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REVIEW

Gut innervation and enteric nervous system development: a spatial, temporal and molecular tour de force

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ABSTRACT

During embryonic development, the gut is innervated by intrinsic (enteric) and extrinsic nerves. Focusing on mammalian ENS development, in this Review we highlight how important the different compartments of this innervation are to assure proper gut function. We specifically address the three-dimensional architecture of the innervation, paying special attention to the differences in development along the longitudinal and circumferential axes of the gut. We review recent information about the formation of both intrinsic innervation, which is fairly well-known, as well as the establishment of the extrinsic innervation, which, despite its importance in gut-brain signaling, has received much less attention. We further discuss how external microbial and nutritional cues or neuroimmune interactions may influence development of gut innervation. Finally, we provide summary tables, describing the location and function of several well-known molecules, along with some newer factors that have more recently been implicated in the development of gut innervation.

KEY WORDS: Gut, Enteric nervous system, Intrinsic innervation, Extrinsic innervation

Introduction

Proper function of the gastrointestinal (GI) tract relies on many specific cell types at precise locations working together in a tightly coordinated manner. Foremost among these cell types are enteric neurons and glia of the enteric nervous system (ENS), which are organized in two concentric and interconnected nerve plexuses embedded in the gut wall. Within each plexus, enteric neurons and glia are arranged in clusters termed 'ganglia', which are linked by interganglionic fiber tracts to form a characteristic mesh-like network. This extensive neural network supplies the full length of the GI tract, running from the esophagus, stomach, small intestine (i.e. duodenum, jejunum and ileum) and caecum (or appendix in humans), through to the end of the large intestine (i.e. colon). During embryonic development, the esophagus, stomach and initial part of the duodenum arises from the primordial foregut, whereas the rest of the small intestine and cecum arises from the midgut, and the colon derives from the midgut and hindgut (Rao and Gershon, 2018; Geesman et al., 2021). Crucially, the network controls muscular activity for the movement of luminal contents through the tract. The ENS is the largest, most complex division of the peripheral nervous system (PNS), comprising millions of nerve cells and glia that enable it to sense and integrate information, and exert appropriate motor outputs (Furness, 2012; Schneider et al., 2019; Fung and Vanden Berghe, 2020; Li et al., 2020). It is crucial that gut innervation develops correctly and, unsurprisingly, this is not a trivial task. In extreme cases, neural progenitors can fail to colonize the entire gut causing aganglionosis, in which the most distal part of the large intestine is left without innervation (Lake and Heuckeroth, 2013). This condition causes the life-threatening Hirschsprung's disease (HSCR), which occurs in about one in 5000 live births (Heanue and Pachnis, 2007; Lake and Heuckeroth, 2013). Nonetheless, even when enteric ganglia do develop, errors in their assembly can still have severe consequences, such as chronic idiopathic intestinal pseudo-obstruction (Gershon, 2009; Rao and Gershon, 2018). Furthermore, subtle developmental faults in circuit wiring can also have significant implications on GI functionality (Sasselli et al., 2013).

Compared with other components of gut innervation, the development of the myenteric plexus (MP) is by far the best-characterized. The MP mainly controls gut motility and is situated between the circular and longitudinal muscle layers of the GI tract. In mice, vagal neural crest cells first enter the foregut at approximately embryonic day (E) 9.5 and migrate caudally whilst proliferating and populating the gut at the level of the prospective MP (Young et al., 1998) (Fig. 1). As enteric neural crest-derived cells (ENCCs) begin to cluster and form ganglia in the myenteric layer, some of the progenitors undergo radial migration towards the gut lumen and give rise to the submucosal plexus (SMP), which is situated closer to the mucosa and is responsible for regulating fluid secretion, nutrient absorption and intestinal blood flow (Uesaka et al., 2015, 2016; Rao and Gershon, 2018) (Fig. 1). However, our understanding of how the SMP arises is relatively limited. How sensory projections from the MP and SMP are directed to the mucosa to sense information about luminal contents also remains largely unexplored. Furthermore, little is known about the molecular pathways that govern these essential developmental processes.

In this Review, we focus on the most recent advances related to enteric and extrinsic neuron development, highlighting current knowledge about how ENS architecture is established in three dimensions. We first briefly touch on how the ENS develops along the length of the gut and how regional differences in ENS wiring might arise. We then shift our focus to ENS development in the radial serosa-mucosa axis, with a particular emphasis on SMP formation. We also discuss recent findings on the extrinsic innervation of the developing gut. We address how neuroimmune interactions or external microbial and nutritional cues may influence ENS development. Finally, we summarize the signaling pathways and key molecules involved in these developmental processes. The Review is largely focused on mouse ENS development, due to the inherent flexibility of murine genetic models, but we do include other models such as zebrafish and chick where relevant.

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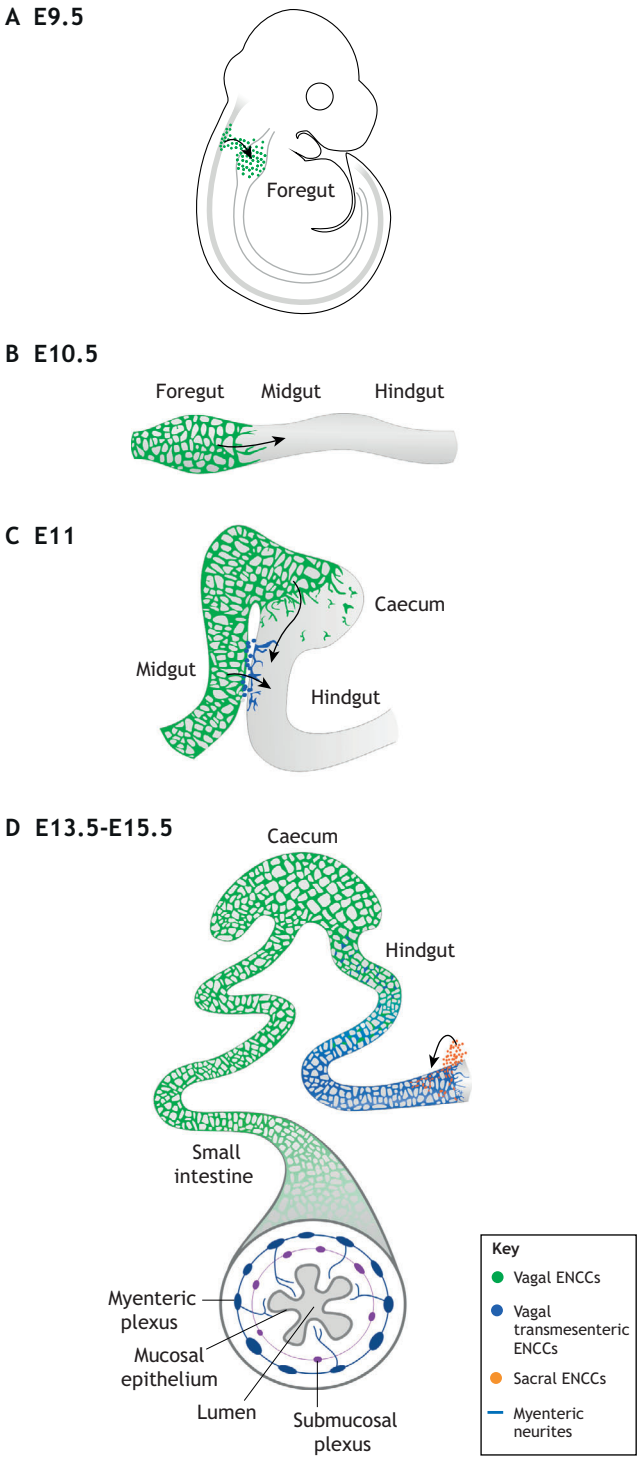

Fig. 1. Timeline of key events during ENS development in the mouse embryonic gastrointestinal tract. (A) At E9.5, a small group of vagal ENCCs invades the foregut and starts to migrate rostrocaudally. (B) ENCCs migrate in chains, proliferate and differentiate into enteric neurons and glia behind the migratory wavefront. (C) As the gut lengthens, it forms a single bend at ∼E11, such that the midgut is transiently apposed to the hindgut. Between E11 and E12, some vagal ENCCs take a shortcut across the mesentery from the midgut to the hindgut and bypass the caecum. The transmesenteric and caecal (vagal) ENCC populations merge to form the ENS in the proximal hindgut. As vagal ENCCs arrive, the hindgut also grows in length. (D) At E13.5, there is a smaller group of sacral ENCCs that enters the hindgut and migrates caudorostrally. Vagal ENCCs reach the end of the gut by ∼E14.5. Meanwhile, myenteric neurites in the small intestine start to project radially towards the mucosa. A subpopulation of ENCCs from the myenteric plexus then migrates inwards to begin forming the submucosal plexus from E15.5 and the submucosal plexus continues to develop postnatally.

Development of intestinal innervation along the longitudinal axis

ENCC migration and colonization

During the early stages of development, vagal and some sacral neural crest-derived progenitors have to migrate and proliferate extensively to colonize the gut as it rapidly grows and elongates. These progenitors first disseminate throughout the gut, migrating in chains along the rostrocaudal axis (Fig. 1A). The process of gut colonization has been extensively discussed in a number of excellent reviews (Lake and Heuckeroth, 2013; Xu et al., 2014; Hao et al., 2016; Nagy and Goldstein, 2017; Rao and Gershon, 2018). Murine, chick and quail, and zebrafish models are commonly used to study ENS development (Hearn et al., 1998; Wallace et al., 2005; Goldstein and Nagy, 2008; Ganz, 2018; Hao et al., 2020), although zebrafish have a simpler ENS that does not have an SMP and lacks defined ganglia (Wallace et al., 2005; Ganz, 2018).

In mice at E9.5, a small group of vagal ENCCs invades the foregut and starts to migrate rostrocaudally, with ENCCs proliferating and differentiating into neurons and glia behind the migratory wavefront (Fig. 1B). As the gut lengthens, it forms a single bend at ∼E11, such that the midgut is briefly closely apposed to the hindgut. Between E11 and E12, some vagal ENCCs directly cross the mesentery from the midgut to the hindgut, bypassing the caecum (Fig. 1C). These ‘transmesenteric’ vagal ENCCs give rise to at least the distal two-thirds of the hindgut ENS, with the distal half being almost exclusively derived from this population (Nishiyama et al., 2012). The transmesenteric and caecal (vagal) ENCC populations merge to form the ENS in the proximal hindgut. As vagal ENCCs arrive, the hindgut also grows in length. At E13.5, there is a smaller group of sacral ENCCs that enters the hindgut and migrates caudorostrally (Wang et al., 2011). Vagal ENCCs then reach the end of the gut by about E14.5. Around this time, myenteric neurites in the small intestine start to project radially towards the mucosa (Hao et al., 2020). A subpopulation of ENCCs from the MP then migrates inwards to begin forming the SMP from E15.5 onwards (Uesaka et al., 2013) (Fig. 1D). Although electrical activity and spontaneous calcium ion (Ca^{2+}) activity is detected in the developing ENS from as early as E11.5 (Hao et al., 2011), neurogenic motor activity is only observed from E18.5 in the duodenum (Roberts et al., 2010) and by P10 in the colon (Roberts et al., 2007). The electrophysiological properties of developing enteric neurons continue to mature postnatally (Foong et al., 2012). The time between the onset of electrical activity and neurally-mediated gut function is likely an important period during which the formation of synaptic connections between different neurons and their various cellular targets occurs (Hao et al., 2013a).

The development of the human ENS occurs through a similar ENCC colonization process, in which ENCCs take a similar migratory route to that in mice and chick embryos between embryonic weeks (EW) 4-7 (Fu et al., 2003; Wallace and Burns, 2005; Obermayr et al., 2013). Subsequently, the development of myenteric ganglia occurs from EW7-EW14 and coincides with the differentiation of the smooth muscle. The SMP develops later than the MP and begins forming from EW8.5-EW14 (Fu et al., 2003, 2004). The human ENS displays electrically-evoked Ca^{2+} responses by EW16, and this timing coincides with the

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expression of key neurotransmitters and synaptic proteins (McCann et al., 2019).

Regional specializations

Recent work by Li and colleagues has demonstrated that there are regional differences in the connectivity of the ENS in the proximal versus distal colon (Li et al., 2019). How such specializations are established is not well understood. One of the factors influencing the axonal projections of early enteric neurons is the directionality of migrating ENCCs (Young et al., 2002). The caecum is a specialized blind-ending pouch structure that develops by budding off the side of the gut tube at the junction between the ileum and colon. In line with its complex form, the pattern of ENCC migration in the caecum differs significantly from that of other intestinal regions (Druckenbrod and Epstein, 2005). Rather than continuing to advance as strands, the ENCC wavefront halts once it reaches the developing caecum at around E11.5. After a pause of ∼8-12 h, isolated ENCCs begin migrating into the caecum and form isolated cell aggregates. These then extend strands to other cell clusters and re-establish an interconnected network before resuming its caudal migration. It is during this time that transmesenteric ENCCs take a shortcut, crossing from the midgut to the hindgut as mentioned earlier (Nishiyama et al., 2012). It remains to be examined whether the distinct migration patterns of these early neuronal progenitors foreshadow differences in the configuration of the enteric circuitry in the caecum and colon.

Regulation of ENCC migration

ENCC migration in the developing gut is influenced by cell number, cell-cell interactions mediated by cell adhesion molecules, and factors secreted by the mesenchyme (Young et al., 2004a,b). It is well established that several signaling pathways are crucially important to establish a fully functional ENS. Two such pathways, the glial cell line-derived neurotrophic factor (GDNF) and its receptor/co-receptor RET/GDNF family receptor alpha-1 (GFRα1), and endothelin-3 (EDN3) and its receptor endothelin-receptor type B (EDNRB), are considered the most dominant in the developmental process (discussed below; Table 1) (Heuckeroth et al., 1998; Young et al., 2001; Gianino et al., 2003; Uesaka et al., 2013; Bondurand et al., 2018). Mutations in either of these pathways converge on an HSCR phenotype that leaves the last segment of the GI tract void of enteric neurons (Puffenberger et al., 1994; Romeo et al., 1994; Schuchardt et al., 1994; Amiel et al., 1996; Shimotake et al., 2001).

Undoubtedly, the RET pathway plays the most crucial role in ENS development. RET, a member of the receptor tyrosine kinase superfamily, is a transmembrane receptor that is present in all ENCCs as they migrate through the gut. The activation of RET is crucial for precursor survival (Heuckeroth et al., 1998; Taraviras et al., 1999), proliferation (Heuckeroth et al., 1998; Gianino et al., 2003), migration (Natarajan et al., 2002), differentiation (Hearn et al., 1998) and neurite growth (Young et al., 2001). Loss of RET

Table 1. Molecules involved in the RET and EDNRB signaling pathways

Molecule	Location (time of expression)	Related receptor/ligand and functions
RET signaling pathway		
Gdnf	Gut mesenchyme	Chemoattractant for enteric neurites (Young et al., 2001); the ligand for receptor RET and co-receptor GFR1; GDNF plays an important role in neuronal differentiation and its signaling is necessary for the radial migration of ENCCs (Uesaka et al., 2016); it is one of the known HSCR causative genes; it has roles in survival or differentiation and possibly enteric neuron subtype specification (Yan et al., 2004; Uesaka and Enomoto, 2010; Wang et al., 2010).
Gfrα1/2	Gfrα2 is expressed in MP and SMP in the newborn intestine	RET co-receptor; conditional knockdown of the RET/GDNF/Gfrα1 pathway at E13.5 results in a lack of submucosal ganglia (Uesaka et al., 2013).
Kif26A	Protein gene product 9.5 (PGP9.5)$^{+}$ clusters in muscular and SMP layer in colon (E12.5, E14.5 and P12)	An atypical kinesin; a negative regulator of RET; *Kif26A*$^{-/-}$ mice show myenteric neuronal hyperplasia, pseudo-obstruction (Zhou et al., 2009).
Neurturin (NRTN)	Gut mesenchyme; the circular muscle layer	A neurotrophin in the GDNF family that binds to RET and its co-receptor Gfrα2; *Nrtn*- or *Gfra2*-deficient mice show a decreased number of substance P-expressing excitatory axons in the circular muscle layer (Heuckeroth et al., 1999; Rossi et al., 1999, 2003; Gianino et al., 2003); one of the known HSCR causative genes.
Ret	ENCCs (as they migrate through the gut)	A transmembrane tyrosine kinase receptor that supports ENS precursor survival (Heuckeroth et al., 1998; Taraviras et al., 1999), proliferation (Heuckeroth et al., 1998; Gianino et al., 2003), migration (Natarajan et al., 2002), differentiation (Hearn et al., 1998) and neurite growth (Young et al., 2001); *RET* dysfunction causes intestinal aganglionosis within the entire gut in mice and human (Schuchardt et al., 1994; Shimotake et al., 2001), and is found to be involved in most of HSCR cases; co-receptor GFR1 and ligand GDNF are the crucial RET activators during fetal development; transcription factors SOX10, RARB, GATA2 and PHOX2B are all crucial to regulate the expression of *RET* (Chatterjee et al., 2017, 2019).
Sprouty2 (Spry2)	Generally expressed during embryogenesis	A negative regulator of receptor tyrosine kinase signaling; homozygous null mice show myenteric neuron hyperplasia, pseudo-obstruction and achalasia (Taketomi et al., 2005).
EDNRB signaling pathway		
Ece1	N/A	An EDN3 processing protease; one of the known HSCR causative genes.
Ednrb	Neural crest derivatives (including ENCCs)	A G protein-coupled receptor that works together with its ligand Edn3, which is expressed in the gut mesenchyme, involved in ENS development in the colon; maintains ENCCs in an uncommitted and proliferative state (Nagy and Goldstein, 2017); one of the known HSCR causative genes.
Edn3	Gut mesenchyme	A ligand of EDNRB that plays crucial roles in ENCC proliferation, differentiation and migration (Bondurand et al., 2018); one of the known HSCR causative genes.

ENCC, enteric neural crest cell; HSCR, Hirschsprung's disease; N/A, not applicable.

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function causes intestinal aganglionosis within the entire gut of RET-null mice (Schuchardt et al., 1994; Shimotake et al., 2001), and similar mutations have been described in patients (Amiel et al., 2008). Apart from RET itself, its co-receptor GFRα1 and the ligand GDNF are also necessary to activate the RET pathway during fetal development (see Table 1 for references). Inactivating mutations in this pathway are responsible for the majority of familial and sporadic HSCR cases (Romeo et al., 1994; Angrist et al., 1996). Conversely, overactivation of this pathway, for example by gain-of-function mutations such as RET-C618F, leads to hyperganglionosis (Okamoto et al., 2019). Although the role of the RET signaling pathway is fairly well established, it remains unclear how specific RET gene mutations bring about some – but not other – pathological phenotypes (e.g. multiple endocrine neoplasia). It is also unclear why single mutations are insufficient to result in a phenotype in some circumstances and therefore require additional mutations in other genes (Nakatani et al., 2020). The importance of GDNF is further highlighted *in vitro*, where the ability of enteric neural progenitors to generate an ENS is enhanced by GDNF (McKeown et al., 2017). A number of transcription factors, including SOX10, RARB, GATA2 and PHOX2B, are all crucial to regulate the expression of *RET* (Chatterjee et al., 2017, 2019), and as such, also play a crucial role in ENS development. In addition, Sprouty2 (an inhibitor of receptor tyrosine kinase signaling) and the kinesin Kif26A serve as negative regulators of RET signaling (Taketomi et al., 2005; Zhou et al., 2009). Finally, perturbation of Hoxb5 causes Ret haploinsufficiency, impaired NCC migration and hypo/aganglionosis in mice (Lui et al., 2008; Carter et al., 2012; Kam et al., 2014; Kam and Lui, 2015), indicating a key role for HOXB5 in ENCC development.

The EDNRB signaling pathway is another crucial element in the control of ENCC proliferation, differentiation and migration (reviewed by Bondurand et al., 2018) (Table 1). Many known HSCR causative mutations are from members of the EDNRB pathway. EDNRB is a G protein-coupled receptor located on ENCCs that works together with the ligand-processing protease endothelin-converting enzyme (ECE) and the ligand EDN3, which are expressed in the gut mesenchyme during ENS development. EDN3 can also affect ENCCs indirectly by enhancing GDNF function, and it maintains ENCCs in an uncommitted and proliferative state (Stone et al., 1997; Hearn et al., 1998; Lahav et al., 1998; Bondurand et al., 2006; Nagy and Goldstein, 2006). EDN3 also modulates the extracellular matrix (ECM) to influence ENCC migration.

Intracellular c-Jun N-terminal kinase (JNK) and cAMP signaling have been proposed to act downstream of GDNF/RET and EDN3/EDNRB signaling (Hao et al., 2019). Recently, pharmacological agents that stimulate or inhibit intracellular JNK or cAMP signaling have been used to identify potential determinants of the speed and directionality of individual ENCCs in E12.5 mouse gut (Hao et al., 2019). Migration speed is modulated by JNK and cAMP signaling, whereas directionality and adhesion appear to be regulated by cAMP signaling, but not JNK, suggesting that these various migratory properties of ENCCs are differentially regulated. Apart from GDNF/RET and EDN3/EDNRB signaling pathways, a number of other molecules also play a crucial part, or have a refining role, in how the ENS is established (Tables 2 and 3). Notably, Sox10 is a key transcription factor with crucial roles in maintaining the progenitor state of multilineage ENCCs, and in glial development (Bondurand and Sham, 2013). Sox10 expression is maintained in glial cells, whereas it is downregulated as progenitors differentiate into enteric neurons (Hao et al., 2017a).

Influence of the gut microenvironment

The microenvironment of the intestinal wall, which is composed of several different ECM components, has been proposed to be another determinant of ENCC migration. A recent study has shown that genes encoding ECM proteins are among the most enriched in mouse intestine at late developmental stages (i.e. at E15.5) (Nishida et al., 2018). Notably, over time, collagen VI (ColVI) expression is upregulated, whereas fibronectin (FN) expression is downregulated. Subsequent functional assays suggest a role for ColVI in inhibiting FN-induced ENCC migration. Another study has shown that both mouse and chick vagal- and sacral-derived ENCCs begin producing collagen type XVIII (Col18) close to the ENCC wavefront early in development and that Col18 is permissive for ENCC migration. In contrast, agrin, which impedes ENCC migration, starts to be expressed at a later stage (i.e. from E10) (Nagy et al., 2018). Collectively, these findings indicate that ENCCs migration is influenced by the microenvironment and ENCCs may be capable of actively shaping this environment through spatiotemporally controlled secretion of specific ECM proteins.

Gangliogenesis

Once a gut region is populated by ENCCs, myenteric neural precursors begin clustering to form ganglia. It is not clear which factors govern this particular organization, nor whether mechanical forces generated by early onset of neural activity play a role. There is indeed some evidence suggesting that gangliogenesis is an activity-dependent process. One study has shown that ganglion formation correlates with intracellular Ca^{2+} transients in the sympathetic nervous system (McKinney and Kulesa, 2011). Similarly, spontaneous propagating Ca^{2+} waves occur between adjacent enteric neural progenitors during embryonic development. These waves involve purinergic signaling, and specific antagonism of purinergic P2 receptors causes defects in ENS network formation (Hao et al., 2017b). Apart from intrinsic activity in the forming ENS, theoretical modeling also suggests that changes in adhesive capacity of the cells may underlie such grouping behavior (Hackett-Jones et al., 2011). The relationship between ganglion formation, their size or the numbers of neurons they contain and their later functionality is completely elusive; several different reports show that both hyper- and hypoganglionosis are associated with ENS functionality defects (Wedel et al., 2002; Breau, 2006; Meier-Ruge et al., 2006; Yin et al., 2006; Hendershot et al., 2007).

Neuronal subtypes

As enteric ganglia form during development, progenitors differentiate into glia and diverse subtypes of enteric neurons (Wallace and Burns, 2005), including sensory neurons, various types of interneurons, and excitatory and inhibitory motor neurons (Fung and Vanden Berghe, 2020). Different functional subsets of enteric neurons can be inferred based on their axonal projection patterns and expression of primary transmitters, their synthesizing enzymes, calcium-binding proteins or cytoskeletal proteins (Qu et al., 2008; Mongardi Fantaguzzi et al., 2009). Based on these properties, 16 different neuronal subtypes have been identified in the mature ENS (Hao and Young, 2009). Different neurochemical subtypes of enteric neurons are born (i.e. exit the cell cycle) over specific developmental time points (Fig. 2). For example, serotonin neurons, which are anally-projecting interneurons, are one of the earliest born neuronal subtypes. They have been shown to exit the cell cycle from as early as E8, with a peak time of cell cycle exit at around E10-E11.5 (Pham et al., 1991; Hao and Young, 2009; Bergner et al., 2014). On the other hand, calcitonin gene-related

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Table 2. Pathways and secreted molecules implicated in the development of gut innervation

Molecule	Location (time of expression)	Related receptor/ligand and functions	Role
Artemin (ARTN)	Developing mesenteric arteries.	*Artn*-deficient mice exhibit defects in the sympathetic innervation of the gut (Honma et al., 2002).	Ex
Brain-derived neurotrophic factor (Bdnf)	Undifferentiated mesenchyme of the fetal gut; the lamina propria and gut muscle.	A neurotrophic factor which supports neuronal survival and differentiation; shows high affinity with TrkB receptor which is essential for maintenance of glial cells in ENS (Levanti et al., 2009); essential for the survival, growth or maintenance of vagal sensory fibers (Uesaka et al., 2016); smooth muscle-derived BDNF acts to reduce sensory innervation density (Biddinger and Fox, 2014).	Ex
Bone morphogenetic protein (Bmp)	Mesenchyme; ENCCs.	Regulates ENCC migration (Fu et al., 2006) and differentiation (Chalazonitis et al., 2008, 2011), and influences neuronal subtype diversity (Nagy and Goldstein, 2017); BMP enhances neuronal aggregation (Goldstein et al., 2005) and induces the fasciculation of neurites (Fu et al., 2006).	L
Cdc42	N/A	A Rho family GTPase; in *Cdc42*-deficient mice, ENCCs fail to proliferate and colonize in distal gut (Fuchs et al., 2009).	L
Celsr3	Neuroectodermal derivatives of the gut (during embryogenesis).	The cadherin EGF LAG seven-pass G-type receptor (Celsr)3; involved in PCP pathway, which is crucial for axonal projection and establishes ENS connectivity (Sasselli et al., 2013).	N/A
Chondroitin sulfate proteoglycans (CSPGs)	Declined in the circular layer of the developing rat mid- and hindgut (E12.5-E16.5).	CSPGs (including versican and collagen type IX) inhibit ENCC migration (Nagy et al., 2016).	L
Collagen VI	Distributed along the length of the developing gut.	ECM component that inhibits GDNF-mediated ENCC migration (Nishida et al., 2018).	L
Deleted in colorectal cancer (Dcc)	Migrating ENCCs.	DCC, a netrin receptor, is required for netrins to attract ENCCs (Jiang et al., 2003).	C
	Vagal nerve fibers.	The netrin/DCC pathway acts as an attraction force for vagal nerve fibers to the gut (Ratcliffe et al., 2006).	Ex
DENN/MADD domain containing 3 (DENND3)	N/A	A guanine nucleotide exchange factor; involved in intracellular trafficking by activating the small GTPase RAB12 in mouse embryonic fibroblasts (Matsui et al., 2014); in zebrafish, *dennd3* loss-of-function disrupted ENS development and caused an HSCR-like phenotype (Gui et al., 2017).	N/A
Dicer	N/A	An miRNA-processing enzyme which is required for survival of differentiating neural crest cells (Zehir et al., 2010).	L
Ece1	Please refer to Table 1.	Please refer to Table 1.	L
Ednrb	Please refer to Table 1.	Please refer to Table 1.	L
Edn3	Please refer to Table 1.	Please refer to Table 1.	L
ErbB2/3	ErbB3: ENS precursors (at E13.5); enteric glia and a subset of neurons (in the developing and mature ENS).	Members in EGF-receptor family; ErbB2 in colonic epithelium is required for postnatal maintenance of the ENS (Crone et al., 2003).	L
Ercc1	N/A	A nucleotide excision repair factor which is also important in recombination repair and the repair of interstrand crosslinks; *Ercc1*-deficient mice have severe colonic obstruction (Selfridge et al., 2010).	N/A
Growth arrest specific 1 (Gas1)	Enteric neural-crest-derived progenitors; enteric neurons; smooth muscle layers in gut.	The binding partner of Shh; *Gas1*-deficient mice have more ENS precursors and neurites in the mucosa at E18.5 (Biau et al., 2013; Jin et al., 2015); the enteric axon growth is only inhibited by Shh together with Gas1 *in vitro* (Jin et al., 2015).	C
Glial cell-derived neurotrophic factor (Gdnf)	Please refer to Table 1.	Please refer to Table 1.	C
Gfrα1/2	Please refer to Table 1.	Please refer to Table 1.	C
Hedgehog (including SHH and IHH)	Produced by intestinal epithelial cells.	Diffusible morphogens that regulate BMP4 expression and activate mesenchymal expression of forkhead (Fox) transcription factors: FOXF1 and FOXF2 (Ormestad et al., 2006); *Hh* mutant mice show multiple GI defects (Ramalho-Santos et al., 2000); Hh signaling is mediated by smoothened (Smo), which acts as a transducer, and the Hh-binding receptor patched homolog 1 (Ptch1) that suppresses Smo (Marigo et al., 1996; Stone et al., 1996); deficiency in this signaling is involved in HSCR (Ngan et al., 2011; Liu et al., 2015); Hh promotes proliferation and inhibits neuronal differentiation (Sukegawa et al., 2000; Fu et al., 2004; Reichenbach et al., 2008); SHH	C

Continued

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Table 2. Continued

Molecule	Location (time of expression)	Related receptor/ligand and functions	Role
		prevents premature radial migration of ENCCs into the future submucosa (Lake and Heuckeroth, 2013); SHH plays an inhibitory role in ENCC migration and does not act directly on ENCCs, which appear to lack the HH receptors, but rather indirectly (Nagy and Goldstein, 2017).	
Integrin family (including α4β1, α5β1, α6β1, αVβ1, αVβ3, αVβ5)	On the surface of ENCCs.	Cell adhesion molecules that modulate their interactions with the ECM; defects in β1 integrin (*Itgb1*) lead to abnormal cellular adhesion and migration and results in an HSCR-like phenotype (Breau, 2006; Nagy et al., 2009; Broders-Bondon et al., 2012).	L
Kif26A	Please refer to Table 1.	Please refer to Table 1.	L
Neural cell adhesion molecule L1 (L1cam)	On the surface of ENCCs.	The cell adhesion molecule that modulates ENCC interactions with the ECM and maintains cell–cell contacts at the migratory wavefront; L1CAM mutations reduce ENCC contact and lead to HSCR (Anderson et al., 2006a).	L
Laminin 111	The epithelial basal lamina; around enteric ganglia.	Promotes neuronal differentiation and converts the effect of netrin–DCC signaling from attraction into repulsion (Rao and Gershon, 2018); it can cease the attraction of vagal nerves toward sources of netrin-1 (Ratcliffe et al., 2008).	C
Lgi4	ENCCs.	Involved in glial development and myelination; *Lgi4*$^{-/-}$ mice show a reduced number of glial cells, impaired glial marker expression and abnormal ENS structure (Nishino et al., 2010).	N/A
Mitogen-activated protein kinase 10 (Mapk10)	N/A	Mapk inhibitors block ENCC migration in cultured guts (Asai et al., 2006); Mapk10 highly expressed in the mammalian ENS; an HSCR susceptibility locus which can be targeted by miR-4516 and causes migration delay *in vitro* (Wang et al., 2020) and in zebrafish (Heanue et al., 2016).	L
Met	Subsets of enteric neurons.	Ablation of MET (the receptor of hepatocyte growth factor, HGF) in the mouse PNS leads to defects in enteric neurite outgrowth and increased vulnerability to intestinal inflammation (Avetisyan et al., 2015).	N/A
N-cadherin	Most ENCCs.	Homophilic cell adhesion molecule; mice deficient in N-cadherin exhibit significantly delayed ENCC colonization of the gut (Broders-Bondon et al., 2012).	L
NCLN (Nicalin)	Unknown.	A transmembrane protein that binds with Nomo (nodal modulator) to form a protein complex that antagonizes Nodal [a subfamily of the transforming growth factor-β (TGFβ) proteins] signaling (Haffner et al., 2004); involved in induction of the mesoderm and endoderm in vertebrates (Schier, 2003).	N/A
Netrin-1	Produced by intestinal epithelial cells; expressed in ENCCs and differentiated enteric neurons (during radial migration of ENCCs).	Netrin-deficient mice do not have an SMP (Jiang et al., 2003); netrin-1 and its receptor DCC can attract vagal sensory axons to the fetal mouse gut (Ratcliffe et al., 2006); important for axon guidance in the CNS (Finci et al., 2015); these indicate that the Netrin/DCC pathway is necessary for secondary/radial migration and axon projection of ENCCs toward submucosa to form SMP (Uesaka et al., 2016).	C
Neuropilin-1	ENCCs.	Co-receptor for Sema3A (Anderson et al., 2007).	L
Neurotrophin-3 (NT-3)	The outer smooth muscle layers of the developing GI tract; the smooth muscle walls of blood vessels that supply gut.	The ligand of TrkC and p75NTR that is required for the survival-differentiation of subsets of developing enteric neurons (Chalazonitis et al., 2001); NT-3 and TrkC are crucial for the survival of the majority of vagal sensory neurons (Ernfors et al., 1994; Fariñas et al., 1994; Liebl et al., 1997; Tessarollo et al., 1997).	Ex
Neurturin (NRTN)	Please refer to Table 1.	Please refer to Table 1.	L
Nerve growth factor (NGF)	In the epithelial layer and enteric ganglia of the developing GI tract.	Plays crucial roles in the survival of small nociceptive sensory neurons as well as sympathetic neurons (Fariñas, 1999).	Ex
Nucleoporin 98 (NUP98)	Nuclear membrane of ENCCs.	A missense *de novo* mutation was identified in the last exon of the *NUP98* gene, affecting the NUP96 protein; NUP96 is involved in the nuclear pore complex and regulates proliferation; NUP96 also interacts with NUP98, which is involved in transcriptional regulation of the HSCR genes	L

Continued

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Table 2. Continued

Molecule	Location (time of expression)	Related receptor/ligand and functions	Role
		(such as SEMA3A, DSCAM, NRG1 and the NRG1 receptor ERBB4) in human neural progenitor cells (Gui et al., 2017).	
Pds5A/B	N/A	Cohesion regulatory factors; *Pds5A*/*B*-deficient mice have delayed colonization of ENCCs and partially penetrant colonic aganglionosis (Zhang et al., 2009).	L
Phactr4	Embryonic ENCCs.	A phosphatase and actin binding protein that regulates the actin cytoskeleton and cell adhesion, and acts to antagonize β1 integrin signaling in regulating *in vivo* migration; homozygous mutant mice show colonic hypoganglionosis (Zhang and Niswander, 2012; Zhang et al., 2012).	L
O-fucosyltransferase 1 (Pofut1)	Neural crest cells.	Involved in Notch signaling; NC-specific Pofut1-knockout mice die within 1 day of birth and show premature neurogenesis (Okamura and Saga, 2008).	L
Prokineticin 1 (PROK1)	Unknown.	A secreted protein that induces ENCC proliferation and differentiation; PROK1 mutations can be found in some HSCR cases (Ngan et al., 2007; Carter et al., 2012).	L
Pten	ENCCs.	A tumor suppressor protein that reverses the reaction catalyzed by PI3K; serves as a 'brake' on ENCC migration, proliferation and growth (Fu et al., 2010).	L
Rac1	N/A	One GTPase in Rho family; ENCCs fail to proliferate and colonize the distal gut in *Rac1*-deficient mice (Fuchs et al., 2009).	L
Raldh2 (Aldh1a2)	N/A	The retinaldehyde dehydrogenase that synthesizes RA; NC cells never enter the gut in Raldh2$^{-/-}$ mice (Niederreither et al., 2003).	L
Ret	Please refer to Table 1.	Please refer to Table 1.	L
Retinoic acid (RA)	The paraxial mesoderm.	Produced by Raldh; is required for the efficient migration and colonization of ENCCs (Niederreither et al., 2003; Fu et al., 2010), but excess RA can cause defects in ENS development (Pitera et al., 2001); induces shorter neurites in enteric neurons (Lake and Heuckeroth, 2013).	L
Robo1/2	The crest-derived cells at somites 8 and below; nodose ganglia.	Avoid the proximal gut, where the repellent ligand SLIT2 is expressed (Rao and Gershon, 2018); Robo/Slit pathway determines which cell types in the gut wall will be innervated by vagal nerves (Goldberg et al., 2013).	Ex
S100B	Various cell types, including astrocytes and enteric glia.	Important for maintaining Sox10 and proliferation of the developing enteric glial lineage (Hao et al., 2017a).	N/A
Semaphorin 3A (Sema3A)	The inner mesenchyme (during development of colon and caecum); transiently expressed in the outer layers of the distal hindgut of mice (E11.5 to E14.5).	Sema3A is secreted from target tissue and serves as a repulsive cue for sacral ENCCs (Anderson et al., 2007).	L
Slc6a2 (NET)	On the plasma membranes of noradrenergic neurons.	Norepinephrine reuptake transporter; homozygous mutant mice show decreased neuronal numbers and selective decreases in numbers of serotonin and calretinin-immunoreactive neurons (Li et al., 2010).	L
Slit2	Fetal stomach and intestine.	The repellent ligand for ROBO receptors (Rao and Gershon, 2018).	Ex
Sprouty2	Please refer to Table 1.	Please refer to Table 1.	L
TBATA (Spatial)	Early differentiating neurons (during differentiation).	Required for neurite outgrowth and dendrite patterning in mouse hippocampal neurons (Yammine et al., 2014).	N/A
Tcof1	Unknown.	Nucleolar factor; in *Tcof1*$^{+/-}$ mice gut, ENCC colonization is delayed; however, the migration continues between E14 and E18 to colonize the entire bowel (Lake and Heuckeroth, 2013).	L
Tenascin-C	Distributed along the length of the developing gut.	ECM molecule that supports ENCC migration *in vitro* (Akbareian et al., 2013).	L
Tryptophan hydroxylase 2 (Tph2)	Enteric neurons.	Neuronal serotonin biosynthesis enzyme; in homozygous null mice, the number of myenteric neurons, specifically dopaminergic and GABAergic neurons, is decreased (Li et al., 2011).	L
Tropomyosin receptor kinase B (TrkB; Ntrk2)	Vagal afferents (during ENS development).	The receptor of BDNF (Ernfors et al., 1992; Wetmore and Olson, 1995).	Ex
Tropomyosin receptor kinase C (TrkC; Ntrk3)	A subpopulation of ENS neurons.	Binding of NT-3 to TrkC receptor promotes the survival and differentiation of neurons, and deletion of NT-3 or TrkC reduces enteric neuron number in the ENS (Chalazonitis et al., 2001); NT-3 and TrkC are crucial for the survival of	Ex

Continued

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Table 2. Continued

Molecule	Location (time of expression)	Related receptor/ligand and functions	Role
		the majority of vagal sensory neurons (Ernfors et al., 1994; Fariñas et al., 1994; Liebl et al., 1997; Tessarollo et al., 1997).	
Vitronectin	Distributed along the length of the developing gut.	ECM that supports ENCC migration *in vitro* (Breau et al., 2009).	L
Wnt	Mesenchyme; epithelium.	Wnt signaling is crucial for the proliferation of epithelial cells in the intestine (Gregorieff et al., 2005); Wnt-3, Wnt-6 and Wnt-9b are highly expressed in crypt epithelial cells, whereas Wnt-2b, Wnt-4, Wnt-5a and Wnt-5b are expressed in differentiated epithelial and mesenchymal cells of the small intestine and colon (Gregorieff et al., 2005); Wnt-11 is expressed in the epithelium of the colon (Lickert et al., 2001); Wnt-4 was also found in the mesenteric anlage (Lickert et al., 2001).	N/A
	Wnt-1: NC.	Often used as a model for ENS-specific expression in mice (Danielian et al., 1998; Boesmans et al., 2013); works together with its receptor Frizzled 3 in PCP pathway, which is important for neurite directionality (Sasselli et al., 2013); Wnt activation increases the number of enteric neurons in *in vitro* systems in mouse and human (Zhang et al., 2017).	N/A
	Wnt-5a: mesenchyme; expressed in a graded manner along the embryonic gut (Lickert et al., 2001).	Noncanonical morphogen required for gut organogenesis; acts upstream of Fzd3 and Celsr3 to establish enteric neural circuits (Sasselli et al., 2013).	N/A
Zfhx1B (Zeb2)	NC.	Zinc-finger/homeo-domain protein; *Zfhx1b*$^{-/-}$ mice have failure of vagal NC delamination (Van de Putte et al., 2003); NC without Zfhx1b prevents ENCC migration beyond the proximal duodenum (Van de Putte et al., 2007); one of the known HSCR causative genes.	L

For each molecule, the text is assigned a letter in the last column according to their role (if clearly established and unique), with 'L' indicating a role in the establishment of the longitudinal axis, 'C' the circumferential axis and 'Ex' the development of extrinsic nerves.
ECM, extracellular matrix; EGF, epidermal growth factor; ENCC, enteric neural crest cell; GI, gastrointestinal; Hh, hedgehog; HSCR, Hirschsprung's disease; IHH, Indian hedgehog; LAG, laminin G; N/A, not applicable; NC, neural crest; PCP, planar cell polarity.

peptide (CGRP) neurons (i.e. putative sensory neurons; Qu et al., 2008) are born later, from around E10-E11.5 (Pham et al., 1991; Bergner et al., 2014). Recent work using human fetal gut samples has shown that the development of several key enteric neuronal subtypes occurs between EW12 and EW14 (McCann et al., 2019). The mechanisms responsible for neuronal differentiation and specification of different neuronal subtypes remain to be elucidated.

Spatiotemporal differences in neuronal subtypes

Although the pool of progenitors is considered fairly homogenous, the mesh-like pattern of the myenteric ganglia, as well as the size of the ganglia, can vary considerably between gut regions (Furness, 2006). The neurochemical coding of enteric neurons can also differ along the gut: for example, calretinin-immunoreactive neurons in the mouse small intestine are predominantly cholinergic, whereas in the colon, ∼25% of calretinin^{+} neurons are non-cholinergic (Sang and Young, 1998). How regional differences in the proportions of specific neuronal subtypes arise requires further investigation. It is likely that these differences reflect the intrinsic wiring and/or composition of the enteric circuitry in a given region, and that this in turn mirrors the specialized functions of different gut regions, such as storage or emptying (Rao, 2020).

A recent study has compared the transcriptional profiles of all ENS cells (isolated using a *Wnt1:Cre* line), ENS progenitors (isolated using a *Sox10:Cre* line) and non-ENS cells of the mouse gut at E11.5 and E15.5, to reveal that a substantial number of novel as well as previously identified transcription and signaling factors vary in spatial and temporal expression in the ENS between these developmental ages (Memic et al., 2018). This comprehensive dataset will undoubtedly provide important clues to understanding the transcription factors and signaling mechanisms that contribute to the development of neuronal and glia diversity (Boesmans et al., 2019).

Postnatal neuronal subtype development

ENS development continues postnatally, where the proportions of different enteric neuronal subtypes are still being established and their connections are plastic (Hao et al., 2013b). Postnatal development is considered a crucial period for establishing the gut microbiome (Box 1) and shaping gut health, which can have significant implications for later life (Foong, 2016; Hao et al., 2016). For example, neonatal antibiotic administration is associated with increased susceptibility to various diseases, such as inflammatory bowel disease (Ananthakrishnan, 2013). Although not well understood, this is inevitably linked to the immune system (Box 2). Recent work shows that the administration of vancomycin during the neonatal period, as well as during the post weaning period, leads to structural and functional alterations in the enteric circuitry and disrupted colonic motility (Hung et al., 2019, 2020). The transition from a liquid to a solid diet during weaning presents another substantial environmental change that corresponds with a period of ENS maturation, whereby significant changes in synaptic contacts and neurochemistry were observed over this time (Parathan et al., 2020). The extent to which alterations in diet (Box 3) and/or microbiota (Box 1) may influence developmental changes and the involvement of neuroimmune interactions (Box 2) is yet to be unraveled.

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Table 3. Transcription factors (TFs) involved in ENS development

Molecule	Location (time of expression)	Functions	Role
Ascl1 (MASH1)	ENCCs (upon arriving in the foregut).	The basic helix-loop-helix TF that is required for the development of subsets of autonomic neurons (Blaugrund et al., 1996); suppresses SOX10 (Kim et al., 2003).	L
Dlx1/2	ENCCs (as they migrate, proliferate and differentiate).	Highly conserved homeobox TFs that are crucial for subpallial interneuron differentiation and migration in the CNS; *Dlx2* enhances expression of the TF *Zfhx1b* in the CNS; *Dlx1/2*$^{-/-}$ mice show severe intestinal motility dysfunction at P0 (McKinsey et al., 2013; Wright et al., 2020).	N/A
Enx (Hox11L1; Tlx2)	Unknown expression in GI.	Homeodomain TF; homozygous mice show myenteric neuronal hyperplasia and pseudo-obstruction (Shirasawa et al., 1997).	N/A
Forkhead (include Foxf1 and Foxf2)	The intestinal mesenchyme.	Inactivation of TF *Foxf* expression leads to a severe reduction of collagen types I and IV and causes colorectal aganglionosis (Ormestad et al., 2006).	L
Hand2	ENCCs (once ENCCs colonize the intestine).	A basic helix-loop-helix TF required for the development of subsets of autonomic neurons (Hendershot et al., 2007); *Hand2* haploinsufficiency results in a reduction of nNOS neurons in embryos (D'Autréaux et al., 2011).	L
Hoxb5	The migratory vagal and trunk NCCs.	A member of the Antennapedia homeobox family; a molecule upstream of the RET pathway; perturbation of Hoxb5 causes Ret haploinsufficiency, impaired NCC migration and hypo/aganglionosis in mice (Fu et al., 2003; Lui et al., 2008; Carter et al., 2012; Kam et al., 2014; Kam and Lui, 2015).	L
Pax3	NCCs.	Paired-box TF; homozygous *Pax3* mice show total intestinal aganglionosis (Lang et al., 2000).	L
Phox2B	Undifferentiated ENCCs and ENCCs entering the gut mesenchyme.	The TF that is required for Ret expression in mouse pre-ENCCs (Pattyn et al., 1999); SOX10 and PHOX2B suppress each other (Nagy and Goldstein, 2017); one of the known HSCR causative genes.	L
Sox10	NCCs (as NCCs delaminate from the neural tube); migratory ENCCs; mature enteric glia.	The SRY-related HMG box TF that is required for the survival of ENCCs (Kapur, 1999); activates expression of RET (Lang et al., 2000) and EDNRB (Zhu et al., 2004); maintains ENCCs in an undifferentiated state; plays a role in neuronal-subtype specification in the small intestine (Rao and Gershon, 2018); one of the known HSCR causative genes.	L
T-box transcription factor 1 (Tbx1)	The pharyngeal endoderm and the mesodermal core of the pharyngeal arches, but not in the NC-derived mesenchyme of the pharyngeal arches.	May participate in Slit/Robo signaling; it is required for not only the projection of vagal axons to the gut but also the development of vagal ganglia (Uesaka et al., 2016).	Ex
Transcription factor 4 (Tcf4)	Unknown expression in GI.	Regulates differentiation of fibroblast to myofibroblast (Noizet et al., 2016); one of the known HSCR causative genes.	L
Transcription factor A (Tfam)	Mitochondria.	A mitochondrial transcription factor; *Tfam*-deficient ENS shows severe GI motility problems that may result from defects in specific subpopulations of enteric neurons and regions of the GI tract (Viader et al., 2011).	N/A

For each TF, the text is assigned a letter in the last column according to their role (if clearly established and unique), with 'L' indicating a role in the establishment of the longitudinal axis, 'C' the circumferential axis and 'Ex' the development of extrinsic nerves.
ENNC, enteric neural crest cell; GI, gastrointestinal; N/A, not applicable; NC, neural crest; NCCs, neural crest cells.

Additional sources of enteric neurons

It remains contended whether adult neurogenesis occurs in the ENS, the circumstances under which it may occur and the source of such potential progenitors (Joseph et al., 2011; Kulkarni et al., 2017; El-Nachef and Bronner, 2020; McCallum et al., 2020). It has been proposed that enteric neurogenesis is maintained in the mature ENS by enteric glia (Laranjeira et al., 2011; McCallum et al., 2020) or Sox10-expressing multipotent progenitors (Kulkarni et al., 2017), with convincing evidence for the ability of glial cells to serve as a source of new neurons when lesioned areas need to be repopulated (Laranjeira et al., 2011). In addition, Uesaka and colleagues have shown that, in mice, Schwann cell precursors (SCPs) can contribute to adult neurogenesis in the ENS. SCPs likely enter the gut via the extrinsic nerves that act as a scaffold onto which they migrate towards the gut (Uesaka et al., 2015). Recent work in mouse models of HSCR show that SCPs situated within extrinsic nerves play a role in GDNF-induced neurogenesis, providing a source of enteric neurons and glia (Soret et al., 2020). In zebrafish, neural crest-derived cells at the trunk level, likely to be SCPs, can also contribute to postnatal neurogenesis during development and following injury (El-Nachef and Bronner, 2020). Yet another source of enteric neurons, based on pancreatic duodenum homeobox 1 (Pdx1), has been proposed by Brokhman and colleagues. Pdx1 expression is first detected in the fore-midgut junction at ∼E8.5 and later in all the mucosa cells in the duodenum (Offield et al., 1996). *Pdx1::Cre* recombination experiments suggest that a fraction of enteric neurons is of mesoderm origin derived from Pdx^{+} progenitors that start migrating at E11.5 (Brokhman et al., 2019).

New molecular players

Although the number of additional pathways or molecules revealed in recent years remains limited, some interesting advances have been made in relation to mitogen-activated protein kinase (MAPK) activity, and distal-less homeobox (Dlx)/vasoactive intestinal peptide (VIP) signaling in relation to ENS development. A recent *in vitro* study has shown that miR-4516, a cell migration suppressor

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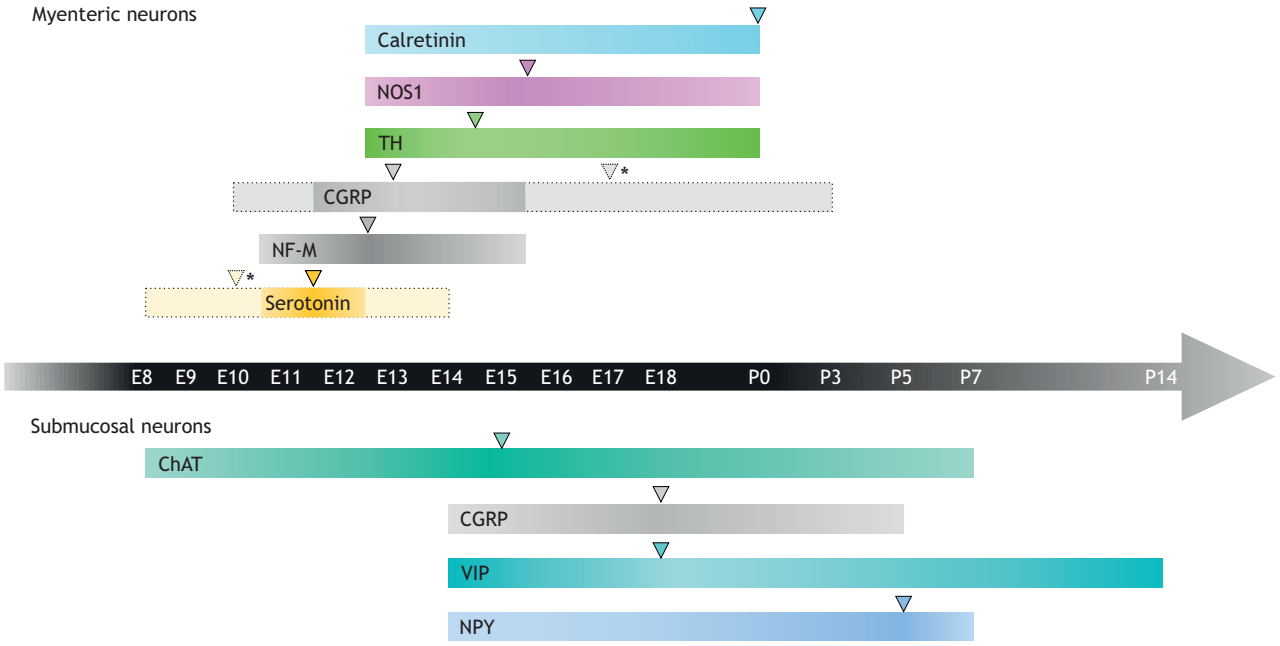

Fig. 2. Timeline of the birthdate of different neurochemical subtypes of myenteric and submucosal neurons in the developing mouse gastrointestinal tract. The colored bars indicate the embryonic (E) and postnatal (P) ages during which different neuronal subtypes are born, with the peak time of cell cycle exit marked by the arrowhead above each bar. In the myenteric plexus, there are some discrepancies in the birthdates of serotonin and calcitonin gene-related peptide (CGRP) neurons reported in the study by Bergner and colleagues and in that of Pham and colleagues (Bergner et al., 2014; Pham et al., 1991). The reason for the disparity is unclear; the findings of the latter study are indicated by asterisks and faded bars. Serotonin neurons, which are descending interneurons, are the first to be born and are detected as early as E8 and peak cell cycle exit is at ∼E10-E11.5. Neurofilament-M (NF-M) and CGRP, which are markers of sensory neurons in the adult MP, have a peak time of cell cycle exit at ∼E13 (Bergner et al., 2014). For tyrosine hydroxylase (TH) neurons, the peak time for cell cycle exit is ∼E14. Nitrergic nitric oxide synthase 1 (NOS1)-containing neurons include inhibitory motor neurons and interneurons and are born from E12.5, with a peak time of cell cycle exit at E15.5, although, there is also some suggestion that NOS may be expressed transiently at E11.5 (Bergner et al., 2014). Calretinin neurons include excitatory motor neurons, interneurons and sensory neurons, and their peak birthdate is at P0. Generally, submucosal neurons are born later than myenteric neurons and continue to be born postnatally (Pham et al., 1991). In the submucosal plexus, cholinergic (choline acetyltransferase, ChAT) neurons are detected from E8 and their peak time of cell cycle exit is at E15. Submucosal CGRP neurons, which are cholinergic and include secretomotor neurons, are born from E14 and their peak birthdate is at E18. VIP and NPY submucosal neurons are non-cholinergic and include secretomotor and vasodilator neurons. The peak cell cycle exit for VIP neurons is at E18, whereas the peak birthdate for NPY neurons is at P5.

(Chowdhari et al., 2017), directly targets Mapk10 and alters HSCR susceptibility (Wang et al., 2020). Dysfunction of Mapk10, which is highly expressed in the mammalian ENS, also causes migration delay in zebrafish (Heanue et al., 2016). Another recent study links ENS development-related gene *Dlx* to the expression of the VIP neurotransmitter (Wright et al., 2020). The transcription factors *Dlx1* and *Dlx2* are expressed during ENS development and, although *Dlx1/2*$^{-/-}$ mice show no defects on the topography of the ENS, they do show severe intestinal motility dysfunction at postnatal day (P) 0. RNA sequencing of E14.5/P0 *Dlx1/2*$^{+/+}$ and *Dlx1/2*$^{-/-}$ enteric neural progenitors has identified dozens of dysregulated genes, including a downregulation of *Vip*. The *Dlx1/2*$^{-/-}$ ENS shows a significant decrease in VIP-lineage neurons and neuronal *Vip* expression, which may contribute to gut dysmotility.

Development of intestinal innervation along the radial axis

Submucosal plexus development

As we have discussed, in mice the full length of the gut is colonized by vagal neural crest cells at ∼E14.5. Only after this process is complete does the radial migration of a subpopulation of cells from the myenteric region into the submucosa to form the SMP begin. In the proximal small intestine, ENCCs are observed in the submucosal region from E15.5 (Hao et al., 2020) (Fig. 1D). The entry of SCPs associated with ingrowing extrinsic fibers coincides with the inward migration of submucosal ganglia precursors from the myenteric region. SCPs also contribute to a small population (less than 5%) of submucosal, but not myenteric, neurons and glia in the small intestine (Uesaka et al., 2015, 2016).

As in the small intestine, development of the SMP in the large intestine also follows that of the MP and continues postnatally in mice (McKeown et al., 2001). Exceptionally, in the avian hindgut the SMP develops before the MP (Burns and Douarin, 1998). Unlike the small intestine, in which neural progenitors are predominantly derived from vagal ENCCs, sacral-level ENCCs also contribute to progenitors in the large intestine in mammalian and avian systems (Fig. 1D). In mice, one population of these enters the hindgut between E13.5 and E15.5, following the arrival of the vagal NCCs. From E16.5, a separate population of SCP cells enters the large intestine alongside extrinsic fibers and first settles in the MP. From E18.5, individual sparsely distributed neuronal cell bodies are observed in the presumptive submucosa and the arrangement of these cells into ganglia begins postnatally at ∼P3 (McKeown et al., 2001). Ultimately, SCPs contribute to ∼20% of myenteric and submucosal neurons in the colon (Uesaka et al., 2015, 2016). Hence, a subpopulation of SCPs must also migrate later perinatally, from the MP towards the submucosa. Although SCPs also give rise to enteric glia, it is unclear whether they differentiate into specific morphological-defined subtypes (Boesmans et al., 2015; Uesaka et al., 2016).

In the developing avian hindgut, endothelial cells can be seen to populate the gut in two concentric rings, seemingly delineating the presumptive myenteric and submucosal layers, even before the actual

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Box 1. Microbial influences

Recent work has demonstrated that the gut microbiome can modulate intestinal physiology. Gut microbiota can influence the transcriptional profile of enteric neurons via acryl hydrocarbon receptor signaling, and ultimately affect colonic motility (Obata et al., 2020). Microbes can also regulate sympathetic activation of a gut-brain circuit to influence gastrointestinal transit (Muller et al., 2020b). Further, microbiota can alter the number of specific populations of neuropeptidergic enteric neurons, which are capable of modulating blood glucose (Muller et al., 2020a). The influence of microbiota on ENS development is less clear. Some studies suggest that bacteria are present in amniotic fluid, placenta and/or embryonic gut (Aagaard et al., 2014; Stinson et al., 2019, 2020; Younge et al., 2019). Other studies propose a sterile fetal environment, arguing that detected bacteria were likely to be contaminants (Rowlands et al., 2017; de Goffau et al., 2019; Kuperman et al., 2020; Theis et al., 2020). Although a fetal microbiome remains unclear, metabolites from maternal gut microbiota, particularly short chain fatty acids (SCFAs), influence neural development *in utero* (Kimura et al., 2020). These SCFAs cross the placenta via the bloodstream to promote the differentiation of GPR41-expressing sympathetic neurons and their projections to the heart (Kimura et al., 2020), and GLP1-expressing enteroendocrine cells via GPR43 in intestinal organoids. Neural GPR43 and GPR41 expression in embryonic gut has not been examined; however, adult enteric neurons express GPR41, and lamina propria leukocytes express GPR43 (Nøhr et al., 2013), provoking interesting questions regarding the influence of maternal microbial metabolites on ENS development and neuroimmune interactions (Box 2).

arrival of ENCCs (Goldstein and Nagy, 2008). It has been suggested that the established vasculature serves as a route for ENCC migration and that the interaction between ENCCs and endothelial cells is mediated by β1 integrin signaling (Nagy et al., 2009). However, later studies in embryonic chick and mouse hindgut have indicated that ENCC migration, at least in the rostrocaudal axis, occurs independently of vascularization (Delalande et al., 2014; Hatch and Mukouyama, 2015). Furthermore, the perturbation of intestinal vasculature formation in a *Vegfa*$^{120/120}$ mouse model does not prevent colonization of the full length of the intestine by ENCCs (Delalande et al., 2014). Nonetheless, these mice do show a notable disruption in the organization of ENS architecture at E16.5, suggesting that the vasculature may play a role in forming myenteric ganglia. The potential involvement of vascular cues in the clustering of enteric ganglia and patterning in the radial axis requires further investigation.

A number of signaling pathways are important in SMP development (Uesaka et al., 2016) (Fig. 3). In mice, netrin signaling plays a role in radial migration; netrins are expressed in the mucosal epithelium and act as chemo-attractants. Ablating the netrin receptor deleted in colorectal cancer (DCC) in mice leads to a failure in the formation of the SMP (Jiang et al., 2003). Similarly, GDNF signaling is also required for radial migration, as ablating the GDNF receptors (GFRα1 or RET) once the rostrocaudal colonization of the gut is complete (E15.5) severely disrupts the development of submucosal ganglia (Uesaka et al., 2013). Furthermore, conditional knockdown of RET at E13.5 results in a retention of GFRα1-expressing ENS precursors in the MP and these cells fail to migrate to the submucosal region by E18.5 (Uesaka et al., 2013). On the other hand, sonic hedgehog (SHH) signaling in the epithelium appears to play an important role in demarcating the SMP by restricting inward-migrating ENCCs from advancing beyond the submucosa (Sukegawa et al., 2000). There is some suggestion that this may involve the hedgehog cell surface receptor growth arrest specific 1 (Gas1), expressed on ENCCs (Biau et al., 2013). Indeed, both SHH$^{-/-}$ and Gas1$^{-/-}$ mutant mice show ectopic enteric neurons in the mucosa (Ramalho-Santos et al., 2000; Biau et al., 2013). However, it is difficult to delineate whether these ENS abnormalities are direct consequences of the loss of SHH signaling or secondary to the additional defects in the smooth muscle and mesenchyme (Jin et al., 2015). SHH may also inhibit GDNF-induced ENCC migration via the induction of BMP4 expression in the mucosal mesenchyme (Fu et al., 2004, 2006). This BMP4 signal is refined by a BMP antagonist, noggin, which acts to both shield inward-migrating ENCCs in the submucosa from the inhibitory effect and promote GDNF-induced migration in mice (Fu et al., 2006). A recent study has also identified cerebral dopamine neurotrophic factor (CDNF) as a key factor for keeping submucosal neurons viable, because submucosal neurons of CDNF-knockout animals are vulnerable to aging effects more than myenteric neurons (Lindahl et al., 2020). Furthermore, avian studies indicate that SHH can induce the expression of specific ECM proteins in the mesenchyme, such as collagen IX, to impede ENCC migration (Nagy et al., 2016; Nagy and Goldstein, 2017). Thus, development of the ENS in the radial axis requires an intricate coordination of numerous chemoattractive and inhibitory factors.

Mucosal innervation

In the proximal small intestine, the projection of neurites from the MP towards the mucosa begins as early as E13.5, preceding the development of the SMP and even villi (Hao et al., 2020). By E15.5, these neurites in the developing villi are already able to transmit information back to the MP (Hao et al., 2020). This also overlaps with the beginning of the differentiation of different epithelial cell types, including enteroendocrine cells (specialized sensor cells in the mucosa), which occurs from ∼E14-E15 in the proximal small intestine (Desai et al., 2008). Following this, from E16.5, mature ENS synapses can be detected at the ultrastructural level (Vannucchi and Faussone-Pellegrini, 2000). Thus, the prenatal gut may already be equipped to begin detecting luminal contents at this stage. Enteroendocrine cells possess specialized processes termed 'neuropods' that form synaptic connections with extrinsic sensory nerves that project into the mucosa and enable the communication of luminal information to the central nervous system (CNS) (Kaelberer et al., 2018). It remains to be determined whether such neuro-epithelial connections between the neurites projecting into the mucosa and the neuropods of enteroendocrine cells are

Box 2. Neuroimmune interactions

The coordinated actions of the enteric immune system and ENS serve crucial roles in maintaining intestinal homeostasis and host defense. The complex interaction between microbes, immune cells and neural elements during ENS development and in adulthood have been subject to insightful reviews (Kabouridis and Pachnis, 2015; Margolis et al., 2016; Obata and Pachnis, 2016; Veiga-Fernandes and Pachnis, 2017). Nonetheless, this remains an area of which we still have limited understanding. Muscularis macrophages (MM), which reside in the muscularis externa, have been of particular interest because they engage in crosstalk with enteric neurons to regulate gastrointestinal motility (De Schepper et al., 2018), and this interaction is in turn tuned by the microbiota (Muller et al., 2014). Notably, it has been recently demonstrated that enteric innervation is not necessary for the colonization and patterning of MM in the embryonic gut, using Ret-deficient mice that lack an ENS in the small or large intestine (Avetisyan et al., 2018). This study further shows that MM colonization of the gut precedes the arrival of ENCCs, suggesting that maturation of this neuroimmune interaction begins postnatally, upon exposure to external microbial and dietary factors in the lumen.

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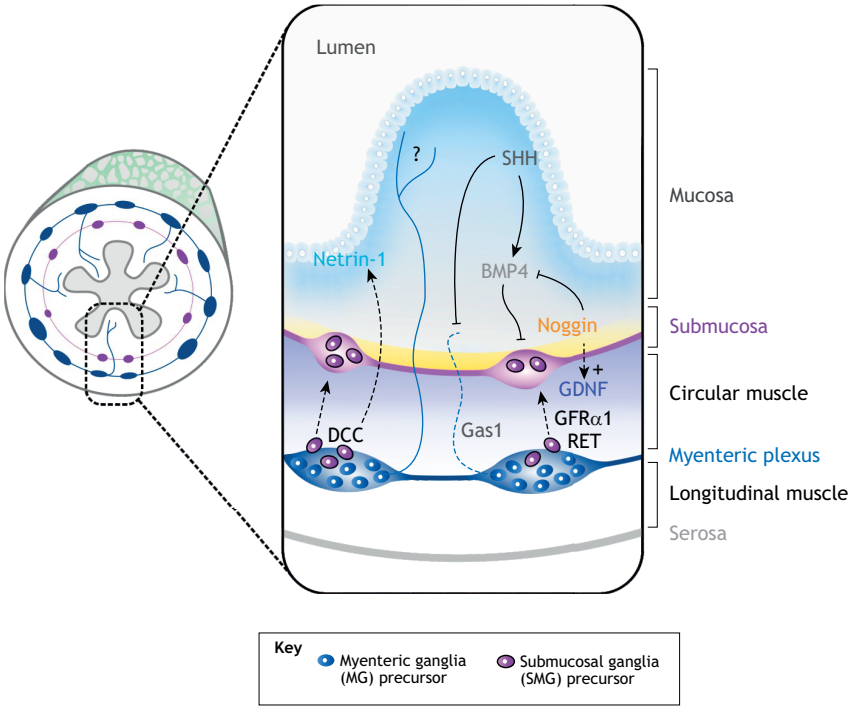

Fig. 3. Simplified schematic depicting key signaling mediators involved in the radial patterning of the developing mouse ENS. Netrin-1 secreted from mucosal epithelial cells attracts ENCCs that express the netrin receptor DCC, from the myenteric layer to the submucosa. Although netrin-1 also plays a role in attracting vagal sensory fibers into the gut, whether it also guides enteric neurites into the mucosa is unclear. GDNF-GFRα1-RET signaling is required for radial migration. Following the primary rostrocaudal migration of ENCCs, GDNF expression shifts from the outer mesenchyme to the submucosal side of the circular muscle to promote the secondary inward migration of ENCCs. SHH signaling in the epithelium prevents myenteric neurites from prematurely projecting into the mucosa. This may be mediated by the hedgehog cell surface receptor Gas1. SHH further acts by inducing BMP4 expression in the mucosal mesenchyme to inhibit ENCC migration into the mucosa. Noggin (a BMP antagonist) is produced by a thin layer of cells situated between the BMP4-expressing mesenchyme and submucosal ganglia precursors, protects the submucosa from the effects of BMP4 and promotes GDNF-induced migration. ENCCs ultimately give rise to submucosal and myenteric neurons and glia in the respective plexus layers.

established at this point in development, as well as the mechanisms underlying the formation of this specialized innervation (Bohórquez et al., 2015).

Developing submucosal ganglia precursors, scouting intrinsic axons and extrinsic fibers (see below) appear to follow similar guidance cues as they migrate towards the mucosa, although this is yet to be fully established. It is unclear whether they may interact throughout this process (Hao et al., 2016; Uesaka et al., 2016). For example, netrin/DCC signaling is involved in the radial migration of ENCCs and also attracts vagal sensory afferent fibers towards the submucosa (Jiang et al., 2003; Ratcliffe et al., 2006). However, it remains to be shown whether netrins similarly guide enteric neurites towards the mucosa. In culture, netrin-1 promotes enteric neurite outgrowth, but this effect required the presence of non-crest derived cells of the gut wall, suggesting that co-factors produced from these cells are necessary (Jiang et al., 2003). SHH signaling, via Gas1, is also involved in directing chemorepulsion of myenteric neurites away from the mucosa (Jin et al., 2015) (Fig. 3). Whether the development of enteric mucosal innervation and the migration of ENCCs towards the submucosa are indeed governed by similar chemoattractant and chemorepellent factors needs to be further examined.

Box 3. Nutritional influences

In addition to the microbial factors (Box 1), the developing ENS may also be influenced by other environmental factors, such as maternal and neonatal nutrition (Van Haver et al., 2008; Lake and Heuckeroth, 2013). For example, vitamin A deficiency causes aganglionosis in the mouse hindgut (Fu et al., 2010; Heuckeroth and Schäfer, 2016). Furthermore, retinoic acid (RA) signaling (a vitamin A metabolite) is required for RET expression in vagal ENCCs and promoting chain migration (Simkin et al., 2013; Uribe et al., 2018). Meis3, a downstream effector of RA signaling, promotes neuronal survival and efficient chain migration of ENCCs (Uribe et al., 2018). A study in preterm pigs has shown that neonatal diet can influence the expression of neuronal and glial markers in their ENS (Van Haver et al., 2008). In another murine study, a Western (high fat) diet has been shown to halt the decline in the number of nitrergic myenteric neurons that occurs in the stomach antrum over the first 4 months of life – an effect partly involving GDNF (Baudry et al., 2012).

Connecting the nerve plexus layers

The myenteric and submucosal plexuses are interconnected, but little is known about how this connectivity is formed (Rao and Gershon, 2018). Neurites in the MP are seen to begin projecting towards the colonic mucosa at ~E16.5, before the appearance of submucosal neurons (McKeown et al., 2001). Therefore, connections between myenteric and submucosal neurons must develop at a later stage. In an elegant study by Lasrado and colleagues, various genetic tools have been employed to map the fate of individual *Sox10*$^{+}$ ENCCs in mouse embryos from E12.5 (Lasrado et al., 2017). Labeled ENCCs gave rise to clones that consisted of only neurons, only glia, or both neurons and glia. Lineally related cells of glial and bipotential neuroglial progenitors are distributed in columns along the radial axis. Furthermore, clonally related myenteric neurons display a greater degree of synchrony in their calcium responses to electrical stimulation, compared with unrelated neurons (Lasrado et al., 2017). It will be interesting to further investigate whether lineally related myenteric and submucosal neurons within a radial column show similar synchronization, as this may provide insights into how the wiring of these different elements is established.

Extrinsic innervation

Extrinsic neurons innervate the ENS via projections from the CNS to the gut. They include vagal afferent neurons, spinal afferent neurons, pre-/postganglionic sympathetic neurons and parasympathetic motor neurons (Fig. 4). Vagal afferent cell bodies reside in the nodose or jugular ganglia (i.e. the inferior or superior ganglia of the vagus nerve) and extend their projections centrally to the brainstem, whereas spinal afferent cell bodies reside in dorsal root ganglia and extend their projections to the spinal cord. Both vagal and spinal afferents direct their other projections

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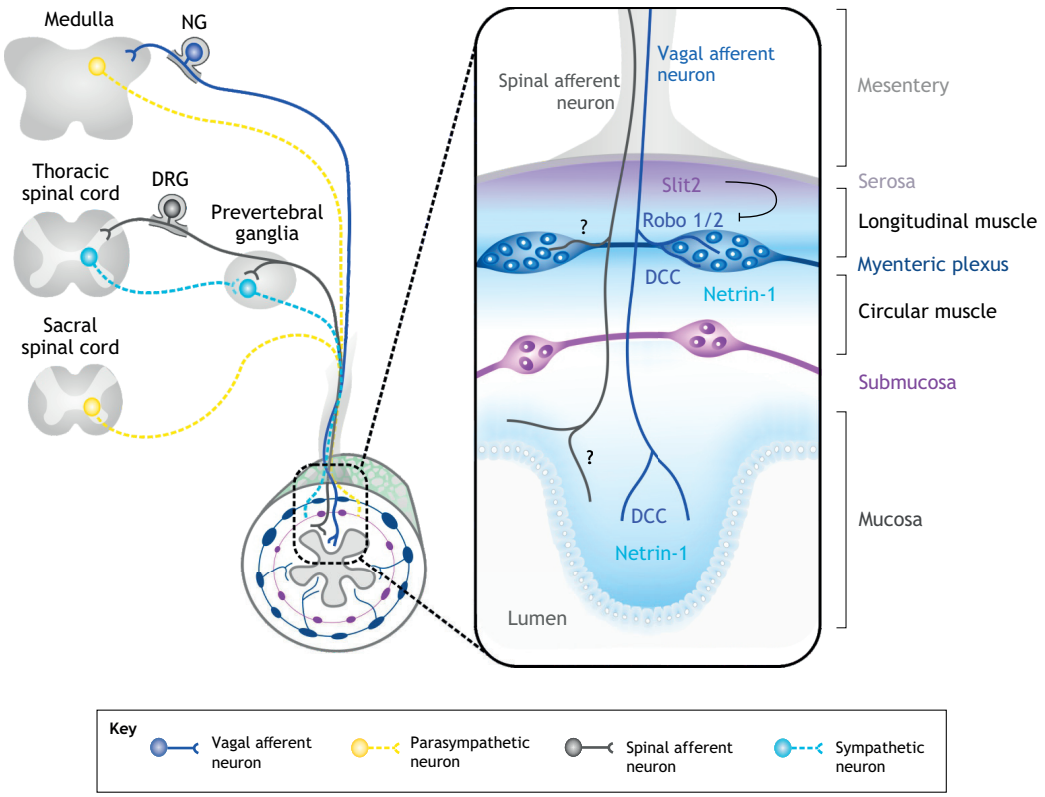

Fig. 4. Simplified schematic illustrating the extrinsic innervation of the gut. The expanded gut cross-section highlights the developing projections of vagal afferent neurons in the nodose ganglia (NG) to the myenteric plexus and mucosa, as well as their guidance cues. Vagal afferent axons that express DCC are first drawn towards netrin-1, expressed in the outer gut mesenchyme, and then later to the high concentration of netrin-1 secreted by myenteric ganglia. Vagal afferent neurons also express the receptors Robo1 and Robo2, which can interact with the chemorepellent Slit2. The overlapping expression of Slit2 with netrin-1 in the outer mesenchyme subsequently counters the attraction effects of netrin once the vagal afferents enter the gut wall, to promote projections towards the myenteric layer. Vagal afferents grow further inwards towards the mucosa where they are again attracted to the netrin-1 gradient that is produced by the mucosal epithelium. The signals involved in the development of gut innervation by spinal afferent neurons in the dorsal root ganglia (DRG) are yet to be established.

towards the gut, penetrating and ramifying into the multiple layers of intestinal wall (Brookes et al., 2016). Previous studies have shown that vagal nerve fibers share the same pathway with migrating vagal NCCs in mice, although they move slower than vagal NCCs from the hindbrain to the foregut (Baetge and Gershon, 1989; Anderson et al., 2006b).

Latest work by Niu and colleagues provides an extensive account of the timing and trajectories of the development of extrinsic nerves that supply the murine GI tract (Niu et al., 2020). Briefly, vagal afferent axons project along the esophagus at E10.5. Following a short delay, parasympathetic neurons in the dorsal motor nucleus of the hindbrain project their axons along pioneering vagal afferent fibers and together they enter the stomach by E11.5. Vagal afferent axons then converge with spinal afferent axons at E12.5, while sensory afferent and parasympathetic axons also split into a gastric branch to the stomach and a celiac branch to the intestine. Post-ganglionic sympathetic neurons in the prevertebral ganglia begin projecting towards the gut at E12.5 and divide into a gastric and a celiac trajectory at E14.5. From E14.5 to E16.5, vagal afferent, spinal afferent, post-ganglionic sympathetic and parasympathetic axons project in close association along the gut mesentery and innervate the full length of the gut by E16.5.

Another recent study has documented the development of the sympathetic innervation of the gut in human embryos and fetuses between EW4 and EW9.5 (Kruepunga et al., 2020): NCCs migrate to the para-aortic region and then the pre-aortic region from EW5 to EW6, in which they begin forming the sympathetic trunk and prevertebral ganglia, respectively. Extrinsic nerve fibers then migrate along the intestinal arteries to merge with the intrinsic innervation between EW7 and EW9. By EW9.5, the extrinsic innervation has yet to reach the distal midgut. After arriving in the gut, extrinsic nerves serve as an important route of information exchange between the GI tract and the CNS. Vagal and spinal afferents are responsible for communicating different aspects of sensation. Vagal afferents convey sensations of fullness and satiety, whereas spinal afferents signal high-threshold sensations such as bloating, pain and urgency.

In addition to the netrin/DCC pathway described earlier, a number of other pathways and molecules have been shown to be involved in the development of extrinsic gut innervation (Tables 2 and 3; Fig. 4). For example, Tbx1 is a transcription factor required for the vagal projections into the gut wall and for the development of vagal ganglia (Uesaka et al., 2016). Once vagal fibers reach the gut, Robo/Slit signaling is important for determining which cell types within the GI tract are innervated by vagal nerve fibers (Goldberg et al., 2013) (Fig. 4). Other important molecules that have been identified relate to the survival and maintenance of extrinsic neurons. For vagal afferents, these molecules include brain-derived neurotrophic factor (BDNF) (Jones et al., 1994; Erickson et al., 1996; Murphy and Fox, 2010), neurotrophin-3 (NT-3) (Ernfors et al., 1994; Fariñas et al., 1994; Liebl et al., 1997; Tessarollo et al., 1997), and their receptors tropomyosin receptor kinase B (TrkB) and tropomyosin receptor kinase C (TrkC), respectively. On the

other hand, nerve growth factor (NGF) has a crucial role in the survival of small nociceptive sensory neurons, as well as sympathetic neurons (Fariñas, 1999). Espinosa-Medina and colleagues have shown that parasympathetic ganglia derived from SCPs make their way along already established preganglionic fibers (Espinosa-Medina et al., 2014). Further studies are necessary to better characterize the molecules involved.

Conclusion and future perspectives

Despite the striking advances made over the past 30 years in our knowledge of the development and function of the ENS, a number of key aspects remain to be addressed. For example, we still have a limited grasp on the development of innervation in the radial axis – how the SMP arises, how sensory projections from the MP and/or SMP sense information about luminal contents, how these sensory circuits develop and how extrinsic nerves are integrated all remain elusive. Future efforts towards a better understanding of ENS development are multifaceted, ranging from further investigations into gangliogenesis to studying how regional differences in ENS connectivity may be established, as well as microbiota-neuroimmune crosstalk and more extensive characterization of the underlying molecular pathways. Importantly, given that the majority of ENS development studies still heavily rely on murine models owing to the limited accessibility of human fetal gut, efforts towards better understanding the developing human ENS are crucial. There has been substantial progress in this area, with recent work conducted by McCann and colleagues providing key insights into the development of the functional enteric circuitry and the emergence of electrically-evoked neural activity in the human fetal intestine (McCann et al., 2019). In addition, human pluripotent stem cells and induced pluripotent stem cells (iPSCs) have proven to be valuable alternatives for studying human neural development, circumventing the limited availability of human samples (Frith et al., 2018). Such work will be informative for identifying novel approaches to repairing the ENS in disease and also reciprocally feeds back into advancing iPSC technology (Burns et al., 2016; McCann et al., 2017). Identifying the molecules involved in orchestrating ENS development, their concentrations and timing of expression, will facilitate iPSC-based and stem cell-based regenerative therapies for enteric neuropathies, such as HSCR (Hotta et al., 2013; Fattahi et al., 2016; McCann et al., 2017; Frith et al., 2018; Barber et al., 2019). Theoretically, achieving an optimal ENS differentiation protocol for patient-derived iPSCs would be an ideal outcome, which could pave the way to better therapies for many GI neuropathies. However, for any such approach to be effective and efficient, research into iPSC and repair approaches will have to go hand in hand with continuing basic research to refine our knowledge about molecular factors (and their timing) involved in the differentiation of different subtypes of enteric neurons and glia, the generation of the typical concentric organization and interaction with the extrinsic nerves.

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Competing interests

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