Published in final edited form as:

Neurogastroenterol Motil. 2011 October ; 23(10): 898–911. doi:10.1111/j.1365-2982.2011.01764.x.

Development of the Vagal Innervation of the GUT: Steering the Wandering Nerve

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Abstract

Background—The vagus nerve is the major neural connection between the gastrointestinal tract and the central nervous system. During fetal development, axons from the cell bodies of the nodose ganglia and the dorsal motor nucleus grow into the gut to find their enteric targets, providing the vagal sensory and motor innervations respectively. Vagal sensory and motor axons innervate selective targets, suggesting a role for guidance cues in the establishment of the normal pattern of enteric vagal innervation.

Purpose—This review explores known molecular mechanisms that guide vagal innervation in the gastrointestinal tract. Guidance and growth factors, such as netrin-1 and its receptor, deleted in colorectal cancer (DCC), extracellular matrix molecules, such as laminin-111, and members of the neurotrophin family of molecules, such as brain-derived neurotrophic factor (BDNF) have been identified as mediating the guidance of vagal axons to the fetal mouse gut. In addition to increasing our understanding of the development of enteric innervation, these studies may also reveal clinically relevant insights into the underlying mechanisms of vago-vagal signaling from the gastrointestinal tract.

Keywords

vagus nerve; gastrointestinal tract; axon guidance; enteric nervous system; nodose ganglia; dorsal motor nucleus

Introduction

The vagus nerve wanders throughout regions of the head, neck, thorax and abdomen to innervate numerous viscera, and thus it is aptly named; the Latin word *vagus* literally means “wandering”. Among its many roles, the vagus nerve is the main conduit of 2-way communication between the central (CNS) and the enteric nervous systems (ENS) (1). While the ENS is capable of independent function, normal gastrointestinal (GI) function requires the ENS and the vagal innervation to operate in coordination. The vagal innervation is essential for normal homeostasis, satiety, nausea, detection of visceral pain and operations of sphincters (2-5). Vagal sensory and motor fibers are found throughout the length of the GI tract, from the esophagus to the colon (6, 7), but the functions of vagovagal signaling have been best described in the gastroesophageal region (8). Given the importance of normal

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The authors have nothing to disclose.

vagal function in humans, it is conceivable that disorders in gastroesophageal motility, such as gastroesophageal reflux, delayed gastric emptying and feeding intolerance, may be rooted in developmental abnormalities of the enteric vagal innervation.

The vagus nerve descends to the bowel during fetal life. While the vagus is a mixed nerve, containing both sensory, or afferent, and motor (preganglionic) or efferent fibers, the majority of axons are afferent (1). The cell bodies of vagal sensory neurons are found primarily in the nodose ganglia, but also in the jugular ganglia. The cell bodies of motor neurons are located in the dorsal motor nucleus and in the nucleus ambiguus (1). Once in the bowel wall, vagal sensory axons are found in 3 different configurations (Fig. 1)(1): i) long intramuscular arrays (IMAs) between the cells of the external muscle; ii) large intraganglionic laminar endings (IGLEs) which are associated with the connective tissue sheaths of ganglia and are mechanosensitive (9); iii) lamina propria of the mucosa. In contrast, vagal motor fibers synapse directly on a subset of neurons within myenteric and submucosal ganglia (7, 10). The importance of reaching these specific targets is highlighted by the correlation of the location of vagal endings to their function: IMAs have structural features consistent with stretch receptors (11), IGLEs are known to respond to tension in the gut wall and the mucosal projections in the lamina propria correspond to functional mucosal receptors (2).

The development of vagal terminals, the focus of this review, is best understood in the context of what is known about their structure and distribution within the adult GI tract. IMAs are found in the forestomach and lower esophageal and pyloric sphincter. They consist of variable length axonal telodendria that are connected by crossbridge fibers and run parallel to, and interact with, smooth muscle fibers and with intramuscular interstitial cells of Cajal (12-16). IGLEs are distributed throughout the esophagus, stomach and intestine, but are most dense in the stomach and duodenum (14, 15, 17). They are characterized as laminar aggregates of fine terminal puncta that cover all or a portion of a myenteric ganglion, sandwiched between its sheath and neuron layer. Vagal mucosal afferents and vagal efferents are both found throughout the GI tract. Vagal sensory axons, which include both mechano- and chemoreceptors, travel through the muscle wall and submucosa to reach the mucosa where they form two types of terminal arborizations, one that encircles crypts and another that ramifies within the lamina propria of villi (18, 19). In contrast, mature efferent terminals consist of a network of characteristic rings of fiber arborizations and terminal complexes that encircle neurons within the myenteric ganglia, and are similar in structure, but less dense within submucosal ganglia (10, 20, 21).

While numerous investigations have and continue to characterize the role of axon guidance molecules during the development of the CNS, the roles of axon guidance molecules in the development of the extrinsic and intrinsic innervations of the GI tract is a relatively new and growing area of interest (22, 23). The majority of investigations related to guidance factors during ENS development have focused on the intrinsic innervation and specifically on the migration and colonization of the developing GI tract by neural crest-derived cells, with a few studies identifying factors that appear to influence axon outgrowth and/or guidance within the developing ENS (24-26). While more limited in number, recent investigations have begun to identify guidance factors required for the normal development of the vagal innervation, which is the focus of this review. We first provide a brief background on axon guidance, then focus on the molecules that contribute to the guidance of vagal axons as they innervate the developing GI tract, and conclude by highlighting potential future directions of this emerging field.

Growth Cones and Axon Guidance

At the end of a growing neurite process is a small, conical expansion of the cytoplasm, which is the growth cone. Growth cones are highly motile structures that are responsible for detecting guidance cues in their microenvironment as they navigate towards their synaptic targets. Guidance cues, which can be membrane-bound, extracellular matrix-bound or diffusible, interact with receptors on the membrane of the growth cone, leading to the initiation of a signaling cascade. These signaling cascades ultimately impinge on the cytoskeleton leading either to an attractive response in which the growth cone turns towards the source of the chemical cue, or to a repulsive response in which the growth cone orients itself away from the source of the guidance cue (Fig. 2) (27, 28). While the number of molecules that have either been shown to be involved or implicated in the guidance of growing neuronal processes continues to expand, studies to date relevant to the vagal innervation of the GI tract have focused on the netrin, Slit, semaphorin and neurotrophin families (representative examples of their roles in the developing nervous system are provided in Table 1).

Netrins

The word “netrin” is derived from the Sanskrit word “*netr*” meaning “one who guides” and, as such, the netrin family of proteins are best known for their roles in cell migration and axon guidance. Netrins exhibit a high degree of evolutionary conservation with respect to function. While the secreted netrins -1, -3 and -4, as well as the glycosyl phosphatidylinositol (GPI)-anchored netrin-G1 and -G2 have all been characterized in mammals, netrin-1 has been the most studied and its roles during development the most investigated (29). Secreted netrins can function in both an attractive and repulsive capacity (30-33). Deleted in colorectal cancer (DCC) has been shown to mediate the attractive response to secreted netrins while UNC5 homologues A, B, C and D in mammalian species can mediate repulsion. In some cases, UNC5 mediates repulsion when co-expressed with DCC (29, 34). This change in responsiveness is dynamic; for example, if UNC5A is internalized, allowing plasmalemmal DCC to operate independently of UNC5, netrin-mediated repulsion is lost and the response is converted to attraction (35). Netrin-1 is well known for its role in neurodevelopment, guiding commissural axons at the midline of the spinal cord as well as axons at the corpus callosum and hippocampal commissure (32). The essential role of netrin/DCC signaling, furthermore, is clearly demonstrated by the defects in axonal projections observed in mice lacking functional DCC (36). Similar to *netrin* ^{-/-} mice, *DCC* ^{-/-} mice have defects in the projections of commissural axons in the spinal cord and brain, characterized by a failure of these axons to cross midline (36).

Slits

Slit proteins, signaling through Roundabout (Robo) receptors, are able to mediate chemorepulsion (37). The Robo receptor gene, a member of the immunoglobulin superfamily, was first identified in *Drosophila*, and was later identified in mammals (38, 39). Within the mammalian nervous system, there are three Robo receptors (Robo1-3), which can exist in alternatively spliced isoforms, as well as three Slit glycoproteins (Slit1-3), which also appear in alternately spliced isoforms (40, 41). Slits are cleaved into two fragments, the larger of which, the N-terminal fragment, interacts with Robo receptors (41). Slits appear to lack a clear preference for a Robo receptor and their binding behavior has thus been described as “promiscuous” (41). In the context of nervous system development, Slit/Robo signaling is best known for modulating axon guidance at midline structures within the CNS (40). Slit triple knockout mice have defects in commissural axon guidance within the spinal cord; mice lacking Robo1 or Robo2 have similar defects at the spinal cord midline (42).

Semaphorins

Semaphorins, a large family of proteins now divided into 8 classes, were first discovered in the grasshopper (43) and since identified in invertebrates (classes 1 and 2), vertebrates (classes 3 through 7) and viruses (class 8) (44). Of the vertebrate semaphorins, class 3 are secreted, classes 4 through 6 are transmembrane proteins, and class 7 are GPI-anchored proteins. The secreted semaphorins bind the neuropilins, while the transmembrane semaphorins interact with the plexins. Semaphorin signaling involves the recruitment of co-receptors to the signaling complex (44). *Sema3A* mutant mice have been found to have multiple defects in their nervous systems, including sensory axon guidance, cortical neurite orientation, odor map formation and abnormal defasciculation (45-48). While semaphorins tend to function in a repulsive capacity in the developing nervous system, as in the case of *Sema3A* (49), some exceptions are known and function in an attractive capacity, such as *Sema3C* (50). Semaphorins can thus be considered as bidirectional guidance cues.

Neurotrophins

Neurotrophins are secreted proteins with numerous roles in neural development, maintenance and function. The prototypical member of the neurotrophin family of secreted proteins, nerve growth factor (NGF), was named for the first prominent effect of NGF-secreting sarcoma on explanted dorsal root ganglia observed, a dense outgrowth of nerve fibers (51). The ability of NGF to stimulate axon outgrowth is shared with the subsequently identified neurotrophins, brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 (52), which have similar structures to each other and to NGF (~50% homology of amino acid sequences) (53). Neurotrophin effects are mediated by high affinity tyrosine kinase receptors selective for NGF (trkA), BDNF and NT-4 (trkB), and NT-3 (trkC), and a common low-affinity p75 neurotrophin receptor (p75^{NTR}) that acts alone or in concert with trk receptors or other co-receptors (54). Many effects of neurotrophins require retrograde transport of neurotrophin-receptor complexes from nerve terminals to the cell body or dendrites (55, 56), although at least some instances of axon outgrowth may be regulated by local signaling (57). In addition to promoting axon growth, *in vitro* experiments have suggested neurotrophins act as axon guidance molecules, as evidenced by the orienting of growth cones toward a neurotrophin gradient (58). Moreover, while *in vivo* experiments involving gene knockouts of neurotrophins or their receptors, transgenic over-expression of neurotrophins, or neurotrophin receptor blockers have been consistent with an axon growth role for peripheral sensory neurons, it has been difficult to prove an axon guidance function of neurotrophins *in vivo* (52).

Timeline of Vagal Axon Growth During Development

Timeline of vagal sensory innervation of the GI tract

In order to place the molecular mechanisms of vagal innervation into context, it has first been necessary to establish a timeline of the normal descent and innervation pattern of vagal axons. To study the vagal sensory innervation, a lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was applied to the nodose ganglia of fetal mice on embryonic days (E) 12, 14 and 16 (59). To do so, fetal mice were harvested and a paste of DiI with Triton-X was applied to the presumptive nodose ganglia bilaterally with an entomology pin. The fetuses were fixed for 24 hours at 4°C in phosphate-buffered formaldehyde and subsequently incubated in phosphate-buffered saline for 4 (E12, E14) or 6 (E16) weeks at 37°C. Applying DiI to the nodose ganglion in this manner is thought to restrict DiI-labeling to vagal sensory neurons and their axons (59). These studies revealed that vagal sensory fibers reach the stomach by E12 and the celiac ganglion and duodenum by E14. At E16, vagal sensory fibers can be found in the distal small intestine, and at the same stage, forming a plexus that ramifies within the musculature of the esophagus (59).

Similar findings have been reported in earlier work, some of which involved labeling of both afferent and efferent vagal axons. For example, vagal axons are already present and innervating the mouse esophagus at E12 (60), and are seen entering the mouse stomach at E11 (61), and the rat stomach at E12 (62). Once vagal axons have reached the mouse stomach, DiI-labeled axon bundles forming the vagal gastric branches extend from the esophagus toward the greater curvature of the stomach, with progression beyond the greater curvature by E14.5/E15.5 and by E16.5 have the appearance of an adult-like distribution throughout the stomach wall (63).

Once vagal sensory axons reach the GI tract, vagal fibers are subsequently guided to specific locations in the smooth muscle layers, enteric plexi and mucosa (1). Developmental studies in mice have shown that IGLE-like terminals are first observed in the esophagus at E15 and become more differentiated by E18 (60). In the stomach, putative precursors to IGLEs and IMAs are first detected by E16.5/E17.5 (14, 63). Specifically, an IGLE consists of (1) a laminar (2) aggregate of fine terminal puncta (3) within the neuropil of a myenteric ganglion and (4) covering all or part of the ganglion (17, 18, 64, 65). In developmental studies, criterion (4) is determined by locating terminal processes in the z-plane immediately above or below the planes containing myenteric neurons, the myenteric plexus, and vagal efferent terminals. In contrast, an IMA consists of (1) an array of parallel axonal telodendria (2) in close proximity and (3) interconnected by bridging axonal elements (4) located in either the longitudinal or circular muscle layer (12, 14, 15). In the developmental studies, criterion (4) is determined by locating terminal processes in the series of z-planes that include the longitudinal or circular muscle layers, respectively identified above or below the planes containing the myenteric plexus. In addition to the above criteria, changes in IGLE and IMA morphology over the course of development are further characterized by selecting examples on postnatal day 8 (P8) that are sufficiently mature to be matched to adult profiles, then working backwards sequentially one postnatal/fetal day at a time to the stage of interest (63, 66). In the studies discussed in this review, DiI was applied directly to vagal gastric branches and thus both afferent and efferent axons are labeled. The afferent and efferent endings can be distinguished using the methods described above for characterizing IGLEs and IMAs as well as by using the specific criteria for identifying efferent fibers, as described in the next section of this review.

Using the criteria defined above, IGLEs, at their initial stage of development, were found to consist of a loosely packed planar cluster of a small number of terminal puncta or growth cone-like structures resting immediately above or below a myenteric ganglion. As their development progresses IGLE precursors gradually became larger in size, consisting of greater numbers of terminal puncta that became more tightly packed and by P8 their structure appears close to maturity (Fig. 3). In contrast to the developmental progression of IGLEs, the initial stage of IMA development involves neurite formation. Parent axons arise from the myenteric plexus to innervate the circular or longitudinal smooth muscle layer where they give off one or more branches that form short processes, some led by growth cone-like structures. As development progresses the neurites grow parallel to muscle fibers to begin the formation of IMA processes, known as telodendria, and the first bridging collateral branches that connect separate telodendria of an IMA precursor form. Telodendria growth continues until P8 and interconnecting cross-bridge fibers increase in number as IMA precursors approach their mature form (Fig. 4). It should be noted that although similar profiles to those of the putative IGLE and IMA precursors illustrated in Figures 3 and 4 can be observed in adults, the ones depicted in these figures are at least half the size of their adult counterparts, and unlike in adults where they comprise a minority of the forms present, the illustrated fetal IGLEs and IMAs are representative of the majority of the precursors present.

Timeline of vagal motor (preganglionic) innervation of the GI tract

Studies of the development of the vagal motor innervation have been more limited to date than of the vagal sensory innervation. In fetal rats, vagal motor axons have been identified by inserting DiI into the proximal gut and determining the age at which DiI labeling is seen reaching cell bodies in the nucleus ambiguus (NA) and dorsal motor nucleus (DMV) (62). Using this method, the vagal motor axons from the proximal gut are first observed in the NA at E13 and motor neurons from the DMV are found to first project to the gut at E14 (62). In more detailed studies in fetal mice, in which DiI was applied to the anterior gastric branch of the vagus, putative efferent vagal terminals are first observed at E16.5 (63). Similar to the identification of developing IGLEs and IMAs, criteria have been adapted to identify efferent terminal precursors. Specifically, developing efferent terminals are located in the focal plane of the myenteric neurons, between the focal planes in the smooth muscle layers, and consist of (1) a network of characteristic rings of fiber arborizations and terminal complexes (2) circling the myenteric neurons (3) within the myenteric ganglia (10, 20, 21). Using these criteria, vagal efferent terminals are found to develop in all the major stomach compartments and are present throughout most of the stomach wall at all ages studied, similar to the mature efferent terminal distribution. Putative vagal efferent terminals, moreover, are seen encircling myenteric neurons, similar to the adult structure, and can be found throughout most of the stomach wall (48). At E16.5 single or small numbers of encircled neurons are present within myenteric ganglia. Between E17.5 and P3, putative efferent terminals increase in number dramatically with an increase in the density of terminal fibers that surrounded each neuron. In parallel, the terminals surrounding each neuron become clustered with those surrounding other neurons as increasing proportions of myenteric neurons became innervated. By P3, putative efferent terminals appear similar in density, distribution and morphology to terminals in adult animals. Vagal efferents thus appear to develop more rapidly than IMAs and IGLEs, which in contrast, continue to mature structurally and to increase in density after P3 (63).

In examining the timing of descent of vagal sensory and motor axons, a pattern emerges in which vagal fibers reach the foregut at time points later in gestation than what has been described for neural crest-derived cells in fetal mice. Specifically, neural crest-derived cells initially enter the mouse foregut at E9.5-E10 (67, 68) while vagal fibers have not been described entering the stomach wall until E11 (61). This sequence of events has raised the question as to how the development of the ENS might influence that of the vagal innervation. In recent work, the vagal sensory innervation was found to be dependent on enteric neurons (69). While studies suggest that the mechanism for dependence involves the ability of enteric neurons to synthesize netrins, the extent to which netrin secretion accounts for the requirement of enteric neurons for the normal development of the vagal sensory innervation remains an area of further study (69).

Roles of Guidance Molecules In Enteric Vagal Development

While the basic pattern of vagal innervation to the GI tract is characterized, the axon guidance pathways by which these projections become established are not well delineated. Studies to date support important roles for axon guidance cues in ensuring that vagal fibers both find the gut as well as their correct locations within it, thus establishing the normal vagal wiring pattern of the developing GI tract.

Attractive guidance signaling

Netrins, and their receptor, DCC, are not only expressed in the developing CNS, but are also present in the developing GI tract (70, 71). Functionally, netrin-1 has been demonstrated to play a physiologically significant role in the guidance of crest-derived neural and glial

precursors to the submucosal and pancreatic plexuses through DCC (70). Previous work has established that netrin-1 and its receptor, DCC, mediate the attraction of vagal sensory axons to the fetal bowel (59). In these studies, DCC was expressed and developmentally regulated in the nodose ganglia, and a subset of the descending vagal axons were found to be DCC-immunoreactive (Fig. 5). Netrin-1 was expressed in the outer mesenchyme and mucosa of the developing foregut, while vagal axons, visualized with DiI applied to the fetal nodose ganglion as described above, were found to extend within and toward both netrin-1 producing zones. *In vitro*, neurites extending from explants of nodose ganglia were attracted towards both co-cultured explants of foregut and netrin-1 secreting cells. Function-blocking antibodies against DCC abolished the selective growth toward each of these targets (59). One advantage of these *in vitro* studies is that when explants of nodose ganglia are cultured, only sensory neurons are present. Mice lacking either netrin-1 or DCC do not survive beyond the perinatal period, thus limiting functional studies in more mature animals.

Neurotrophins may also play a role in attraction of vagal sensory axons to specific regions of the developing GI tract. BDNF and neurotrophin-3, which have been most extensively studied, are expressed during development in several gut tissues in close apposition to growing vagal sensory and motor axons (72-74). These tissues include blood vessels that supply the gut, mesentery adjacent to these blood vessels where present, smooth muscle of the stomach and intestines, and lamina propria of the stomach antrum and corpus. Knockout of BDNF in mice resulted in alterations in developing vagal afferents shortly after birth, some of which were consistent with an axon growth or guidance role for BDNF (66). The effects of BDNF seemed to be specific to vagal afferents, since no alterations were seen in the numbers of vagal efferent terminals in BDNF knockout compared to wild-type animals. First, IGLE numbers were reduced by 50% at P0. This could be explained by loss of BDNF-mediated growth or attraction of a subpopulation of IGLE axons to the stomach muscle wall. Second, aberrant patterning of axons and fiber bundles in the antrum of BDNF knockout mice occurred, suggestive of defects in axon growth/guidance or fasciculation, which could involve motor as well as sensory axons. Since BDNF is normally expressed by antrum smooth muscle immediately adjacent to the affected vagal fibers, it is possible that loss of BDNF-mediated axon growth or guidance contributed to the disorganization of antral vagal axons. Similarly, loss of BDNF from the antral lamina propria could have removed a cue necessary to attract vagal axons from the myenteric plexus to the mucosa, leaving vagal axons to sprout or wander in the myenteric plexus in search of this cue. While not conducted in the GI tract, studies in mice that have a double knockout of both BDNF and BAX, in which the knockout of BAX prevents programmed cell death, provide further evidence of an axon guidance role for BDNF (55). In these investigations, vagal baroreceptor afferents that supply the cardiac outflow tract were found to approach, but not enter their target blood vessel walls that normally express BDNF, but lack BDNF in the knockout model (75). Studies of baroreceptor afferents are facilitated by the presence of only sensory axons in the peripheral nerve branches and only sensory terminals in the target tissue, which makes it possible to label them with standard methods of immunohistochemistry using antibodies to neural-specific antigens. *In vitro* experiments similar to those carried out for netrin and laminin, described above and below respectively, would add additional evidence supporting a guidance role for BDNF. Further studies are also needed to characterize the function of the BDNF receptors, trkB and p75^{NTR}, in the context of vagal axon guidance.

Unfortunately, most neurotrophin knockout mouse models are lethal in the perinatal period, precluding functional studies of their effects on sensory nerve development. The most obvious phenotype in the rare NT-3 and BDNF mutant mice that survive for a short period of time after birth is an uncoordinated gait. This lack of coordination is related both to the loss of proprioceptive afferent nerves as well as to the loss of peripheral sensory neurons, such as the vestibular afferent nerves, which lead to an overall deficit in coordination and

balance (76). A targeted approach to knocking out neurotrophin function that overcomes this lethality has been developed (72), and is beginning to yield interesting results for studying the roles of BDNF and NT-3 in vagal development (77, 78). These targeted models have the potential to extend developmental studies in neurotrophin knockout mice to the early postnatal stages and enable examination of sensory receptor formation, growth and differentiation.

One exception to the need for a targeted approach is the neurotrophin-4 (NT-4) knockout mouse, which survives into adulthood with apparently normal health and ability to breed. These mice exhibit a selective and dramatic loss of intestinal IGLEs, whereas gastric IGLEs appear normal (79). This altered intestinal innervation phenotype was associated with normal daily food intake, but altered patterns of consumption with increased meal duration noted in mice fed a solid diet and increased meal size in mice fed a liquid diet (79). The available evidence suggests this loss of intestinal IGLEs could have resulted from loss of NT-4 axon guidance or survival functions. Consistent with this interpretation, NT-4 supports survival of cultured developing nodose ganglion neurons (80) and induces axon outgrowth from the adult nodose ganglion *in vitro* (81). NT-4, moreover, is expressed in visceral tissues (82) traversed by vagal sensory axons growing toward the GI tract. NT-4 deficiency has effects in other neural systems, including loss of a large population of P2X3-expressing vagal sensory nerves that innervate the neuroepithelial bodies in the lung and may be mechanoreceptors (83), loss of adult down hair cutaneous mechanoreceptors (84), loss of sympathetic neurons that innervate the adrenal medulla (85), and a partial loss of gustatory innervation (86). NT-4 deficient mice have, furthermore, been found to have an increase in mean arterial blood pressure (87). Conversely, the addition of NT-4 can promote axon growth, as demonstrated by the ability of implanted NT-4 in the adrenal medulla of NT-4 knockout mice to stimulate terminal sprouting of surviving sympathetic innervation (85). Similar to NT-3 knockout mice, NT-4 knockout mice have abnormal coordination. It has been suggested that the reduced coordination in NT-4 knockout mice is a consequence of reduced conversion of somatosensory neuron precursors from an NT-3 dependent proprioceptive neuronal fate to a BDNF-dependent mechanosensitive neuronal fate (88). It thus follows that one of the main types of neuron that depends on NT-4 for normal development may be a mechanoreceptor of vagal or somatosensory origin, although sympathetic and taste chemosensory neurons are also affected.

Repulsive guidance signaling

Vagal sensory axons, while being attracted to the gut and to specific locations within it, also appear to be restricted from entering the majority of the bowel wall. It is highly probable, therefore, that both attractive and repellent cues participate in the guidance of vagal sensory fibers to their correct enteric targets.

Slits1-3, like netrin-1, have been identified in the developing GI tract, specifically in the mesoderm of the chick bowel (89). Within the fetal mouse gut, transcripts encoding Robo1 have been identified in the nodose ganglia and mRNA encoding Slits-1-3 have been found in the gut (90). While these data imply that Slit/Robo-mediated chemorepulsion may play a role in restricting vagal sensory axons to specific regions in the bowel wall, future experiments are required to clarify this mechanism.

Semaphorins may also participate in the normal development of the vagus nerve. Vagal sensory axons from the hindbrain have been shown to collapse in response to Semaphorin 3A (91), and work in Semaphorin 3D mutant mice revealed defects to be present in vagal nerve morphology (45). Detailed characterization of these defects, however, as well as the roles of semaphorins in guiding vagal axons in the periphery, has yet to be explored in detail.

Axon pathfinding, in addition to responding to classical guidance cues, can be modulated by interactions with factors present within the extracellular matrix. Laminins, for example, which are adhesion molecules of the extracellular matrix, also play a role in cell signaling (92). Interaction with laminin-111 has been shown to convert netrin-1-mediated attraction to repulsion (93). Laminin-111 is expressed in the fetal mouse gut (94) in locations where vagal sensory axons form endings (95). This observation led to the hypothesis that enteric laminin-111 could act as a stop signal for vagal sensory axons entering the gut wall. To evaluate this hypothesis, tissue preparations from fetal mice, in which vagal sensory axons were labeled by applying DiI paste to the nodose ganglion, were double-labeled with antibodies to laminin. Vagal sensory axons were found to terminate at the laminin-rich sheaths of developing ganglia, consistent with the location of IGLEs (95). In *in vitro* studies, nodose ganglia, removed from E14 fetal mice, were co-cultured with netrin-1 secreting and control cells; the preferential growth of nodose axons toward the netrin-1 secreting cells was reversed by the addition of soluble laminin to the culture media. Similarly, the presence of soluble laminin abolished the attractive response of nodose axons towards a co-cultured explant of foregut. These *in vitro* investigations confirmed that laminin inhibits the netrin-mediated attraction of nodose axons (95).

Technical challenges

Progress in understanding the development of the vagal innervation of the gut has been made possible in large part by recent advances in methods for selectively labeling developing vagal axons and terminals, mainly using DiI as described above, and in some instances combined with immunohistochemistry. Progress, however, is still significantly hindered by several limitations due in part to the small size of the developing nodose ganglion and vagal nerve branches, which makes them difficult to inject, to limitations on the distance DiI can diffuse from the injection site, and to DiI chemistry. For instance, some of these methods label both efferent and afferent vagal axons, so even though the terminals can be located, it may not be possible to determine if a DiI-labeled axon is sensory or motor. This limitation is one of the main reasons why virtually nothing is known about the regulation of vagal motor axon growth. Not all DiI preparations, furthermore, are readily combined with immunohistochemistry, thus making it difficult to study interactions of DiI-labeled neural elements with guidance molecules and their receptors. Of particular consequence for these studies, Murphy and Fox (66) have stated they were unable to identify an antibody that could reliably label growth cones in DiI-labeled wholemount tissue, or even in tissue sections; thus, the frequent use of the term “growth cone-like” in that paper and in this review. An additional limitation is the lack of unique “markers” for vagal neurons generally, or for markers that could be selective for afferent or efferent axons. In the future, it is possible that we might be able to take advantage of recent molecular approaches to selective labeling of specific neuron types with green fluorescent protein or its derivatives (96).

Future Directions

It is anticipated that future studies will not only further elucidate the roles of classical axon guidance molecules in the vagal sensory and motor innervation of bowel, but may also uncover additional guidance roles for molecules that have already been shown to participate in enteric nervous system (ENS) development. It is unlikely that classical axon guidance molecules are the only molecules involved in the patterning of the vagal innervation; candidates for additional modulating factors may be identified by studying molecules already known to be present in the developing GI tract. Retinoic acid (RA), for example, is not only essential for cell-cell signaling during vertebrate organogenesis (97), but is also required for the normal development of the ENS (98). RA has been shown to exert a repulsive effect on the neurites of enteric neurons (99) and to have an attractive effect on

axons from culture spinal cord explants (100). While the effects of RA may differ between the ENS and CNS, these studies are consistent with the idea that RA exerts tropic effects on growing neurites and may play an iterative role in the development of the ENS. Bone morphogenetic proteins (BMPs) may similarly have multiple functions during enteric development. In the CNS, BMPs have previously been demonstrated to act not only as classical morphogens, but also as axon guidance molecules (101). In the developing ENS, BMPs participate in the regulation of enteric neuronal phenotype diversity (102), in the ability of enteric neurons to migrate and form ganglionic aggregates (103); and they have also been found to influence neurite fasciculation and the orientation of neurite outgrowth (104). In summary, molecules previously known to function in ENS development are potential candidates for playing additional roles in guiding axons to their correct targets within the gut wall.

Guidance molecules may contribute not only to how and when vagal axons enter the GI tract, but may also influence how vagal terminals interact with different cell types in the gut wall. For example, *Ret*^{-/-} mice, which lack enteric neurons, have been found to have an absent vagal sensory innervation (69). Enteric neurons synthesize netrins (69) and it is thus possible, but remains to be proven, that the loss of netrin-secreting neurons accounts for the failure of vagal sensory nerves to innervate aganglionic gut. In addition to forming IGLEs within the ENS, vagal sensory axons have shown to be in close contact with interstitial cells of Cajal (ICC) (105). Mice lacking ICC have a marked reduction in IMAs (16, 106); it is possible that ICC secrete a factor that promotes the development of IMAs and future studies might investigate whether this factor is a guidance molecule. Regardless of the target, it is likely that axon guidance molecules do not act individually, but rather act in coordination with other molecules, such as another guidance molecule, or a growth factor or the extracellular matrix. Such interactions could help account not only for the intricate morphology of IGLEs and IMAs but also for the potential specificity of how a vagal axon “chooses” to innervate one enteric cell type versus another. Earlier in this review, we present data consistent with possibility that interactions of netrin-1 with laminin-111 participate in the formation of IGLEs. It is also possible, but remains to be investigated, that netrin-1 interacts with BDNF. Netrin-1 and BDNF appear to exhibit spatially and temporally overlapping expression patterns in the developing GI tract. Since these molecules have been shown to be capable of interacting to influence axon growth through activation of overlapping sets of intracellular signals and targets (107-111), they are poised to modulate each other's ability to reach a target. Such interactions could change attraction to repulsion, or could alter the sensitivity of certain axons to other factors.

One of the challenges in the field of axon guidance is to understand how a vast set of connections can be established by, as is currently known, a relatively small set of factors (112). Future studies, therefore, will not only need to focus on identifying additional candidate molecules, such as has been done in the context of ENS development (113), but will also need to examine how known and future factors function individually and together both during the timeline of ENS development as well as in specific locations in the bowel wall. We anticipate that in gaining an understanding of how vagal afferent and efferent fibers innervate the fetal gut, we can not only elucidate new mechanisms in the field of axon guidance, but also gain clinically relevant insights into the underlying mechanisms of vago-vagal signaling from the GI tract.

Acknowledgments

E.M.R. is supported by the Canadian Association of Gastroenterology (CAG)/Astra Zeneca/Canadian Institutes of Health Research (CIHR) Operating Grant and by the McMaster Children's Hospital. N.F. is supported by the National Science and Engineering Research Council of Canada (NSERC). E.A.F. is supported by the National

Institutes of Health (NIH) R01 NS46716. We thank Dr. Neil Dufton (Farncombe Family Digestive Health Research Institute) for his assistance in preparing the schematic figures.

References

1. Powley TL, Phillips RJ. Musings on the wanderer: what's new in our understanding of vago-vagal reflexes? I. Morphology and topography of vagal afferents innervating the GI tract. *American journal of physiology*. 2002; 283:G1217–1225. [PubMed: 12388183]
2. Blackshaw LA, Brookes SJ, Grundy D, Schemann M. Sensory transmission in the gastrointestinal tract. *Neurogastroenterol Motil*. 2007; 19:1–19. [PubMed: 17280582]
3. Berthoud HR. Vagal and hormonal gut-brain communication: from satiation to satisfaction. *Neurogastroenterol Motil*. 2008; 20 1:64–72. [PubMed: 18402643]
4. Goyal RK, Chaudhury A. Physiology of normal esophageal motility. *Journal of clinical gastroenterology*. 2008; 42:610–619. [PubMed: 18364578]
5. Ramkumar D, Schulze KS. The pylorus. *Neurogastroenterol Motil*. 2005; 17 1:22–30. [PubMed: 15836452]
6. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci*. 2000; 85:1–17. [PubMed: 11189015]
7. Berthoud HR, Carlson NR, Powley TL. Topography of efferent vagal innervation of the rat gastrointestinal tract. *The American journal of physiology*. 1991; 260:R200–207. [PubMed: 1992820]
8. Browning KN, Travagli RA. Plasticity of vagal brainstem circuits in the control of gastric function. *Neurogastroenterol Motil*. 2010
9. Zagorodnyuk VP, Chen BN, Brookes SJ. Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. *The Journal of physiology*. 2001; 534:255–268. [PubMed: 11433006]
10. Kirchgeßner A, Gershon MD. Identification of vagal efferent fibers and putative target neurons in the enteric nervous system of the rat. *J Comp Neurol*. 1989; 285:38–53. [PubMed: 2568999]
11. Phillips RJ, Powley TL. Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology. *Brain research*. 2000; 34:1–26.
12. Berthoud HR, Powley TL. Vagal afferent innervation of the rat fundic stomach: morphological characterization of the gastric tension receptor. *J Comp Neurol*. 1992; 319:261–276. [PubMed: 1522247]
13. Kressel M, Berthoud HR, Neuhuber WL. Vagal innervation of the rat pylorus: an anterograde tracing study using carbocyanine dyes and laser scanning confocal microscopy. *Cell and tissue research*. 1994; 275:109–123. [PubMed: 7509721]
14. Fox EA, Phillips RJ, Martinson FA, Baronowsky EA, Powley TL. Vagal afferent innervation of smooth muscle in the stomach and duodenum of the mouse: morphology and topography. *The Journal of comparative neurology*. 2000; 428:558–576. [PubMed: 11074451]
15. Wang FB, Powley TL. Topographic inventories of vagal afferents in gastrointestinal muscle. *The Journal of comparative neurology*. 2000; 421:302–324. [PubMed: 10813789]
16. Fox EA, Phillips RJ, Martinson FA, Baronowsky EA, Powley TL. C-Kit mutant mice have a selective loss of vagal intramuscular mechanoreceptors in the forestomach. *Anatomy and embryology*. 2001; 204:11–26. [PubMed: 11506430]
17. Neuhuber WL. Sensory vagal innervation of the rat esophagus and cardia: a light and electron microscopic anterograde tracing study. *Journal of the autonomic nervous system*. 1987; 20:243–255. [PubMed: 3693803]
18. Berthoud HR, Kressel M, Raybould HE, Neuhuber WL. Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI-tracing. *Anatomy and embryology*. 1995; 191:203–212. [PubMed: 7771683]
19. Powley TL, Spaulding RA, Haglof SA. Vagal afferent innervation of the proximal gastrointestinal tract mucosa: chemoreceptor and mechanoreceptor architecture. *The Journal of comparative neurology*. 2011; 519:644–660. [PubMed: 21246548]

20. Holst MC, Kelly JB, Powley TL. Vagal preganglionic projections to the enteric nervous system characterized with Phaseolus vulgaris-leucoagglutinin. *The Journal of comparative neurology*. 1997; 381:81–100. [PubMed: 9087421]
21. Phillips RJ, Baronowsky EA, Powley TL. Long-term regeneration of abdominal vagus: efferents fail while afferents succeed. *The Journal of comparative neurology*. 2003; 455:222–237. [PubMed: 12454987]
22. Young HM, Anderson RB, Anderson CR. Guidance cues involved in the development of the peripheral autonomic nervous system. *Auton Neurosci*. 2004; 112:1–14. [PubMed: 15233925]
23. Hao MM, Young HM. Development of enteric neuron diversity. *Journal of cellular and molecular medicine*. 2009; 13:1193–1210. [PubMed: 19538470]
24. Rossi J, Luuko K, Poteryaev D, et al. Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR α 2, a functional neurotrophin receptor. *Neuron*. 1999; 22:243–252. [PubMed: 10069331]
25. Young HM, Hearn CJ, Farlie PG, Canty AJ, Thomas PQ, Newgreen DF. GDNF is a chemoattractant for enteric neural cells. *Developmental biology*. 2001; 229:503–516. [PubMed: 11150245]
26. Metzger M, Conrad S, Skutella T, Just L. RGMa inhibits neurite outgrowth of neuronal progenitors from murine enteric nervous system via the neogenin receptor in vitro. *J Neurochem*. 2007
27. Bouquet C, Nothias F. Molecular mechanisms of axonal growth. *Advances in experimental medicine and biology*. 2007; 621:1–16. [PubMed: 18269207]
28. Geraldo S, Gordon-Weeks PR. Cytoskeletal dynamics in growth-cone steering. *Journal of cell science*. 2009; 122:3595–3604. [PubMed: 19812305]
29. Moore SW, Tessier-Lavigne M, Kennedy TE. Netrins and their receptors. *Advances in experimental medicine and biology*. 2007; 621:17–31. [PubMed: 18269208]
30. Astic L, Pellier-Monnin V, Saucier D, Charrier C, Mehlen P. Expression of netrin-1 and netrin-1 receptor, DCC, in the rat olfactory nerve pathway during development and axonal regeneration. *Neuroscience*. 2002; 109:643–656. [PubMed: 11927147]
31. Colamarino SA, Tessier-Lavigne M. The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell*. 1995; 81:621–629. [PubMed: 7758116]
32. Serafini T, Colamarino SA, Leonardo ED, et al. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell*. 1996; 87:1001–1014. [PubMed: 8978605]
33. Keino-Masu K, Masu M, Hinck L, et al. *Deleted in colorectal cancer (DCC)* encodes a netrin receptor. *Cell*. 1996; 87:175–185. [PubMed: 8861902]
34. Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, S E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell*. 1999; 97:927–941. [PubMed: 10399920]
35. Bartoe JL, McKenna WL, Quan TK, et al. Protein interacting with C-kinase 1/protein kinase C α -mediated endocytosis converts netrin-1-mediated repulsion to attraction. *J Neurosci*. 2006; 26:3192–3205. [PubMed: 16554470]
36. Fazeli A, Dickinson SL, Hermiston ML, et al. Phenotype of mice lacking functional *Deleted in colorectal cancer (Dcc)* gene. *Nature*. 1997; 386:796–804. [PubMed: 9126737]
37. Stein E, Tessier-Lavigne M. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science (New York, NY)*. 2001; 291:1928–1938.
38. Brose K, Bland KS, Wang KH, et al. Slit proteins bind Robo receptor and have an evolutionarily conserved role in repulsive axon guidance. *Cell*. 1999; 96:795–806. [PubMed: 10102268]
39. Seeger M, Tear G, Ferres-Marco D, Goodman CS. Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. *Neuron*. 1993; 10:409–426. [PubMed: 8461134]
40. Chedotal A. Slits and their receptors. *Advances in experimental medicine and biology*. 2007; 621:65–80. [PubMed: 18269211]
41. Ypsilanti AR, Zagar Y, Chedotal A. Moving away from the midline: new developments for Slit and Robo. *Development (Cambridge, England)*. 2010; 137:1939–1952.

42. Long H, Sabatier C, Ma L, et al. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. *Neuron*. 2004; 42:213–223. [PubMed: 15091338]
43. Kolodkin AL, Matthes DJ, O'Connor TP, et al. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron*. 1992; 9:831–845. [PubMed: 1418998]
44. Roth L, Koncina E, Satkauskas S, Cremel G, Aunis D, Bagnard D. The many faces of semaphorins: from development to pathology. *Cell Mol Life Sci*. 2009; 66:649–666. [PubMed: 18953684]
45. Taniguchi M, Yuasa S, Fujisawa H, et al. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron*. 1997; 19:519–530. [PubMed: 9331345]
46. Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature*. 1996; 383:525–528. [PubMed: 8849723]
47. Taniguchi M, Nagao H, Takahashi YK, et al. Distorted odor maps in the olfactory bulb of semaphorin 3A-deficient mice. *J Neurosci*. 2003; 23:1390–1397. [PubMed: 12598627]
48. Kitsukawa T, Shimizu M, Sanbo M, et al. Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron*. 1997; 19:995–1005. [PubMed: 9390514]
49. Rabacchi SA, Solowska JM, Kruk B, Luo Y, Raper JA, Baird DH. Collapsin-1/semaphorin-III/D is regulated developmentally in Purkinje cells and collapses pontocerebellar mossy fiber neuronal growth cones. *J Neurosci*. 1999; 19:4437–4448. [PubMed: 10341245]
50. Bagnard D, Lohrum M, Uziel D, Puschel AW, Bolz J. Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Development (Cambridge, England)*. 1998; 125:5043–5053.
51. Levi-Montalcini R, Meyer H, Hamburger V. In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer research*. 1954; 14:49–57. [PubMed: 13126933]
52. Tucker KL. Neurotrophins and the control of axonal outgrowth. *Panminerva medica*. 2002; 44:325–333. [PubMed: 12434114]
53. Hallböök F, Ibnez CF, Persson H. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron*. 1991; 6:845–858. [PubMed: 2025430]
54. Schecterson LC, Bothwell M. Neurotrophin receptors: Old friends with new partners. *Developmental neurobiology*. 2010; 70:332–338. [PubMed: 20186712]
55. Grimes ML, Zhou J, Beattie EC, et al. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. *J Neurosci*. 1996; 16:7950–7964. [PubMed: 8987823]
56. Sharma N, Deppmann CD, Harrington AW, et al. Long-distance control of synapse assembly by target-derived NGF. *Neuron*. 2010; 67:422–434. [PubMed: 20696380]
57. Kuruvilla R, Zweifel LS, Glebova NO, et al. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. *Cell*. 2004; 118:243–255. [PubMed: 15260993]
58. Paves H, Saarma M. Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. *Cell and tissue research*. 1997; 290:285–297. [PubMed: 9321690]
59. Ratcliffe EM, Setru SU, Chen JJ, Li ZS, D'Autreaux F, Gershon MD. Netrin/DCC-mediated attraction of vagal sensory axons to the fetal mouse gut. *The Journal of comparative neurology*. 2006; 498:567–580. [PubMed: 16917820]
60. Sang Q, Young HM. The origin and development of the vagal and spinal innervation of the external muscle of the mouse esophagus. *Brain Res*. 1998; 809:253–268. [PubMed: 9853118]
61. Baetge G, Gershon MD. Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. *Devel Biol*. 1989; 132:189–211. [PubMed: 2563710]
62. Rinaman L, Levitt P. Establishment of vagal sensorimotor circuits during fetal development in rats. *Journal of neurobiology*. 1993; 24:641–659. [PubMed: 7686963]

63. Murphy MC, Fox EA. Anterograde tracing method using DiI to label vagal innervation of the embryonic and early postnatal mouse gastrointestinal tract. *Journal of neuroscience methods*. 2007; 163:213–225. [PubMed: 17418900]
64. Rodrigo J, Hernandez J, Vidal MA, Pedrosa JA. Vegetative innervation of the esophagus. II. Intraganglionic laminar endings. *Acta anatomica*. 1975; 92:79–100. [PubMed: 1163197]
65. Rodrigo J, de Felipe J, Robles-Chillida EM, Perez Anton JA, Mayo I, Gomez A. Sensory vagal nature and anatomical access paths to esophagus laminar nerve endings in myenteric ganglia. Determination by surgical degeneration methods. *Acta anatomica*. 1982; 112:47–57. [PubMed: 7080798]
66. Murphy MC, Fox EA. Mice deficient in brain-derived neurotrophic factor have altered development of gastric vagal sensory innervation. *The Journal of comparative neurology*. 2010; 518:2934–2951. [PubMed: 20533354]
67. Rothman TP, Gershon MD. Phenotypic expression in the developing murine enteric nervous system. *J Neurosci*. 1982; 2:381–393. [PubMed: 7062117]
68. Anderson RB, Stewart AL, Young HM. Phenotypes of neural-crest-derived cells in vagal and sacral pathways. *Cell and tissue research*. 2006; 323:11–25. [PubMed: 16133146]
69. Ratcliffe EM, Fan L, Mohammed TJ, Anderson M, Chalazonitis A, Gershon MD. Enteric neurons synthesize netrins and are essential for the development of the vagal sensory innervation of the fetal gut. *Developmental neurobiology*. 2011; 71:362–373. [PubMed: 21485011]
70. Jiang Y, Liu M, Gershon MD. Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas. *Developmental biology*. 2003; 258:364–384. [PubMed: 12798294]
71. Seaman C, Anderson R, Emery B, Cooper HM. Localization of the netrin guidance receptor, DCC, in the developing peripheral and enteric nervous systems. *Mech Dev*. 2001; 103:173–175. [PubMed: 11335129]
72. Fox EA. A genetic approach for investigating vagal sensory roles in regulation of gastrointestinal function and food intake. *Auton Neurosci*. 2006; 126-127:9–29. [PubMed: 16677865]
73. Fox EA, Murphy MC. Factors regulating vagal sensory development: potential role in obesities of developmental origin. *Physiology & behavior*. 2008; 94:90–104. [PubMed: 18234244]
74. Fox EA, McAdams J. Smooth-muscle-specific expression of neurotrophin-3 in mouse embryonic and neonatal gastrointestinal tract. *Cell and tissue research*. 2010; 340:267–286. [PubMed: 20387078]
75. Hellard D, Brosenitsch T, Fritzsche B, Katz DM. Cranial sensory neuron development in the absence of brain-derived neurotrophic factor in BDNF/Bax double null mice. *Developmental biology*. 2004; 275:34–43. [PubMed: 15464571]
76. Ernfors P, Van De Water T, Loring J, Jaenisch R. Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron*. 1995; 14:1153–1164. [PubMed: 7605630]
77. Fox EA, Biddinger JE, Jones KR, Worman A, McAdams J. Smooth muscle-specific knockout of brain-derived neurotrophic factor results in hyperphagia and obesity. 2010
78. Biddinger JE, Fox EA. Meal pattern and microstructure changes underlying hyperphagia and obesity in mice with smooth muscle-specific brain-derived neurotrophic factor knockout. 2010
79. Fox EA, Phillips RJ, Baronowsky EA, Byerly MS, Jones S, Powley TL. Neurotrophin-4 deficient mice have a loss of vagal intraganglionic mechanoreceptors from the small intestine and a disruption of short-term satiety. *J Neurosci*. 2001; 21:8602–8615. [PubMed: 11606648]
80. Hertzberg T, Fan G, Finley JC, Erickson JT, Katz DM. BDNF supports mammalian chemoafferent neurons in vitro and following peripheral target removal in vivo. *Developmental biology*. 1994; 166:801–811. [PubMed: 7813797]
81. Wiklund P, Ekstrom PA. Axonal outgrowth from adult mouse nodose ganglia in vitro is stimulated by neurotrophin-4 in a Trk receptor and mitogen-activated protein kinase-dependent way. *Journal of neurobiology*. 2000; 45:142–151. [PubMed: 11074460]
82. Timmusk T, Belluardo N, Metsis M, Persson H. Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. *The European journal of neuroscience*. 1993; 5:605–613. [PubMed: 8261135]

83. Oztay F, Brouns I, Pintelon I, et al. Neurotrophin-4 dependency of intraepithelial vagal sensory nerve terminals that selectively contact pulmonary NEBs in mice. *Histology and histopathology*. 2010; 25:975–984. [PubMed: 20552548]
84. Stucky CL, Shin JB, Lewin GR. Neurotrophin-4: a survival factor for adult sensory neurons. *Curr Biol*. 2002; 12:1401–1404. [PubMed: 12194821]
85. Schober A, Wolf N, Huber K, et al. TrkB and neurotrophin-4 are important for development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla. *J Neurosci*. 1998; 18:7272–7284. [PubMed: 9736648]
86. Patel AV, Huang T, Krimm RF. Lingual and palatal gustatory afferents each depend on both BDNF and NT-4, but the dependence is greater for lingual than palatal afferents. *The Journal of comparative neurology*. 2010; 518:3290–3301. [PubMed: 20575060]
87. Roosen A, Schober A, Strelau J, et al. Lack of neurotrophin-4 causes selective structural and chemical deficits in sympathetic ganglia and their preganglionic innervation. *J Neurosci*. 2001; 21:3073–3084. [PubMed: 11312292]
88. Liebl DJ, Klesse LJ, Tessarollo L, Wohlman T, Parada LF. Loss of brain-derived neurotrophic factor-dependent neural crest-derived sensory neurons in neurotrophin-4 mutant mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:2297–2302. [PubMed: 10681461]
89. De Bellard ME, Rao Y, Bronner-Fraser M. Dual function of Slit2 in repulsion and enhanced migration of trunk, but not vagal, neural crest cells. *J Cell Biol*. 2003; 162:269–279. [PubMed: 12876276]
90. Ratcliffe EM, Chen JJ, Gershon MD. Chemorepulsion in the guidance of vagal sensory axons that innervate the fetal gut: expression of Slit-1 in the gut and its receptor, Robo-1 in nodose ganglia. *Gastroenterology*. 2005; 128:A-6.
91. Kobayashi H, Koppel AM, Luo Y, Raper JA. A role for collapsin-1 in olfactory and cranial sensory axon guidance. *J Neurosci*. 1997; 17:8339–8352. [PubMed: 9334408]
92. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. *Dev Dyn*. 2000; 218:213–234. [PubMed: 10842354]
93. Höpker V, Shewan D, Tessier-Lavigne M, Poo M, Holt C. Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature*. 1999; 401:69–73. [PubMed: 10485706]
94. Simon-Assmann P, Lefebvre O, Bellissent-Waydelich A, Olsen J, Orian-Rousseau V, De Arcangelis A. The laminins: role in intestinal morphogenesis and differentiation. *Ann N Y Acad Sci*. 1998; 859:46–64. [PubMed: 9928369]
95. Ratcliffe EM, D'Autreaux F, Gershon MD. Laminin terminates the Netrin/DCC mediated attraction of vagal sensory axons. *Developmental neurobiology*. 2008; 68:960–971. [PubMed: 18418846]
96. Gautron L, Lee C, Funahashi H, Friedman J, Lee S, Elmquist J. Melanocortin-4 receptor expression in a vago-vagal circuitry involved in postprandial functions. *The Journal of comparative neurology*. 2010; 518:6–24. [PubMed: 19882715]
97. Duester G. Retinoic acid synthesis and signaling during early organogenesis. *Cell*. 2008; 134:921–931. [PubMed: 18805086]
98. Niederreither K, Vermot J, Le Roux I, Schuhbaur B, Chambon P, Dolle P. The regional pattern of retinoic acid synthesis by RALDH2 is essential for the development of posterior pharyngeal arches and the enteric nervous system. *Development (Cambridge, England)*. 2003; 130:2525–2534.
99. Sato Y, Heuckeroth RO. Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth in vitro. *Developmental biology*. 2008; 320:185–198. [PubMed: 18561907]
100. Dmetrichuk JM, Spencer GE, Carlone RL. Retinoic acid-dependent attraction of adult spinal cord axons towards regenerating newt limb blastemas in vitro. *Developmental biology*. 2005; 281:112–120. [PubMed: 15848393]
101. Butler SJ, Dodd J. A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons. *Neuron*. 2003; 38:389–401. [PubMed: 12741987]
102. Chalazonitis A, Pham TD, Li Z, et al. Bone morphogenetic protein regulation of enteric neuronal phenotypic diversity: relationship to timing of cell cycle exit. *The Journal of comparative neurology*. 2008; 509:474–492. [PubMed: 18537141]

103. Faure C, Chalazonitis A, Rheaume C, et al. Gangliogenesis in the enteric nervous system: roles of the polysialylation of the neural cell adhesion molecule and its regulation by bone morphogenetic protein-4. *Dev Dyn.* 2007; 236:44–59. [PubMed: 16958105]
104. Fu M, Vohra BP, Wind D, Heuckeroth RO. BMP signaling regulates murine enteric nervous system precursor migration, neurite fasciculation, and patterning via altered Ncam1 polysialic acid addition. *Developmental biology.* 2006; 299:137–150. [PubMed: 16952347]
105. Powley TL, Wang XY, Fox EA, Phillips RJ, Liu LW, Huizinga JD. Ultrastructural evidence for communication between intramuscular vagal mechanoreceptors and interstitial cells of Cajal in the rat fundus. *Neurogastroenterol Motil.* 2008; 20:69–79. [PubMed: 17931338]
106. Fox EA, Phillips RJ, Byerly MS, Baronowsky EA, Chi MM, Powley TL. Selective loss of vagal intramuscular mechanoreceptors in mice mutant for steel factor, the c-Kit receptor ligand. *Anatomy and embryology.* 2002; 205:325–342. [PubMed: 12136263]
107. Song HJ, Ming GL, Poo MM. cAMP-induced switching in turning direction of nerve growth cones. *Nature.* 1997; 388:275–279. [PubMed: 9230436]
108. Song H, Poo M. The cell biology of neuronal navigation. *Nature cell biology.* 2001; 3:E81–88.
109. Dontchev VD, Letourneau PC. Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. *J Neurosci.* 2002; 22:6659–6669. [PubMed: 12151545]
110. Gehler S, Shaw AE, Sarmiere PD, Bamberg JR, Letourneau PC. Brain-derived neurotrophic factor regulation of retinal growth cone filopodial dynamics is mediated through actin depolymerizing factor/cofilin. *J Neurosci.* 2004; 24:10741–10749. [PubMed: 15564592]
111. Marsick BM, Flynn KC, Santiago-Medina M, Bamberg JR, Letourneau PC. Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin-1. *Developmental neurobiology.* 2010; 70:565–588. [PubMed: 20506164]
112. Bonanomi, D.; Pfaff, SL. Cold Spring Harbor perspectives in biology. Vol. 2. Motor axon pathfinding; p. a001735
113. Vohra BP, Tsuji K, Nagashimada M, et al. Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. *Developmental biology.* 2006; 298:259–271. [PubMed: 16904662]
114. Dillon AK, Fujita SC, Matise MP, et al. Molecular control of spinal accessory motor neuron/axon development in the mouse spinal cord. *J Neurosci.* 2005; 25:10119–10130. [PubMed: 16267219]
115. Sabatier C, Plump AS, Le M, et al. The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. *Cell.* 2004; 117:157–169. [PubMed: 15084255]
116. Nguyen-Ba-Charvet KT, Plump AS, Tessier-Lavigne M, Chedotal A. Slit1 and slit2 proteins control the development of the lateral olfactory tract. *J Neurosci.* 2002; 22:5473–5480. [PubMed: 12097499]
117. Huber AB, Kania A, Tran TS, et al. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron.* 2005; 48:949–964. [PubMed: 16364899]
118. Cohen S, Funkelstein L, Livet J, et al. A semaphorin code defines subpopulations of spinal motor neurons during mouse development. *The European journal of neuroscience.* 2005; 21:1767–1776. [PubMed: 15869472]

Figure 1.

Schematic diagram illustrating the locations of vagal sensory and motor endings in the gastrointestinal tract. Intramuscular arrays (blue) are found in the longitudinal and circular smooth muscle layers, intraganglionic laminar endings (red) are predominantly located in the myenteric plexus, and mucosal projections (green) extend into the lamina propria. Vagal motor endings (brown) synapse directly on neurons in the myenteric and submucosal plexi.

Figure 2.

Growth cone responses to chemical guidance cues. Upon sensing a gradient of netrin-1, growth cones expressing DCC are attracted towards the attractive cue (green shading). This is first seen as an elongation of filopodia towards the source followed by the reorientation of the growth cone towards the gradient. In contrast, upon sensing a repulsive cue such as Slit2, growth cones expressing Robo receptors collapse, thereby drawing back from the repulsive gradient (red shading).

Figure 3.

Three stages of IGLE development are illustrated with confocal images of DiI-labeled vagal profiles in the myenteric plexus of the mouse forestomach. **A.** The initial stage of IGLE development. This example is a putative IGLE precursor shown at P0 that consisted of a small number of vagal fiber terminals arising from a bifurcating axon bundle in the myenteric plexus. The terminals had the appearance of growth cone-like structures or puncta that straddled the top or bottom surface of a myenteric ganglion. **B.** The second stage of IGLE development is illustrated by this putative IGLE precursor shown at P3 (at center of image). Maturation to this stage consisted of increased numbers of terminal puncta. **C.** The third stage of putative IGLE precursor maturation is illustrated here at P8. The continued development of putative IGLE terminals consisted of a further increase in numbers of terminal puncta, an increase in their packing density, and their arrangement in several leaf-like patterns or groups. Some IGLEs at this stage exhibited one or more groups of terminal puncta lying below the surface of a myenteric ganglion, and one more above it. Scale bars = 10 μm . This figure was previously published as Fig. 5B, D, F (48), copyright Elsevier (2007).

Figure 4.

Four stages of IMA development are illustrated with confocal images of DiI-labeled vagal profiles in the muscle layers of the mouse forestomach. **A.** The initial stage of IMA formation is neurite formation, shown here by a putative IMA precursor at P0. Small vagal axon bundles in the myenteric plexus gave off a single axon (lower left), which entered the smooth muscle layer (as it continued diagonally to the upper right) and gave rise to three neurites (arrows; neurite furthest to the right is enlarged in inset). Some neurites extended small growth cone-like structures. These neurites may represent the initial formation of an IMA or a telodendrion. **B.** The second stage of IMA maturation is telodendria formation, illustrated here by a putative IMA precursor at P0. Two axons that exited from the myenteric plexus are shown here coursing within the longitudinal muscle layer where they branched and distributed short rectilinear fibers to begin formation of IMA telodendria (arrows) that ran parallel to the muscle fibers, including one pair that was interconnected by a crossbridge fiber (arrowheads). **C.** The third stage of IMA development is telodendria extension, demonstrated here by a putative IMA precursor at P0 within the circular muscle layer. The main features of this stage were lengthening of the telodendria and an increase in the numbers of telodendria connected by cross-bridge fibers. **D.** The final stage of IMA maturation is shown here in a montage of confocal images of putative IMA precursors at P4. This stage was dominated by further lengthening of IMA telodendria as compared with earlier stages (e.g., A-C) as well as by continued formation and growth of telodendria. Scale bars = 10 μm (A-D) and 25 μm (inset, A). This figure was previously published as Fig. 5A, C, E (48) and Fig. 3D (52), copyright Elsevier (2007, 2008).

Figure 5.

DCC immunoreactivity is found in fetal mouse paraesophageal vagal nerve trunks at E16. (A-C) Esophagus. Only fasciculated vagal nerve trunks are DCC immunoreactive. The same section of esophagus has been labeled by DiI applied to the nodose ganglia (A; red) and with antibodies to DCC (B; green). (C) The images in panels A and B are merged. Note the absence of co-localization in the ramifying fibers, suggesting that other molecules may be involved in guiding vagal endings to their correct enteric targets. Bar = 50 m. This figure was previously published at Fig. 4F-H (44), copyright John Wiley & Sons, Inc. (2006).

Table 1

Summary of classical axon guidance families, as discussed in this review, with known receptors and representative examples of functions in the developing vertebrate nervous system.

Guidance Molecule	Receptors	Examples of Known Roles	Reference
Netrins			
Netrin-1, (-2*), -3, -4 *only in chicken	DCC, neogenin, UNC5A-D	Guidance of axons at the midline of the spinal cord (netrin-1; attractive) Guidance of spinal accessory motoneurons (netrin-1; repulsive)	(32, 114)
Slits			
Slit-1, -2, -3	Robo-1, -2, -3	Guidance of commissural axons at the midline of the spinal cord (repulsive) Guidance of olfactory bulb axons (slit-1, -2; repulsive)	(115) (116)
Semaphorins			
Sema class III	Formation of receptor complexes involving neuropilins (NRP 1, 2), plexins (A-D) and other co-receptors	Guidance of spinal motor axons (sema3A-F; repulsive) Guidance of cortical efferent nerves (sema3C; attractive)	(117, 118) (50)