/+} mice exhibit distal intestinal aganglionosis, they die shortly after birth, and they are a naturally occurring model of Hirschsprung disease. *Sox10*^{Dom/sox10}^{Dom} embryos die pre- or perinatally with severe abnormalities of NC derivatives, including complete aganglionosis of the alimentary canal. *Sox10*^{Dom} is the only gene mutation identified in rodents that produces a lethal ENS phenotype in heterozygous animals (11).

The *sox10*^{Dom} mutation is a single bp insertion that alters the normal mRNA reading frame and leads to a truncated protein product. It is not yet clear whether the phenotype is the result of haploinsufficiency (production of an insufficient amount of the normal gene product) or a transdominant negative effect (interaction of the truncated product with other proteins) (17). The failure of ENS development in sox10-deficient mice appears to result from excessive cell death in the mutant NC cells early in their migratory pathway (18).

Significant *Sox10* mutations have been identified in individuals with pigment abnormalities, deafness, and Hirschsprung disease (the Waardenburg-Shah syndrome or Waardenburg syndrome type IV) (19–23).

Phox2B. Phox2b is a homeodomain-containing transcription factor whose expression is limited to a subset of neuronal precursors. Enteric *phox2b* expression begins in vagal and truncal NC-derived cells as they invade the foregut mesenchyme and continues in the adult submucosal and myenteric plexus (24, 25).

Table 1. Rodent models of abnormal ENS development

Gene product	Gene alteration	Phenotype observed
Sox 10	Naturally occurring point mutation results in a truncated protein in DOM mice	Sox10 ^{Dom/sox10} ^{Dom} : complete aganglionosis of gut Sox10 ^{Dom/+} : distal hindgut aganglionosis
Phox2b	Targeted gene disruption	Homozygous mice exhibit NC colonization of foregut only. Subsequently, the foregut NC-derived cells undergo apoptosis. At birth, animals exhibit complete aganglionosis of the gut.
Mash1	Targeted gene disruption	Homozygous mice exhibit NC colonization of stomach and distal gut but lack ENS in the esophagus. The distal gut is deficient in serotonergic neurons.
Ret/gdnf/gfra1	Targeted gene disruption	Mice homozygous for disruption of any one of these genes exhibit a failure of NC colonization of the gut distal to the gastric cardia. The esophagus and gastric cardia also exhibit a reduced population of neurons and glia.
Ednrb/edn3/ece1	Targeted gene disruption and naturally occurring null mutations in <i>piebald lethal</i> mice (ednrb), <i>lethal spotting</i> mice (edn3), and <i>spotting lethal</i> rats (ednrb)	Mice homozygous for disruption of any one of these genes exhibit a failure of NC colonization of the hindgut. The length of aganglionosis is variable.
Ncx	Targeted gene disruption	Homozygous mice exhibit increased number of neurons and ganglia in the colon postnatally and develop distal intestinal obstruction.
Hoxa-4	Transgenic overexpression with ectopic expression in the colon	Homozygous mice exhibit abnormal ganglia in the colon and short-segment hypoganglionosis in the distal colon.
Ret/ntn/gfra2	Targeted gene disruption	Mice homozygous for disruption of ntn of gfra2 exhibit decreased number of ganglia and decreased ganglion size in the myenteric plexus. Specific subsets of nerve fibers are reduced in the small intestine.

Phox2b^{-/-} mouse embryos die *in utero*, probably as a result of catecholamine deficiency. Analysis of midgestation embryos demonstrates ENS precursors (expressing *sox10*) in the foregut with a sharp demarcation at the proximal stomach (blue in Fig. 2). ENS precursors arrive at the foregut but fail to migrate further. This phenotype is very similar to that of the *ret*^{-/-}, glial cell line-derived neurotrophic factor (*gdnf*)^{-/-}, or *gfra1*-deficient mice. In addition, the foregut ENS in *phox2b*^{-/-} embryos undergoes apoptosis after colonization. No foregut (or midgut) ENS is identified in *phox2b*^{-/-} embryos after midgestation (26).

The ENS precursors in *phox2b*^{-/-} mice fail to express TH or DβH. Further, there is no *ret* expression in ENS precursors in the mutant embryos. Regulation of *ret* by *phox2b* could account for the failure of the postesophageal ENS (red in Fig. 2) to develop and explain the phenotypic similarity between

phox2b^{-/-} and *ret*^{-/-} mice at midgestation. *Phox2b* also appears to be required for maintenance but not induction of *mash1* expression in sympathetic ganglia. *Mash1* expression is necessary for the development of the foregut ENS. Although the foregut ENS initially develops in *phox2b*^{-/-} embryos, it apparently cannot be maintained in the absence of ongoing *mash1* expression (26).

***Mash1*.** *Mash1* is a basic helix-loop-helix transcription factor that is essential for development of the autonomic nervous system. *Mash1* was originally identified in an immortalized sympathoadrenal progenitor cell line. *In vivo*, *mash1* is expressed in apparent precursors to sympathetic neurons before overt neuronal differentiation. *Mash1* is expressed by the ENS precursors as they invade the foregut mesenchyme (26, 27).

The role of *mash1* in ENS development was identified through targeted disruption of the *mash1* gene in mice.

Figure 2. Schematic presentation of the NC-derived cell contribution to the mammalian ENS. Truncal NC-derived cells (shown in blue) populate the esophagus and gastric cardia. These cells are able to colonize the gut in the absence of *ret* activation. However, survival of these cells appears to be dependent upon *mash1* expression. Vagal NC-derived cells (shown in red/orange) populated the entire gut. These cells are unable to colonize the gut in the absence of *ret* activation and *phox2b* expression, likely due to the regulation of *ret* expression by *phox2b*. Development of a subset of these cells in the distal gut also appears to be dependent upon *mash1* expression. Vagal NC-derived cells fail to completely colonize the hindgut in the absence of *ednrb* activation in the region of the cecum. The length of aganglionosis resulting from *ednrb*-deficiency ranges from total colonic to short distal segment, depending on unidentified modifying genes. *Ncx* appears to be important for postnatal apoptosis in the colonic ENS. In the absence of *ncx*, the ENS exhibits a physiologically important increased number of neurons. The final contribution to the ENS by sacral NC-derived cells is unclear, as the hindgut ENS fails to develop in the absence of hindgut colonization by the vagal NC-derived cells.

Mash1^{-/-} mice have no ENS in the esophagus. The ENS is present in the distal stomach and intestine (red in Fig. 2) but is either delayed in development or missing specific subsets of neurons. Cells in the *mash1*^{-/-} distal ENS exhibit *scg10* mRNA expression, a marker of sympathetic development (28). In the developing sympathetic nervous system, *mash1* expression precedes TH expression (27), and Blaugrund *et al.* (29) report an absence of TH immunoreactivity in the intestines of *mash1*^{-/-} mice shortly after midgestation. They also report a significant reduction in serotonergic neurons in the gut of near-term *mash1*^{-/-} mouse embryos compared with wild-type. No reduction was noted in calcitonin gene-related peptide (cgrp) immunoreactive cells in near-term ENS of *mash1*^{-/-} mice. Together with *in vitro* data suggesting that elimination of transiently catecholaminergic immunoreactive cells from the rat intestine shortly after midgestation leads to selective deficiency of serotonergic neurons, this observation has led to the hypothesis that *mash1* defines a lineage of ENS precursors. *Mash1*-dependent ENS precursors are suggested to include the truncal NC-derived ENS of the foregut (blue in Fig. 2) and a lineage of transiently catecholaminergic vagal NC-derived ENS precursors that are destined to form the serotonergic cells of the gut distal to the esophagus (29, 30).

Ret/Gdnf/Gfra1. The ret receptor is the signaling component of receptor complexes with four ligands (artemin, gdnf, neurturin (ntn), and persephin). The complete receptor complex includes the ret receptor tyrosine kinase and a glycosylphosphatidylinositol-anchored binding component (gfra1, gfra2, gfra3, or gfra4). Each binding component preferentially interacts with specific ligands *in vitro* (31–35). *In vivo*, the absence of gdnf/gfra1-mediated signaling leads to failure of ENS development, whereas absence of ntn/gfra2-mediated signaling leads to more subtle abnormalities in ENS development. Ret and gfra1 are expressed by the NC-derived ENS precursors before infiltration of the foregut wall. Ret is expressed by both vagal and truncal NC-derived cells (36–38). Gdnf is expressed by the mesenchyme of the developing gut (39–41).

Mice homozygous for null mutations in *ret*, *gdnf*, and *gfra1* have almost identical phenotypes characterized by failure of ENS development distal to the esophagus and gastric cardia (red in Fig. 2). They also have renal abnormalities and die shortly after birth (42–46). A reduced population of neurons and glia survive in the foregut of *ret*^{-/-} embryos. Noncatecholaminergic ENS precursors in the foregut of *ret*^{-/-} embryos undergo apoptosis around midgestation (9, 47).

Ret-expressing cells are multipotential progenitors of the mammalian ENS. Organ culture studies demonstrate that ret-positive cells purified from the intestine of mouse embryos just past midgestation proliferate extensively and differentiate into both neurons and glia. Organ culture experiments also demonstrate that the effects of the *ret*^{-/-} mutation are cell autonomous, and the primary cell type in which ret function is required is the ENS progenitor cell (48). That is, failure of colonization of the fetal gut by NC cells in ret-deficient embryos does not result from abnormalities in the gut mesenchyme.

In vitro, ret signaling promotes survival, proliferation, and differentiation of ENS progenitors present in young fetuses.

The effects are developmental stage-specific as similar cultures from later-stage fetuses show diminished response to ret signaling (48, 49). This may explain in part why mutation of the gene encoding another ret ligand, ntn, leads to a dramatically different ENS phenotype.

Ret expression is not detected in the developing ENS of *phox2b*^{-/-} embryos, suggesting that *phox2b* directly or indirectly regulates *ret* expression (49). *Ret*^{-/-} and *phox2b*^{-/-} mice have similar ENS defects distal to the gastric cardia, *i.e.* an apparent failure of ENS precursors to colonize. *Phox2b*- and *ret*-null mutations do not appear to affect colonization of the truncal NC-derived cells in the foregut. Instead, evidence suggests that although all cells in this region are dependent on *phox2b* (and *mash1*) expression for survival, there is at least a subset of cells (transiently catecholaminergic cells) that is independent of ret expression for survival (9).

RET mutations are the most commonly found mutations in individuals with Hirschsprung disease. Thirty to 50% of individuals with familial Hirschsprung disease carry mutations in RET. A population-based study documents RET mutations in 3% in individuals with isolated (*i.e.* nonfamilial) Hirschsprung disease (11, 50). Polymorphisms in RET may predispose to Hirschsprung disease in a complex low-penetrance fashion (51). RET mutations are more common in long-segment Hirschsprung disease (52). A few GDNF mutations and no GFRα1 mutations have been identified in humans with Hirschsprung disease (53–55). Whereas mice heterozygous for *ret* and *gdnf* mutations are healthy, heterozygosity for RET and GDNF mutations in humans significantly increases the risk of having Hirschsprung disease. That is, Hirschsprung disease resulting from RET and GDNF mutations is inherited in an autosomal dominant fashion with incomplete penetrance.

ret mutations in mice and humans causing distal intestinal aganglionosis are generally loss-of-function mutations. The multiple endocrine neoplasia type 2 syndrome (MEN2) is caused by gain-of-function mutations in RET. Interestingly, Hirschsprung disease is rarely found in families with MEN2. The implications for cellular signaling of the RET mutations carried by these families are a fascinating area of ongoing research (56–59).

Ednrb/Edn3/Ece1. There are three edn isopeptides, edn1, edn2, and edn3, encoded by separate genes. There are two G protein-coupled membrane receptors for endothelin (edn), endothelin-A receptor (ednra), and endothelin-B receptor (ednrb) (60, 61). Ednra shows high-affinity binding for edn1 and does not bind edn3 at physiologic concentrations. Ednrb accepts all three ligands with equal high affinity. Edn is produced as an inactive prepropeptide. PreproEdn undergoes two proteolytic processing steps to produce the active peptide (62). The first cleavage produces biologically inactive big edn. Big edn is cleaved by a specific protease, endothelin-converting enzyme (ece), to produce biologically active edn. Two ece genes are identified, *ece1* and *ece2* (63, 64).

During embryonic development, *ednrb* is expressed in premigratory and migratory NC cells. Enteric expression follows a rostral-to-caudal progression in a time course consistent with expression by the colonizing ENS precursors, and it colocalizes with TH expression. *Ednrb* expression is also

detected in enteric non-NC-derived cells (the epithelium of the foregut diverticulum) before arrival of NC cells to the foregut. It is expressed later in development, after NC colonization, by mesenchymal cells of the gut (65, 66). *In vitro*, *ednrb* expression is reported beginning shortly before vagal NC-derived cells complete colonization of the hindgut in mice in enteric mesenchymal cells (67). *Edn3* and *ece1* are expressed by the developing gut mesoderm (65, 68). *Edn3* expression is highest in the cecum (69).

Mice with homozygous targeted disruption of *ednrb* or *edn3* have distal intestinal aganglionosis and coat color spotting. The long-studied naturally occurring mutations in mice, piebald lethal and lethal spotting, are mutations in *ednrb* and *edn3*, respectively (70, 71). In addition, a naturally occurring *ednrb*-null mutation exists in rats (spotting lethal) (72, 73). This mutation leads to total colonic aganglionosis (66, 74). The similarity between the phenotypes of *ednrb*- and *edn3*-deficient animals demonstrates that *edn1* and *edn2* cannot compensate for the missing *edn3*. That is, *edns* act strictly in a paracrine fashion during development.

The critical *ece* for activation of both *edn1* and *edn3* during embryonic development is *ece1*. *Ece1*^{-/-} mice die *in utero* or shortly after birth with defects in NC-derived structures of the heart and craniofacial tissues. Histologic examination of these pups demonstrates that they also exhibit distal intestinal aganglionosis (68).

Using a *DβH*-β-galactosidase (*lacZ*) transgene to visualize the colonizing ENS precursors, abnormalities in ENS colonization of the developing gut are first noted in the cecum of *ednrb*-deficient mice just past midgestation (75). Elegant genetic studies allowing for time-controlled expression of *ednrb* in mice found that the critical period for *ednrb* expression is during the period when the vagal NC-derived cells are colonizing the distal ileum through proximal colon (76). Organ culture experiments demonstrate failure of colonization by NC cells when mouse embryonic gut explants are treated with an *ednrb* antagonist during a similar time frame, whereas no effect on colonization is noted when the gut explants are treated later (77). The critical time and/or location for *ednrb* activation in vagal NC-derived cells is illustrated in orange in Figure 2. An absence of *ednrb* signaling as the ENS precursors colonize the periceal region leads the ENS deficits, ranging from distal colonic aganglionosis to total colonic/distal ileal aganglionosis.

Studies using tissue-specific expression of *ednrb* or *edn3* via a transgene in *ednrb*- or *edn3*-null mice and rats demonstrate that activation of *ednrb* on NC-derived cells allows for apparently normal ENS development. These experiments used the same *DβH* promoter used to follow ENS precursor colonization of the mouse gut by *lacZ* staining. In the *ednrb*-null rat, expression of a *DβH*-*ednrb* transgene results in rats that live and grow well into adulthood with no obvious abnormalities in intestinal function (66). Similar experiments in *ednrb*-null mice demonstrate the formation of *cgrp*, substance P, and serotonin-positive neurons in the distal gut (M. Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX, unpublished data). These studies suggest that expression of *ednrb* on ENS precursors as they are colonizing the developing gut is sufficient for ENS development. That is, expression on

other cell types is not necessary. A *DβH*-*edn3* transgene has also been used to drive *edn3* expression specifically in NC-derived cells in the developing gut of *edn3*-null mice. Mice expressing *edn3* only under the control of the *DβH* promoter develop a functionally normal ENS and live well into adulthood (78). Thus, ectopic expression of the ligand on the receptor-expressing cells is sufficient to allow complete colonization of the gut.

EDNRB and *EDN3* mutations are described in isolated Hirschsprung disease and in Waardenburg-Shah syndrome. *EDNRB* or *EDN3* mutations account for approximately 10% of Hirschsprung disease. These mutations are generally autosomal dominant with incomplete penetrance (11). There is also a report of an *ECE1* mutation in an individual with Hirschsprung disease (79). Interestingly, a recent report describes decreased *EDN3* expression in the ganglionic and aganglionic gut of individuals with Hirschsprung disease with no detected mutation in the *EDN3* gene (80). This suggests that down-regulation of *EDN3* expression may be a more common abnormality leading to aganglionosis.

Ncx (Enx/Hox11L1). The homeobox transcription factor *ncx* (also known as *enx* or *hox11L.1*) is expressed in a subset of NC-derived tissue including ENS precursors starting just past midgestation in the mouse. Myenteric and submucosal neurons in the distal ileum, colon, and rectum express *ncx* postnatally (81, 82).

Approximately 50% of *ncx*^{-/-} mice on a mixed genetic background develop distal intestinal obstruction and die in the first weeks of life. Histologic examination reveals the presence of an ENS in the distal narrowed segment of colon. Both excitatory and inhibitory neuron numbers and the total number of myenteric ganglia appear to be increased in the colons of symptomatic and asymptomatic *ncx*^{-/-} animals. In contrast, *ncx*^{-/-} mice exhibit a moderate decrease in neurons per ganglia and inhibitory neuron numbers in the distal ileum (81, 83).

The abnormal ENS pattern in *ncx*^{-/-} mice is reportedly not distinct until after 2 wk of age (83). This suggests that *ncx* may play a role in proper neuronal identity or pattern formation after colonization. Indeed, whereas up to 50% of the colonic ENS neurons in wild-type mice undergo apoptosis in the postnatal period, significantly less apoptosis is noted in the colonic ENS of *ncx*^{-/-} mice (M. Hatano, Chiba University Graduate School of Medicine, Chiba, Japan, unpublished data). This suggests that *ncx* is an important regulator of postnatal apoptosis in the ENS and that this postnatal apoptosis is physiologically important.

The *ncx*^{-/-} mouse is proposed as a model for intestinal neuronal dysplasia. The further characterization of these mice may help to better define this controversial disease entity.

Hoxa-4. *Hoxa-4* is another homeobox transcription factor. *Hoxa-4* is expressed during embryonic development in the gut mesoderm. *Hoxa-4* is normally expressed in the mouse gut from the foregut to the small intestine (84–86). Several lines of transgenic mice have been created carrying multiple copies of the *hoxa-4* gene under the control of its native promoter. These transgenic mice overexpress *hoxa-4* and also show some ectopic expression in that the transgene is expressed in mesenchyme throughout the bowel (87).

Megacolon occurs in neonatal and adult transgenic mice that overexpress the *hoxa-4* gene. The phenotype is variable between the different transgenic lines in its time of onset and severity. Mice in the most severely affected line die with intestinal obstruction by postnatal d 10. Mice in the least severely affected line develop megacolon only rarely as adults (88).

The intestine of one of the intermediate lines, in which all individuals develop megacolon but are still able to breed, was studied in detail. Abnormalities in gut development are first noted at a time when vagal NC-derived cells are normally completing colonization of the hindgut. At this time, the mucosa and mesenchyme of the terminal colon are significantly enlarged in *hoxa-4* transgenic mice. Neuron differentiation is advanced, but these neurons do not initially form a myenteric plexus. Pelvic ganglia apparently invade the bowel and form ectopic ganglia within gaps in the longitudinal muscle layer. Neonatal *hoxa-4* transgenic mice exhibit a short segment of hypoganglionic terminal colon. In addition, the ganglia that are present are abnormally located (88, 89). Because this is a gain-of-function transgenic study, it is difficult to infer from these studies the physiologic roles of *hoxa-4* in normal ENS development.

Ret/Ntn/Grf α 2. Gfr α 2 and ntn are, like gfr α 1 and gdnf, capable of forming a ret-activation complex. Gfr α 2 is expressed in NC derivatives in the gut. It is most highly expressed in the gut on postnatal d 14 when gfr α 1 expression is very low. Interestingly, gfr α 2 shows variable levels of expression along the gastrointestinal tract, with highest expression in the small intestine (90). Ntn is expressed by mesenchymal cells of the gut, with highest levels of expression in the postnatal small intestine (31, 36, 91).

Mice with null mutations in the *gfr α 2* and *ntn* have reduced innervation in the myenteric plexus and have abnormal gastrointestinal motility. *Gfr α 2*^{-/-} and *ntn*^{-/-} mice have a reduced number of fine acetylcholinesterase-positive fibers in the small intestine, particularly in the duodenum. Specific subsets of fibers appear to be reduced, including substance P-expressing fibers. Decreased number of ganglia or decreased ganglion size is also reported in these animals. Functionally, abnormalities in neurotransmitter release and abnormal contractile responses to electrical stimulation are also reported. *Gfr α 2*^{-/-} mice exhibit growth failure beginning at 2 wk of age. Reduced intestinal innervation and motility in *gfr α 2*^{-/-} mice may contribute to their growth retardation in which there is relative preservation of brain size and body length. Interestingly, *ntn*^{-/-} mice show no growth abnormalities (90, 92).

The timing of normal expression and the abnormalities seen in *gfr α 2*- and *ntn*-deficient mice suggest that ret activation by *gfr α 2*/ntn may be an important trophic factor for postmitotic enteric neurons and that it may be required for the maintenance of a subset of enteric ganglia. In contrast, ret activation by *gfr α 1*/gdnf may be crucial for the survival, proliferation, and differentiation of NC cells during early stages of ENS development. Thus, the nonoverlapping roles of gdnf and ntn may be a consequence of their stage-specific expression in the mammalian gut, the stage-specific expression of *gfr α 1* and *gfr α 2*, or qualitatively different responses by ret.

A heterozygous *NTN* mutation in a family with multiple members affected with Hirschsprung disease is reported. It appears, however, that the *NTN* mutation reported is not sufficient to cause Hirschsprung disease, as this family also carries a significant *RET* mutation. The addition of an *NTN* mutation to a *RET* mutation may significantly worsen the disease, as individuals with both mutations exhibited aganglionosis beginning in the distal ileum (93).

CONCLUSIONS

Although our understanding of the development and structure of the ENS is still quite limited, rapid advances are being made. We can now divide the ENS into several subsets of neurons defined by origin, region of colonization, and neurotransmitter/biochemical marker expression. We have the first indications of the importance of postcolonization/postnatal remodeling of the enteric plexuses to the formation of a functioning ENS. These findings are important steps in the quest to diagnose and treat subtle abnormalities in the ENS.

Studies examining the developmental potential of enteric NC-derived cells are a direct outgrowth of the developmental studies of the ENS described in this review. Several studies suggest that enteric NC-derived cells remain in a multipotential state for a considerable period after invasion of the mammalian fetal gut. The identification of ENS stem cells may eventually allow for the correction of intestinal neuronal deficiencies.

Currently, mutations of seven genes (*sox10*, *ret*, *gdnf*, *ntn*, *ednrb*, *edn3*, and *ece1*) are implicated in the pathogenesis of Hirschsprung disease. However, there remain many important issues to be resolved related to Hirschsprung disease. For example, no mutation in any of these genes can be identified in the majority of individuals with Hirschsprung disease. Further, mutation in none of these genes yet explains the well-described association of Hirschsprung disease with trisomy 21 or the male predominance of Hirschsprung disease.

Obviously, many genetic defects can affect ENS development, and there are undoubtedly many layers of redundancy in the system, allowing some individuals with clear mutations to develop a functionally normal ENS. We can look forward to more advances in our understanding of ENS development resulting from the study of transgenic and knockout rodents. Disorders described in animals will need to be correlated with detailed pathologic studies in humans, making histologic studies of the ENS in children particularly important. As progress continues, this basic knowledge will be transferred to the clinic to improve the diagnosis and treatment of individuals with both common and rare ENS disorders.

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