19. DEVELOPMENT OF THE ENTERIC NERVOUS SYSTEM (ENS)

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SUMMARY

The ENS is more complex and "brain-like" than other regions of the PNS; therefore, the mechanisms that control ENS development differ from those responsible for the differentiation of non-enteric peripheral ganglia. The ENS is derived from precursor cells that migrate to the bowel from vagal, rostral truncal, and sacral levels of the neural crest. These precursors find their way to the fetal gut by following defined pathways that lead to the bowel from the 3 regions of the crest. The molecules that define these migratory pathways have not yet been identified; however, guidance molecules produced by the gut have been discovered, which attract or repel migrating crest-derived émigrés. The population of crest-derived cells that colonizes the gut is multipotent when it arrives in the bowel. Differentiation of enteric neurons and glia, therefore, depends on signals these cells receive from the microenvironment of the fetal gut and is characterized by a progressive loss of developmental potential and a corresponding increase in commitment. Stages in this progression can be recognized by the developmentally regulated expression of a series of transcription factors and growth factor receptors. Deletion of one of these factors leads to an almost complete absence of neurons and glia in the bowel, and loss-of function mutations in genes that encode them cause Hirschsprung's disease (aganglionosis of the terminal colon) in affected human subjects. Other factors affect the development and differentiation of limited numbers or lineages of enteric neurons and thus may lead to defects that are morphologically subtle, even if they are functionally devastating. The ENS may thus be quite abnormal even if ganglia are found when a pathologist examines the bowel. This problem severely limits current diagnosis and treatment of congenital neuromuscular disorders of the gut.

LEARNING OBJECTIVES

At the end of the lecture you should be able to:

1) Discuss the innervation of the gut and why it is different from that of other organs, what these differences are, and what is known of the developmental mechanisms that are responsible for them.

2) Describe where in the embryo the neurons and glia of the ENS come from and what is known about the functions of guidance molecules in enabling the precursors of enteric neurons and glia to find their way to their correct destinations in the gut and the pancreas.

3) Discuss microenvironmental factors that sculpt the differentiation of enteric neurons and glia.

4) Explain why differentiation involves a progressive decline in potential accompanied by a matching gain in specification.

5) Explain how stages in differentiation can be recognized by demonstrating a number of transcription factors and growth factor receptors that serve as markers.

6) Explain why some of these markers are essential for cell survival, and that loss-of-function mutations in genes that encode them lead to congenital neuromuscular diseases of the bowel, such as Hirschsprung's disease.

GLOSSARY

Big endothelin: the inactive secreted form of the endothelins. Activation requires conversion to an endothelin peptide by an endothelin converting enzyme (ECE), which is present in target tissues.

DCC: Deleted in colorectal cancer; receptor expressed by migrating crest-derived cells that is activated by netrins; knockout leads to absence of submucosal and pancreatic ganglia.

Ectomesenchyme: embryonic connective tissue of neural crest origin.

Endothelin3 (End3): peptide that acts on the **endothelinB** receptor (**EdnrB**) to prevent the premature differentiation of enteric neurons. Required to finish the colonization of the bowel by cells from the neural crest. (End3 = animal peptide; END3 = human peptide; EdnrB =animal receptor; EDNRB = human receptor.)

Enteric nervous system: intrinsic innervation of the gut.

GDNF: growth factor, attracts migrating crest-derived cells to the bowel and promotes their proliferation.

Hirschsprung's disease: Congenital aganglionosis of the terminal colon; also called congenital megacolon because of the dilation of the colon proximal to the aganglionic zone.

IPAN: intrinsic primary afferent neuron (in the ENS).

Mash-1: transcription factor expressed by a subset of crest-derived precursors of enteric neurons and glia; required for the development of early-born enteric neurons, including neurons that use serotonin or nitric oxide as their neurotransmitter.

Netrins: family of guidance molecules that attract crest-derived cells migrating in the bowel to the submucosa and pancreas. Required for the formation of submucosal and pancreatic plexuses of ganglia.

Neurotrophins: family of growth factors that act on receptor tyrosine kinases (Trk).

NT-3: neurotrophin required for the development of particular lineages of enteric neurons, including submucosal IPANs; activates **TrkC**.

$p75^{NTR}$: The common neurotrophin receptor, exerts effects of its own, such as promotion of cell death, but more often acts as a co-receptor, making the actions of Trks more specific and of higher affinity.

Phox2b: transcription factor expressed by crest-derived precursors of enteric neurons and glia.

Ret: receptor tyrosine kinase expressed by crest-derived precursors of enteric neurons and glia; responds to **GFRα1** and **GDNF**. Ret = animal protein; *Ret* = animal gene; RET = human protein; RET = human gene.

Sox10: transcription factor expressed by crest-derived precursors of enteric neurons and glia.

TEXT

Because the ENS is a unique region of the PNS, the mechanisms of its development are different from those of other peripheral ganglia

The arrays of intrinsic ganglia that innervate the bowel are called, collectively, the "Enteric Nervous System" or ENS (Fig. 19-1). The arrays of intrinsic ganglia that innervate other organs are called simply, pulmonary ganglia, bladder ganglia, cardiac ganglia etc. Ganglia within innervated

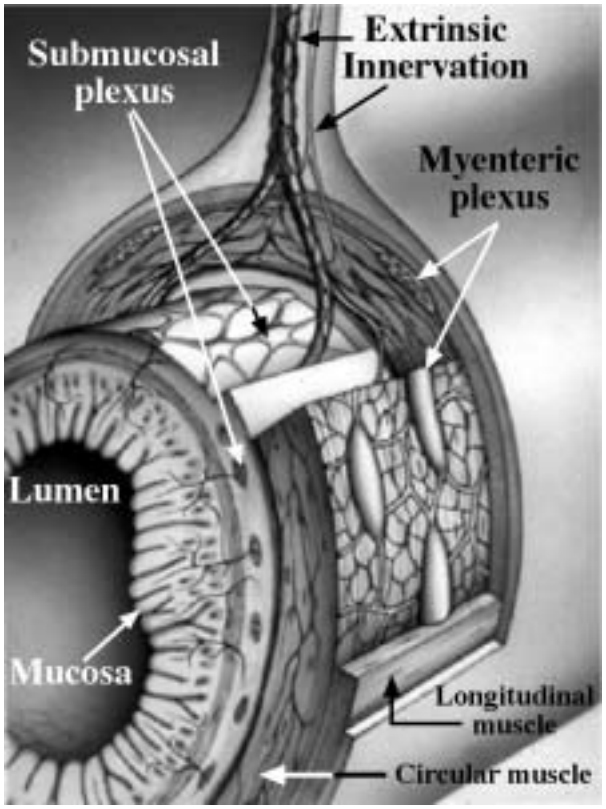

Fig.19-1 Diagram of the wall of the mature small intestine showing the locations of the components of the ENS, submucosal and myenteric plexuses, as well as routes to the bowel of extrinsic nerves that carry impulse traffic to and from the CNS.

organs are characteristic of parasympathetic ganglia. The ENS is located within the gut; why then is it designated as a "system" and not just another collection of parasympathetic relay ganglia? The answer is that the ganglia of the ENS are unique and different from the ganglia found in any other organ. These differences are anatomical, functional, biochemical, and ultimately, therefore, developmental. Anatomically, the ENS resembles the CNS rather than peripheral nerve. Support for the neurons of the ENS is provided by enteric glia, cells that are analogous to CNS astrocytes, rather than by Schwann cells and, as in the CNS, the ENS contains no collagen. The ENS is also very large. It contains more neurons ($> 10^8$) than the spinal cord and more than the combined total of the remainder of the PNS. Functionally, the ENS contains intrinsic primary afferent neurons (IPANs), which function like the sensory neurons in dorsal root and cranial nerve ganglia except that enteric IPANs do not project to the CNS. Instead, they enable the ENS to respond to stimuli even in the total absence of input from the brain or spinal cord. The bowel thus can control its own behavior and, because neurons of the ENS project out the gut, the bowel can also regulate the behavior of neighboring organs, such as the gall bladder and pancreas. These behaviors may consist of simple reflexes or may be quite complex. In fact, many enteric neurons receive no direct input from the CNS and thus cannot be fitted into either the definitions of the sympathetic or parasympathetic nervous systems, which are classified on the basis of their differing outflows of preganglionic fibers from the CNS. Biochemically, the ENS resembles the CNS in that every class of neurotransmitter found in the CNS is also found in the ENS. That means that there are many different types of neurons in the ENS. Clearly, because ganglia of the ENS are different from those of other organs, developmental mechanisms must exist that are responsible for making them so. The ENS, moreover, has to be ready and operative at birth when oral feeding begins. Life in the uterus can be lived in the absence of an ENS. Life out of the uterus requires a functioning ENS. A region of the bowel that lacks an ENS becomes a functional obstruction (pseudoobstruction). The gut thus dilates proximal to a region where the ENS is defective and may perforate or cause a lethal systemic infection.

The ENS develops from a multipotent population of crest-derived precursor cells that migrates to the bowel along defined routes

Conceivably, the ENS could be unique because the precursor cells from which it is derived are genetically determined and specified to enteric fates that are themselves unique. In other words, the

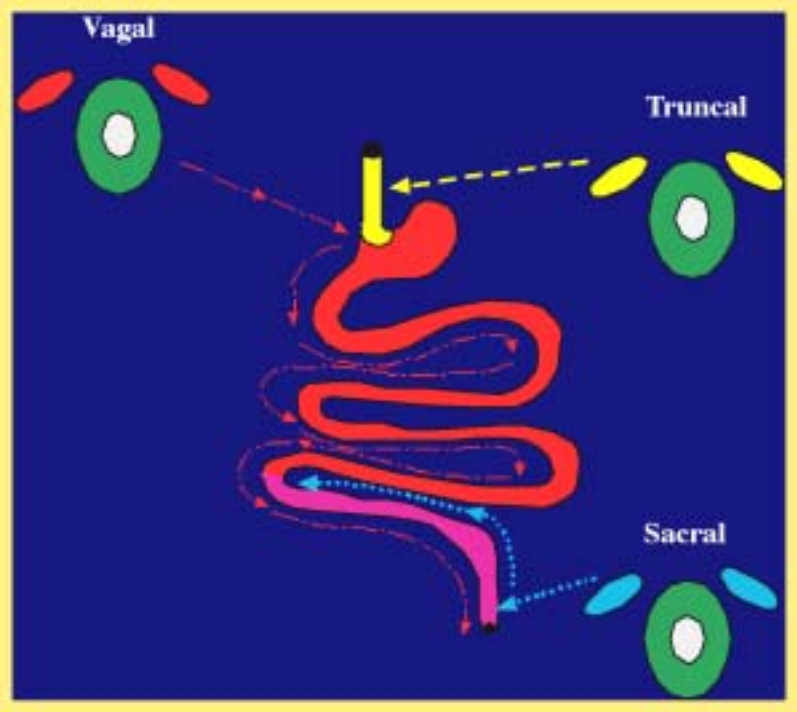

Fig. 19-2 The fetal bowel is colonized by émigrés from vagal (red), sacral (blue) and rostral truncal (yellow) regions of the neural crest. Vagal crest-derived cells colonize the entire bowel, sacral cells only the post-umbilical gut, and truncal cells the esophagus and cardiac stomach.

special properties of the ENS may all be encoded in the genome and represented in developmental programs that neural crest cells that are destined to become enteric neurons and glia go through. Surprisingly, predestination appears not to be the case, or at least not entirely the case. In common with most of the neurons of the PNS, all of the cells of the ENS are émigrés from the neural crest. This origin was originally inferred by experiments of Yntema and Hammond who, in the mid-1950's, deleted what they called the anterior (now known as vagal) neural crest of chick embryos. These deletions cause a failure of enteric ganglia to develop. A more direct confirmation of the neural crest origin of the ENS was provided in the mid-1970's by Nicole LeDouarin and her co-workers. These investigators traced crest-derived cells to the bowel by using quail-chick interspecies chimeras. The nuclei of quail cells exhibit a distinct region of nucleolar-associated heterochromatin that enables these cells to be recognized easily in a field of chick cells (Fig. 19-3). Chimeras are constructed by surgically removing regions of the neural crest from chick embryos and replacing them with quail neural crest (or the reverse, replacing quail crest with that of the chick). Quail crest-derived cells migrate through chick embryos just as they do through quail embryos. Any cell with a quail nucleus that is found in a target organ of a chimeric embryo, which has received a substituting graft of quail neural crest cells, therefore, can be presumed to have migrated to that organ from the neural crest. By constructing a variety of quail-chick chimeras of this type, LeDouarin demonstrated that crest-derived cells migrate to the bowel from the vagal (adjacent to somites 1-7) and sacral neural crest (caudal to somite 28). Since the publication of her studies, more modern investigations, which have used lipophilic dyes or replication-deficient retroviruses expressing a marker to label crest cells, have confirmed that the ENS does indeed originate from the vagal and sacral crest, but they have also revealed that the esophagus and adjacent stomach are colonized by cells that migrate to the gut from the rostral truncal crest (Fig. 19-2). The gut, however, does not have to be colonized by only the correct regions of the crest in order for an ENS to develop. Crest-derived cells from other axial levels will do as well if they are experimentally made

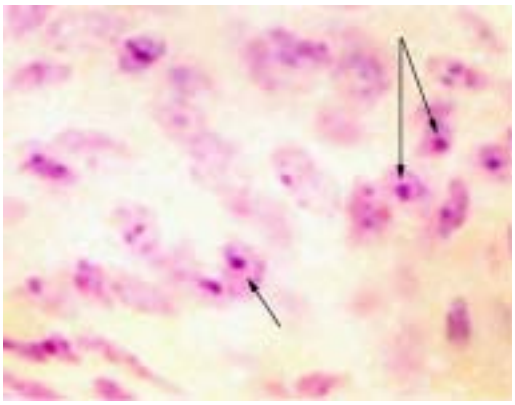

Fig. 19-3 Quail neural crest-derived cells (arrows) can be identified when DNA is stained in quail-chick chimeric embryos.

to colonize the bowel. For example, the region of the crest that ordinarily gives rise to the adrenal medulla and sympathetic ganglia will give rise to an ENS if it is transplanted (heterotopically) to the vagal region of a recipient embryo in which the grafted cells migrate to the host's bowel. The concept has thus become established that crest-derived cells find their way to the bowel from vagal, sacral, and rostral truncal levels by following defined pathways that extend to the gut from these regions of the crest. The population of cells that leaves the crest is not fully determined, but multipotent, and thus dependent on signals that the cells receive, either while they migrate, or after they arrive in gut.

Long-range guidance molecules attract crest-derived cells to the bowel and particular destinations within it

Little is known about the molecular basis of the defined pathways that extend from the neural crest to the gut. There are molecules that enteric mesodermal cells secrete, however, that attract the migration of crest-derived cells that are in the vicinity of the gut. These "guidance factors" may be important in directing the traffic of migrating crest-derived precursors to and within the bowel. One such factor is glial cell line derived neurotrophic factor (GDNF). GDNF is synthesized by the enteric mesenchyme before any crest-derived émigrés approach the gut. GDNF forms a complex with a phosphatidyl inositol-linked protein called GFRα1 and this complex stimulates a receptor tyrosine kinase express by crest-derived cells called Ret (Fig. 19-4). There are 3 other ligand-receptor complexes that activate Ret. The ligands include neurturin (NTN), artemin (ART), and persephin (PSP). Like GDNF, these ligands do no bind directly to Ret, but depend on preferred α co-receptors. GDNF and NTN are expressed in the gut, but only GDNF has the properties of a guidance molecule. *In vitro*, crest-derived cells migrate toward co-cultured explants of bowel and toward sources of GDNF. Secretion of GDNF, therefore, has been postulated to help crest-derived cells migrating in the vicinity of the gut to locate it. The location of crest-derived cells within the gut, however, does not correspond to that of GDNF, which is uniformly expressed throughout the enteric mesenchyme. Crest-derived cells descend within the gut in the outer gut mesenchyme, much like pants descend on a leg. Additional factors are therefore needed to account for the particular intra-enteric distribution of migrating crest-derived cells. These factors must dictate not only where crest-derived precursors migrate, but also where they do not migrate, and where they stop migrating to form ganglia.

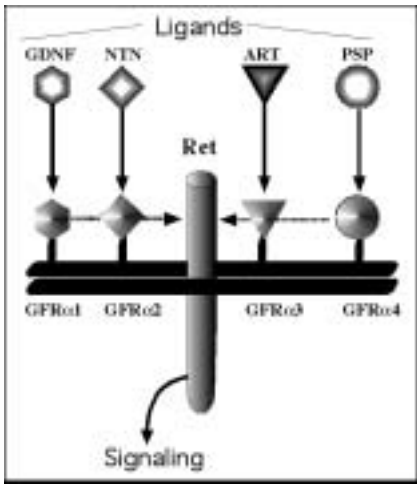

Fig. 19-4 Ligands and receptors that activate Ret.

Guidance in the formation of submucosal and pancreatic ganglia: Netrins

Within the gut, vagal crest-derived cells descend as far as the anal verge. This is a proximo-distal migration. Some crest-derived cells, however, turn perpendicularly to this major pathway and migrate inwardly from the outer gut mesenchyme toward the mucosa. These perpendicularly directed émigrés give rise to the submucosal plexus, which forms later than the myenteric and is thus the result of a secondary migration (Fig. 19-6). Additional members of the vagal crest-derived cell population also turn at a right angle to the main proximo-distal stream of migration, but these cells migrate out of

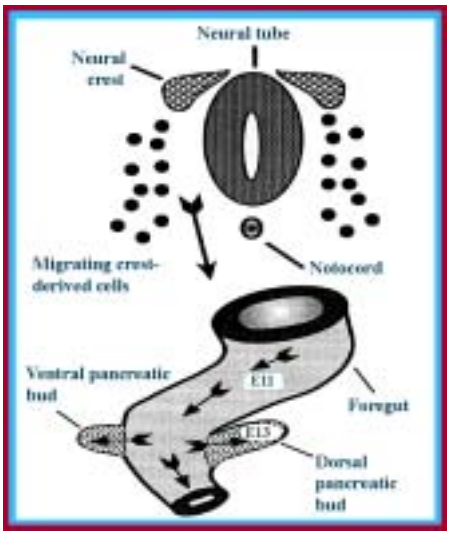

Fig. 19-5 After they colonize the gut, crest derived cells move proximodistally within the gut; subsets then turn perpendicularly to colonize the pancreas.

the gut into the dorsal and ventral pancreatic buds to give rise to pancreatic ganglia (Fig. 19-5). A class of chemoattractant molecules called netrins is responsible for both of these orthogonal migrations of enteric crest-derived cells. Netrins are expressed in the pancreas and by endodermal cells of the intestinal lining. There are 3 netrins. Netrin-1 and –3 are expressed in the mammalian bowel, while netrin-2 is expressed in the avian gut. Netrins stimulate a receptor expressed by subsets of enteric crest-derived cells called deleted in colorectal cancer (DCC). Crest-derived cells that express DCC migrate toward sources of netrins in vitro (Figs. 19-7, 19-8) and this migration can be blocked by antibodies to DCC or by drugs that interfere with intracellular signal transduction pathways activated by DCC. Crest-derived cells that are isolated from within the fetal gut migrate selectively toward a co-cultured explant of fetal bowel or toward a co-cultured explant of pancreas. These migrations too can be blocked by antibodies to DCC or by drugs that interferewith intracellular signal transduction pathways activated by DCC. Finally, if a cross section of bowel is explanted and cultured as a slab with the mucosa in the middle, a subset of crest-derived cells migrates inwardly toward the mucosa from their original position in the outer gut mesenchyme. This inward migration can be blocked by antibodies to DCC, which appear to "freeze" crest-derived cells in the outer gut mesenchyme. Finally, as might be expected from these experimental results, the submucosal plexus and pancreatic

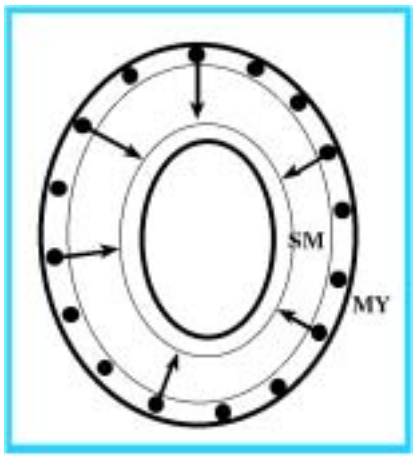

Fig. 19-6 A second subset of crest-derived cells migrating in the outer gut mesenchyme turns perpendicularly to form the submucosal plexus.

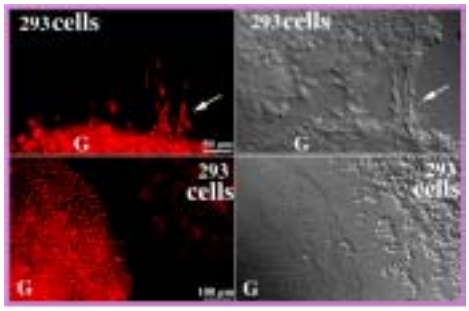

Fig. 19-7 Crest-derived cells migrate out of the primordial gut toward co-cultures cells that express netrin-1.

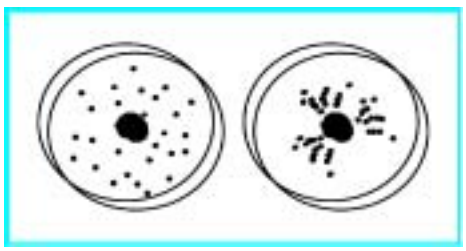

Fig. 19-8 Netrin secreting cells attract co-cultured crest-derived cells isolated from the bowel.

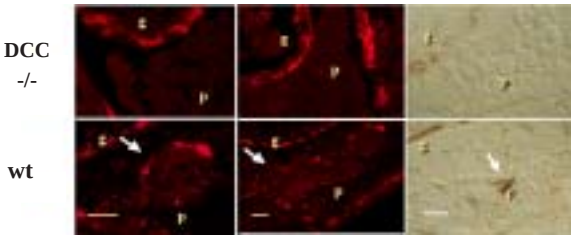

Fig. 19-9 Crest-derived cells are found in the gut (g) of mice that lack DCC; however, there are no submucosal or pancreatic ganglia in these animals.

ganglia fail to form in transgenic mice that lack DCC (Fig. 19-9). The crest-derived cells of these animals lack the ability to respond to netrins and thus colonize the bowel but fail to make the netrin-mediated right turns necessary for the formation of submucosal or pancreatic ganglia.

Progressive commitment defines stages in the development of enteric neurons and glia

As noted above, the population of crest-derived émigrés that colonizes the bowel is multipotent. In fact it is still multipotent when it arrives in the gut. Crest-derived cells that are in the fetal bowel give rise to Schwann cells and sympathetic neurons if they forced, by experimental placement in a younger host embryo, to migrate again. The post-migratory crest-derived cells in the primitive gut, however, cannot form melanocytes or ectomesenchyme (connective tissue). Melanocytes and cells derived from ectomesenchyme, like the crest-derived cells in the bowel, are terminally differentiated cells that develop from precursors that emigrate from in the pre-migratory vagal crest. The routes of migration and sites where terminal differentiation occurs for melanocytes and ectomesenchyme, however, are different from those of enteric crest-derived cells. It has been postulated that the ability to form melanocytes is lost or "filtered" out of the population of crest-derived émigrés bound for the gut as the cells traverse the caudal branchial arches. Uncommitted crest-derived stem cells are retained in the adult ENS, but the developmental potential of these stem cells, like all crest-derived cells in the postmigratory bowel, is limited to neurons and glia; moreover, once a crest-derived émigré does develop into a neuron or glial cell, its differentiation is terminal, that is neurons and glia cannot be induced to develop into other types of cell. The progress of development thus is marked by a progressive and irreversible restriction of potential. As the process of differentiation unfolds, various transcription factors and growth factor receptors are expressed by the cells that undergo developmental regulation. The expression of these factors and the growth factor receptors is often critical and obligatory for cell survival, but even if not, their appearance can be followed to identify stages in the commitment of enteric crest-derived émigrés to their fates.

Transcription factors and growth factor receptors define stages in commitment of enteric neuronal and glia precursors (Fig. 19-11)

The earliest crest-derived enteric neural precursors express a transcription factor called Phox2b. Expression of this factor is seen in early migrating cells and in the émigrés that enter the gut. If expression of Phox2b is knocked out, the ENS fails to form. Sympathetic ganglia, however, also fail to form. Phox2b expression is thus necessary for the formation of an ENS and it is a marker for a primitive cell with a great deal of developmental potential, but Phox2b expression is not gut-specific. Interestingly, a polymorphism has recently been found in the human *PHOX2b* gene. One form of the polymorphic *PHOX2b* gene is associated with the development of Hirschsprung's disease. Hirschsprung's disease, first described by a Danish

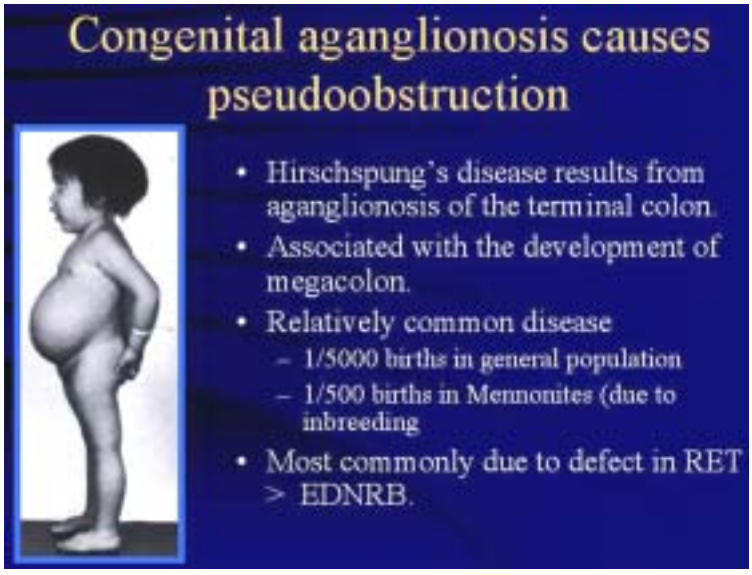

Fig. 19-10

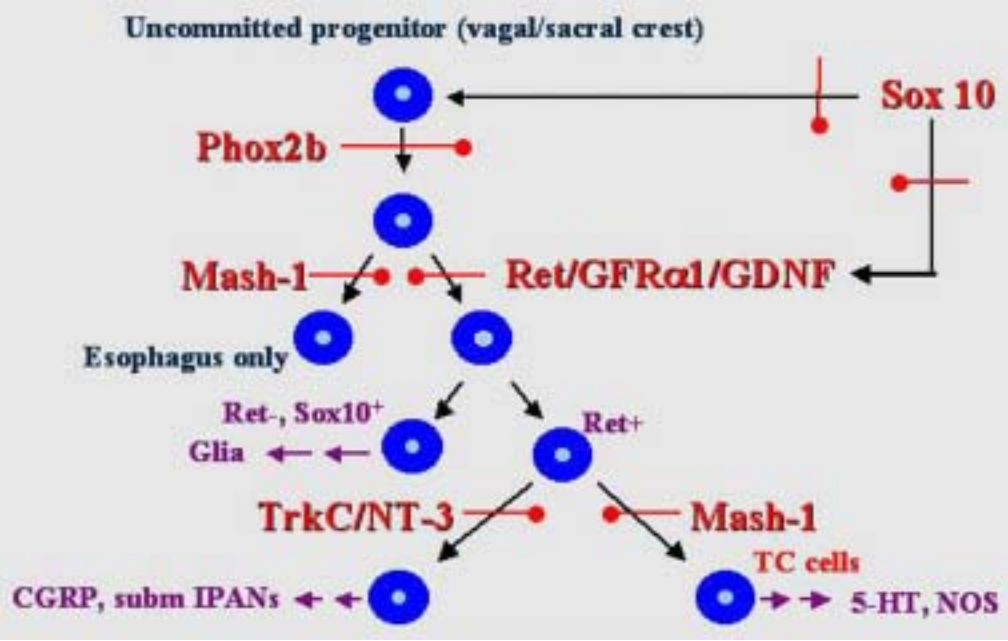

Fig. 19-11 Sequential expression of transcription factors and growth factor receptors mark the stages in the development of enteric neurons and glia. Factors active in the mouse gut are illustrated.

physician (for whom the condition is named), is due to a complete lack of ganglia (aganglionosis) in varying lengths of the terminal colon. As a result of the lack of ganglia, the gut is as obstructed as surely as it would be if it were tied off with a shoelace. The termis pseudoobstruction because there is no anatomical obstruction to the flow of intestinal contents but, in functional terms, the obstruction is quite real and causes the colon to dilate massively (megacolon) rostral to the aganglionic zone. Hirschsprung's disease is, unfortunately, a common congenital defect of the gut. It occurs with a frequency of ~ 1 in 5000 live births (Fig 19-10). It is not clear whether the mutant form of *PHOX2b* actually causes the Hirschsprung's disease but, as we will see below, other genes, which encode factors that must be expressed by enteric neurons, do so. After Phox2b, Sox10 is expressed. The developmental potential of precursors that express Sox10 appears to be more restricted that that of cells that express only Phox2b, but it is still high. Again, if Sox10 is knocked out, the ENS fails to develop, but the defect in the remainder of the PNS is not as widespread as when Phox2b is deleted. More interesting is what happens to the ENS when only one allele of Sox 10 is mutated. In this case, only the terminal bowel becomes aganglionic. The effect is dominant and occurs as a spontaneous mutation in mice called Dom. Animals with the genotype Dom -/- die prior to birth, but Dom +/- mice survive (for a time) with a condition that mimics that seen in humans with Hirschsprung's disease. Some human patients with Hirschsprung's disease have been found to carry loss-of-function mutations in Sox10.

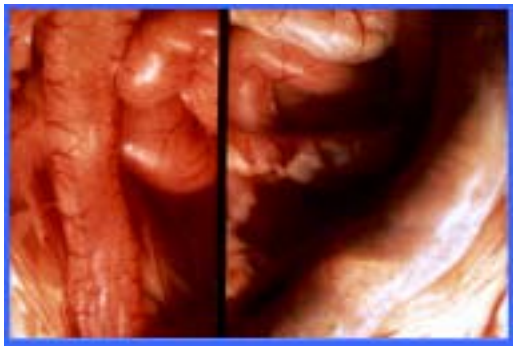

Fig. 19-12 Congenital megacolon. Normal (left); megacolon (right). In this case the condition is shown in a mouse with a spontaneous mutation in *edn3*. The appearance of the colon is similar in humans with Hirschsprung's disease due to any of the genetic defects that cause it.

After Sox 10, Ret must be expressed and the Ret expressing cells have to be stimulated by GFRα1 and GDNF. If genes encoding Ret, GFRα1, or GDNF are deleted in transgenic mice, neurons and glia fail to arise below the level of the esophagus and adjacent stomach. That observation suggests that the growth factor requirements of the rostral truncal crest-derived

cells that colonize the esophagus and adjacent stomach are different from those of the vagal and sacral crest-derived cells that colonize the remainder of the gut. The distal bowel is Ret-dependent, while the most proximal gut is independent of Ret. The gene encoding Ret is large and, in humans, subject to mutation, both sporadic and inherited. When their effect is a gain-of-function, Ret mutations are associated with multiple endocrine malignancies (MEN). When their effect is a subtotal loss-of-function, Ret mutations (and mutations of GFRα1 and GDNF) are associated with Hirschsprung's disease. One interpretation of the effects of loss-of-function mutations of Ret, GFRα1 or GDNF, is that crest-derived émigrés in the gut are not sufficiently driven to proliferate. As a result, the precursor population is depleted before the gut is fully colonized. The terminal colon is the last region of the fetal bowel to receive the colonists from the crest; thus it is the most at risk to suffer aganglionosis as a result of depletion of the crest-derived precursor population. If the starting population is too small or if it does not enlarge adequately, the crest-derived cells fail to reach the end of their road, the terminal colon. About 40% of patients with Hirschsprung's disease have loss-of function mutations in *RET*. These cases tend to be more severe than sporadic occurrences. The *Ret* mutations are commonly associated with a long segment of aganglionosis.

The development of the ENS is regulated by factors that control the rate of differentiation and the extent of proliferation

An odd gene that unexpectedly turned out to cause Hirschsprung's disease is the gene encoding the receptor for a peptide that was previously thought to be involved in the regulation of blood pressure and vascular resistance. Endothelins are peptides that cause intense vasoconstriction in adult animals and humans. There are 3 endothelins, 1, 2, and 3 (Edn1, Edn2 and Edn3). Two endothelin receptors, EdnrA and EdnrB, respond to the endothelins. Edn1 and Edn2 can stimulate EdnrA and EdnrB equally well; however, Edn3 can only activate EdnrB. The endothelins are secreted as large precursor molecules called big endothelins. The big endothelins are converted in tissues to the active small peptides that stimulate the EdnrA and/or EdnrB by endothelin converting enzymes. The big endothelins cannot stimulate EdnrA or EdnrB and are inert. The results of knocking out genes encoding endothelins, the converting enzymes, and the endothelin receptors were highly surprising. The knockout mice lacking the genes did not survive well enough to allow studies of blood pressure regulation to be carried out. Mice that lacked *edn1* or *ednra* had lethal defects of the head and neck, while mice that lacked *edn3* or *ednrb* exhibited aganglionosis of the terminal colon, mimicking that of human patients with Hirschsprung's disease. Lines of mice, which had been known for years, with spontaneous mutations that gave rise to aganglionosis of the colon turned out to have mutations in *edn3* (the lethal spotted mouse) or *ednrb* (the piebald lethal mouse) (Figs. 19-12, 19-13). Mutations in human subjects were then found in genes encoding EDN3, EDNRB, or an endothelin converting enzyme in human patients with Hirschsprung's disease. The endothelin mutations account for about 5% of the occurrences of Hirschsprung's disease. Another surprise came about in how Edn3 affects the development of crest-derived cells. It was anticipated that, like GDNF, Edn3 might promote the proliferation of crest-derived cells. Instead, stimulation of EdnrB by Edn3 inhibits differentiation and retards the

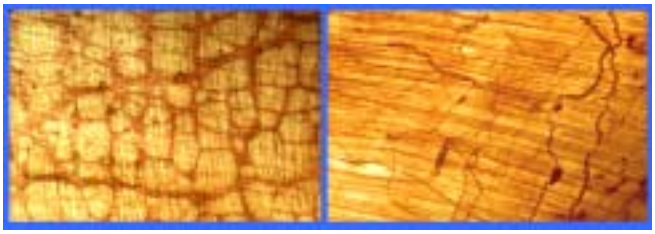

Fig. 19-13 Aganglionosis (right) in the terminal colon of a mouse lacking *Edn3*. Note that, as in the human bowel in Hirschsprung's disease, the gut is not denervated in the aganglionic zone. It contains nerve fibers. Pseudoobstruction is due to the lack of nerve cell bodies and the intrinsic reflexes they mediate.

formation of neurons. Evidently, crest-derived cells can differentiate prematurely. Crest-derived precursors migrate and proliferate; neurons do neither. If the precursor pool becomes a pool of neurons, then the gut that has not yet been colonized when premature differentiation occurs stays that way, uncolonized. Restraint is thus a critical part of development. There is a time and a place for everything. Crest-derived cells have to hold off with terminal differentiation until after they have finished their job of colonization.

Factors that affect the expression of specific lineages of enteric neurons are required late in development (see Fig. 19-11)

Genes that are required at later stages of development tend to be required for the differentiation of particular lineages of enteric neuron (or needed for the development of glia). When they are knocked out, the resulting defects are much smaller than those that occur when Phox2B, Sox10, or Ret are deleted and the resulting defects can be quite subtle. For example, Mash-1 is a basic helix-loop-helix transcription factor that is expressed by subsets of crest-derived cells in the fetal gut. When *mash-1* is knocked out, almost all sympatho-adrenal cells fail to arise and an animal is born without an effective sympathetic nervous system. On the other hand, the gut is aganglionic only in the esophagus. This led to the idea that the rostral truncal crest-derived cells, which colonize the esophagus, are different from vagal or sacral crest-derived cells, which colonize the remainder of the gut, and develop from a common "sympathoadrenal-enteric" lineage of precursor cells. The rostral-truncal crest-derived cells were considered to be Mash-1-dependent while vagal and sacral crest-derived cells were Mash-1-independent. The early studies, however, treated the presence of neurons in the gut below the esophagus in a binary "there or not-there" fashion. The ENS is much too complex to be considered "there or not-there". The bowel is not either aganglionic or normally innervated. It can be partially innervated or defectively innervated. From the standpoint of an animal or an affected human patient, inadequate innervation can be just as devastating as no innervation. When *mash-1* is knocked out, one lineage of enteric neurons, constituting about a third of the ENS fails to develop in the gut below the esophagus. Consequently, the stomach and the intestines contain ganglia, but the gut does not work. It lacks neurons that use serotonin or nitric oxide as their neurotransmitter and most of the motor neurons to smooth muscle. The condition is lethal. The lineage that is *mash-1*-dependent is transiently catecholaminergic and gives rise to neurons that are born early in development. Neurons in the lineage that survives the knockout of *mash-1*, and is thus *mash-1* independent, contain peptides, such as CGRP, and include the intrinsic primary afferent neurons (IPANs) that allow the ENS to mediate reflexes in the absence of input from the brain or spinal cord. The successors of the rostral truncal crest-derived cells in the esophagus are indeed *mash-1*-dependent; so too, however, are substantial numbers of vagal and sacral crest-derived cells. Perhaps there is a common sympatho-adrenal precursor cell; however, if so, its progeny represent only a portion of the ENS, not all of it. A great deal of current research is focused on trying to determine the genetic basis and the factors responsible for the choice made by this putative common progenitor to develop as an enteric neuron in the gut and as a sympathetic neuron out of the gut.

A very well known family of growth factors that is required late in development is that of the neurotrophins. This family is famous becuase it includes nerve growth factor (NGF), the first growth factor to be discovered. The neurotrophin family consists of NGF; brain derived neurotophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT4/5). These molecules activate a common receptor, called $p75^{NTR}$, and a set of receptor tyrosine kinases called TrkA, TrkB, and TrkC. The Trk receptors are responsible for the specificity of the effects of individual neurotrophins. TrkA is the preferred receptor for NGF; TrkB is the preferred receptor for BDNF and NT-4/5; TrkC is the preferred receptor for NT-3. Neurotrophins are target-derived factors that are encountered by the axons of neurons

when they innervate their downstream targets. Many axons arrive in an innervated target and may compete for a limited supply of neurotrophin. Neurons that do not receive enough of a required neurotrophin die. That is a way of getting rid of excessive numbers of neurons by eliminating those that fail to innervate the target cells they should be innervating. BDNF and NT-3 are both expressed in the bowel and subsets of crest-derived cells express TrkC or TrkB. Little is known about the effects of BDNF or TrkB with respect to the formation of the ENS. NT-3, however, has been found to be necessary for the production of many cells of the submucosal plexus, a number of interneurons in the myenteric plexus and, critically, for the IPANs of the submucosal plexus. As a result, the knockout of NT-3 or TrkC is lethal, even though the effects of these deletions on the gut cannot be detected with routine methods.

One lesson to be learned from the examination of the bowel of mice lacking *mash-1*, *NT-3*, or *TrkC* is that methods used routinely by pathologists to diagnose congenital neuromuscular diseases of the gut fail to detect subtle abnormalities of the ENS, even if these abnormalities are lethal. Hirschsprung's disease, if not the defective genes that cause it, is readily diagnosed because Hirschsprung's disease is accompanied by aganglionosis. Ganglia are easy to recognize and their absence is notable. The loss of a particular type of enteric neuron, such as an IPAN, however, is not obvious in routine sections of gut and there has been no systematic investigation of markers that might detect subtle defects. When Hirschsprung's disease has been ruled out, congenital neuromuscular diseases of the bowel are thus defined operationally, by noting what is functionally abnormal. It is likely that the recent explosion of knowledge of ENS development will soon be followed by an equally explosive expansion of knowledge of the pathogenesis of congenital disease of the ENS.

Congenital neuromuscular diseases of the bowel are common and severe. Hirschsprung's disease is the tip of an iceberg in that the defect is, at least, apparent. It can also be treated, although the treatment is often not entirely satisfactory. The aganglionic segment of bowel must be removed. Because the ENS does not innervate the external anal sphincter, that sphincter is still operative, even when the terminal colon is aganglionic. It is thus possible, after removal of the aganglionic colon, to pull the ganglionated bowel down and through the external anal sphincter to establish (hopefully) a relatively normal bowel. This "pull through" operation sometimes does not succeed because motility of the colon that is left behind may be abnormal even when pains are taken by surgeons to coordinate with pathologists and leave no aganglionic tissue behind. Unfortunately, as noted above, the ENS cannot be presumed to be normal just because it contains ganglia; moreover, the external anal sphincter may leak when it is not supported by the ENS-regulated internal anal sphincter, causing incontinence. Fecal incontinence is not fatal but it is not compatible with a decent social life. Better therapy for Hirschsprung's disease is thus needed but the existing therapy, which is often adequate, is better than nothing. Other conditions, such as pseudoobstruction occurring in a gut that contains ganglia, cannot be treated effectively and may require bowel transplantation. Other problems of motility and even inflammation may have their origin in an abnormal development of the ENS. The rapid pace of research on the development of the ENS and a newfound interest of many scientists in it ensures that the diagnosis and treatment of congenital neuromuscular diseases of the gut in the future will be much different and far more effective than they are today.