Developmental Biology 417 (2016) 158–167

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Development of the intrinsic and extrinsic innervation of the gut

Toshihiro Uesaka [a,b,c,*], Heather M. Young [d], Vassilis Pachnis [e], Hideki Enomoto [a,b,c]

[a] Division of Neural Differentiation and Regeneration, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Kobe 650-0017, Japan
[b] Laboratory for Neuronal Differentiation and Regeneration, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan
[c] Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan
[d] Department of Anatomy and Neuroscience, University of Melbourne, 3010 VIC, Australia
[e] Division of Molecular Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

ARTICLE INFO

Article history:
Received 9 February 2016
Received in revised form
9 April 2016
Accepted 21 April 2016
Available online 22 April 2016

Keywords:
Enteric nervous system
Extrinsic nerve innervation
Neuronal projection
Schwann cell precursors
Neuronal and glial differentiation

ABSTRACT

The gastrointestinal (GI) tract is innervated by intrinsic enteric neurons and by extrinsic efferent and afferent nerves. The enteric (intrinsic) nervous system (ENS) in most regions of the gut consists of two main ganglionated layers; myenteric and submucosal ganglia, containing numerous types of enteric neurons and glial cells. Axons arising from the ENS and from extrinsic neurons innervate most layers of the gut wall and regulate many gut functions. The majority of ENS cells are derived from vagal neural crest cells (NCCs), which proliferate, colonize the entire gut, and first populate the myenteric region. After gut colonization by vagal NCCs, the extrinsic nerve fibers reach the GI tract, and Schwann cell precursors (SCPs) enter the gut along the extrinsic nerves. Furthermore, a subpopulation of cells in myenteric ganglia undergoes a radial (inward) migration to form the submucosal plexus, and the intrinsic and extrinsic innervation to the mucosal region develops. Here, we focus on recent progress in understanding the developmental processes that occur after the gut is colonized by vagal ENS precursors, and provide an up-to-date overview of molecular mechanisms regulating the development of the intrinsic and extrinsic innervation of the GI tract.

1. Introduction

The gastrointestinal tract is a complex organ system that performs a range of functions that are essential for life including digestion, absorption, secretion, propulsive movements (peristalsis, migrating motor complexes), mixing, segmentation, excretion, and defense. Many of these gut functions are controlled by intrinsic neurons of the enteric nervous system (ENS) and/or by extrinsic sympathetic, parasympathetic (via the vagus and pelvic nerves), and sensory neurons (in the vagal and spinal pathways) (Fig. 1). Studies on extrinsically denervated intestine have shown that the ENS can act autonomously to control motility reflexes (Bayliss and Starling, 1899; Furness et al., 1995; Langley and Magnus, 1905). However, the central nervous system plays an essential role in controlling esophageal and gastric motility, and in regulating gut function in different emotional states, while extrinsic peripheral nerve pathways coordinate activity between distant regions of the gastrointestinal tract.

The development of the ENS involves a number of processes. Early processes include invasion of the foregut by ENS precursors derived from vagal (caudal hindbrain) neural crest cells (vagal NCCs), and subsequent rostral-to-caudal migration of these vagal NCC-derived ENS precursors to colonize the myenteric (outer) region of the gut. Later processes include colonization of the submucosal (inner) region of the gut by ENS precursors, entry of sacral NCC-derived ENS precursors into the hindgut, and the projection of fibers from extrinsic sensory and visceromotor neurons (from vagal, dorsal root, sympathetic and pelvic ganglia) to the gut. The arrival of extrinsic nerves allows invasion of Schwann cell precursors (SCPs) into the gut. Neuronal and glial differentiation is detectable during early ENS development and continues postnatally. Many of these developmental processes overlap in time (Fig. 2).

There are many excellent reviews on the early processes of ENS development that focus on the mechanisms controlling the migration of vagal NCC into and along the gut (Avetisyan et al., 2015; Goldstein et al., 2013; Heanue and Pachnis, 2007; Lake and Heuckeroth, 2013; Obermayr et al., 2013; Sasselli et al., 2012). Here we will focus on later processes in ENS development, especially on

* Corresponding author at: Division of Neural Differentiation and Regeneration, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Kobe 650-0017, Japan.
E-mail address: toshi@phoenix.kobe-u.ac.jp (T. Uesaka).

http://dx.doi.org/10.1016/j.ydbio.2016.04.016
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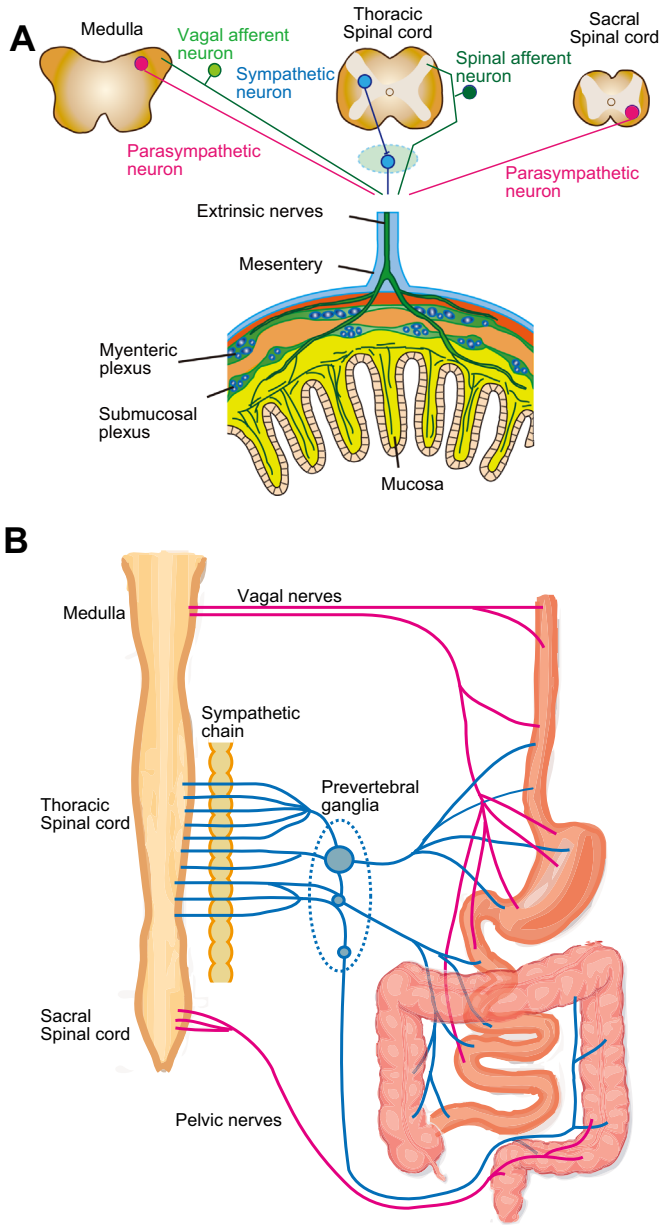

Fig. 1. Intrinsic and extrinsic nerve innervation of gastrointestinal tract. (A) Schematic diagram showing different types of extrinsic neurons projecting to the gut. Postganglionic sympathetic neurons (blue) project to the gut to regulate secretion, gut motility, and blood flow. Parasympathetic extrinsic neurons (pink) in the brainstem and sacral spinal cord run through the vagus and pelvic nerves, respectively, to innervate the gut. Vagal afferent neurons (green) in the nodose and inferior jugular ganglia and spinal afferent neurons (green) in the dorsal root ganglia receive sensory information from the gut. The gastrointestinal tract is composed of two major ganglionated plexuses, myenteric and submucosal plexuses. (B) Sympathetic (blue) and parasympathetic (pink) innervation of the gastrointestinal tract through vagal, sympathetic pelvic pathways (based on Berne et al. (2009), Figure 26-7).

extrinsic gut innervation, formation of submucosal ganglia, role of SCPs in enteric neurogenesis, and neuronal and glial differentiation of ENS precursors.

2. Projection of extrinsic nerves into the gut

2.1. Vagal innervation of the gastrointestinal tract

Vagal sensory and motor fibers are distributed throughout the length of the gastrointestinal tract although the vagal innervation of the distal small intestine and colon is relatively sparse (Berthoud et al., 1991). In mice, migrating vagal NCC and vagal nerve fibers follow the same pathway from the hindbrain to the foregut, but vagal NCC exit the hindbrain and arrive at the gut prior to vagal nerve fibers (Anderson et al., 2006; Baetge and Gershon, 1989); vagal NCC are already present in the foregut at embryonic day 9 (E9) (Natarajan et al., 2002), while vagal nerve fibers first enter the esophagus at E10, the stomach at E11 (Baetge and Gershon, 1989), and the duodenum is innervated by E14 (Murphy and Fox, 2007; Ratcliffe et al., 2006). It is unclear whether the first vagal fibers to enter the mouse foregut are sensory or motor fibers, but in rats, vagal sensory (nodose) axons project to the gut before vagal motor axons arising from the nucleus ambiguous and the dorsal motor nucleus of the vagus (Rinaman and Levitt, 1993). In some circuits involving cranial ganglia, the afferent fibers project to their targets first and provide an essential scaffold for the correct projection of efferent axons (Coppola et al., 2010), but it is unknown whether vagal efferent fibers projecting to the gut require the presence of vagal afferent fibers in order to navigate to the gut.

Tbx1 is a member of an evolutionarily conserved transcription factor family that share a common DNA binding domain called the T-box. In *Tbx1*-null mutant mice, cranial ganglia including the glossopharyngeal and nodose ganglia are malformed (Vitelli et al., 2002). Furthermore, the vagus nerves are severely hypoplastic in the esophageal region, and the vagal innervation of the stomach is absent (Calmont et al., 2011). Although expression of *Tbx1* is not detectable in cranial NCCs (Garg et al., 2001), ablation of this gene in the pharyngeal surface ectoderm causes cranial NCC patterning defects in a non-cell-autonomous manner, which may be due to effects on Slit/Robo signaling (Calmont et al., 2009). In contrast to *Tbx1* null mutants, *Tbx1* heterozygotes display no significant defects in the morphology of cranial ganglia but exhibit a spectrum of defects relating to the vagus innervation of the stomach (Calmont et al., 2011). Thus Tbx1 is required for the projection of vagal axons to the gut as well as the development of vagal ganglia. Further studies are required to identify signaling pathways downstream of Tbx1 that are involved in the vagal innervation of the gut.

Netrin-1 and its receptor, deleted in colorectal cancer (DCC), appear to play a role in attracting vagal sensory nerves into the gastrointestinal tract. Vagal nerve fibers express DCC, while Netrin-1 is expressed by multiple cell types in the developing stomach including the mucosal epithelium, outer mesenchyme and enteric neurons (Ratcliffe et al., 2006). In culture, the neurites of nodose ganglion cells are attracted to explants of foregut, and this attractive response is blocked by DCC function-blocking antibodies (Ratcliffe et al., 2006). Vagal fibers fail to enter the stomach in *Ret* null mutant mice, which lack enteric neurons, and it was therefore proposed that neurons are an essential source of netrin-1 for attracting vagal fibers (Ratcliffe et al., 2011). However, it is also possible that the entry of vagal fibers into the stomach requires Ret signaling. Laminin-111, which is present in the developing gut, has been proposed to terminate the attraction of vagal nerves toward sources of netrin-1 (Ratcliffe et al., 2008).

Slit ligands are also expressed in the fetal stomach and intestine while the Slit receptors, Robo1 and Robo2, are expressed by nodose ganglia (Goldberg et al., 2013). As the neurites extending from nodose neurons are repelled by Slit2 in vitro, it has been suggested that Slit/Robo signaling plays a role in determining which cell types in the gut wall are innervated by vagal fibers (Goldberg et al., 2013). Hence, both attractive and repellent cues probably participate in the guidance of vagal afferent fibers to appropriate targets within the gut wall.

Brain-derived neurotrophic factor (BDNF) is first expressed by

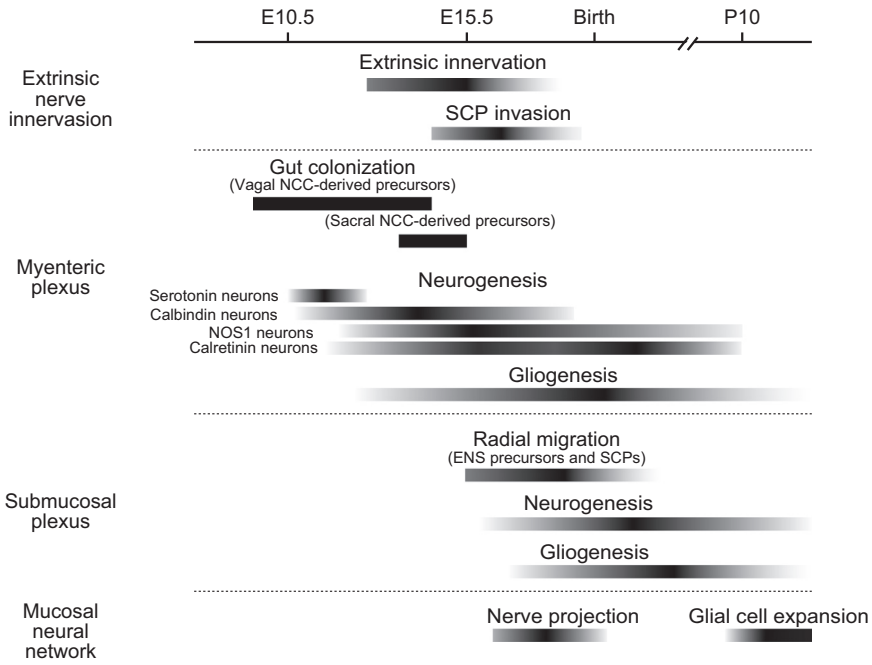

Fig. 2. Timing of developmental events during ENS development in the mouse. There is considerable overlap in the timing and duration of developmental processes. The timing of neurogenesis for different neuron sub-types refers to their time of exit from the cell cycle.

the undifferentiated mesenchyme of the fetal gut and then later by the lamina propria and gut muscle (Fox et al., 2013b; Fox and Murphy, 2008). The BDNF receptor, TrkB, is expressed by vagal afferents during development (Ernfors et al., 1992; Wetmore and Olson, 1995). *Bdnf*-deficient mice display a substantial loss of vagal afferents (Erickson et al., 1996; Jones et al., 1994; Murphy and Fox, 2010), indicating that BDNF is essential for the survival, growth or maintenance of vagal sensory fibers. Surprisingly, conditional ablation of *Bdnf* restricted only to smooth muscle cells, including vascular smooth muscle, results in an increase in the sensory innervation of the small intestine and increased neuron numbers in nodose ganglia (Biddinger and Fox, 2014), suggesting that smooth muscle-derived BDNF acts to reduce sensory innervation density. One mechanism that could account for increased neuron survival in conditional *Bdnf* knockout mice is reduced signaling by a BDNF precursor protein, proBDNF through the p75NTR receptor (Biddinger and Fox, 2014). In sympathetic neurons in vitro, proBDNF can induce neuronal apoptosis via activation of p75NTR (Teng et al., 2005), and sympathetic neuron number is increased in *Bdnf*-deficient mice (Bamji et al., 1998). Numerous studies suggest that binding of proBDNF to p75NTR, and mature BDNF to TrkB, have opposing effects on cell survival (Huang and Reichardt, 2001; Kenchappa et al., 2006; Teng et al., 2005; Volosin et al., 2006). Whether proneurotrophins contribute to naturally occurring cell death remains to be shown. It will be important to elucidate the contribution of proBDNF to the development and maintenance of the vagal innervation of the gut.

Neurotrophin-3 (NT-3, encoded by *Ntf3*) expression is also detected in smooth muscle cells comprising the outer layers of the developing gastrointestinal tract, and in the walls of blood vessels that supply the gut (Fox et al., 2013a; Fox and McAdams, 2010). NT-3, acting in part through activation of the receptor TrkC (encoded by *Ntrk3*), is essential for the survival of a large proportion of vagal sensory neurons: In *Ntf3*-and *Ntrk3*-null mutants, the number of nodose neurons is around 30% and 50% lower respectively than in control mice (Ernfors et al., 1994; Farinas et al., 1994; Liebl et al., 1997; Tessarollo et al., 1997). In dorsal root ganglion neurons, NT-3 not only supports neuron survival but also acts as a chemoattractive cue to guide fibers to target skeleton muscle (Genc et al., 2004), but it is not yet known whether NT-3 in the gut regulates the trajectories of vagal fibers. Smooth muscle-specific ablation of NT-3 causes a decrease in the number of meal-induced, activated (c-fos-positive) neurons in the brainstem but the number and morphology of vagal fibers in the gut was not reported (Fox et al., 2013a). TrkC is also expressed in a subpopulation of enteric neurons, and both *Ntf3*-and *Ntrk3*-deficient mice display a significant decrease in the number of myenteric and submucosal neurons (Chalazonitis et al., 2011). Interestingly, the involvement of the same signaling pathways in the development of both the intrinsic and extrinsic innervation of the gut may be critical for the co-ordinate development of the two systems.

2.2. Spinal afferent innervation of the gastrointestinal tract

Spinal afferents arise from trunk and sacral level neural crest cells and project to the gut via the thoracic and lumbar splanchnic nerves, then via mesenteric nerves (spinal afferent pathways), while sacral afferents project to the distal colon via the pudendal and pelvic nerves (sacral spinal pathways, Costa et al., 2004). Visceral afferents comprise approx. 7% of sensory cell bodies within the dorsal root ganglion (Grundy, 2006) but the development of the spinal sensory innervation of the gut, and the regulatory molecules involved, have not yet been examined.

2.3. Sympathetic innervation of the gastrointestinal tract

The sympathetic innervation of the bowel arises from abdominal prevertebral ganglia comprising the coeliac, superior mesenteric, and inferior mesenteric ganglia. Axons from sympathetic neurons grow towards their targets along arteries (Glebova and Ginty, 2005). In E13.5–15.5 mice, sympathetic axons extend along arteries in the mesentery and enter the gut wall after E15 (Hatch and Mukouyama, 2015). Artemin (ARTN), endothelin (ET), and NT-3 expressed in arteries promote axon extension along arteries (Enomoto et al., 2001; Honma et al., 2002; Kuruvilla et al., 2004; Makita et al., 2008). Indeed, *Artn* is expressed in developing mesenteric arteries, and *Artn*-deficient mice exhibit defects in the sympathetic innervation of the gut (Honma et al., 2002).

Mesenteric arteries not only serve as a pathway to the gut, but they are themselves targets for innervation. Interestingly, the sympathetic innervation of mesenteric arteries develops postnatally after sympathetic axons have projected into the gut (Brunet et al., 2014). There are distinct subpopulations of sympathetic neurons that project to specific targets within the gut (Macrae et al., 1986; Tan et al., 2010). It is unknown whether different subtypes of sympathetic neurons are specified prior to their arrival in the gut and navigate to specific targets, or whether the phenotype of sympathetic neurons in the gut is determined after they have contacted their target cells as occurs in the sweat glands and periosteum (Francis and Landis, 1999).

Nerve growth factor (NGF) is essential for the survival of small nociceptive sensory neurons as well as sympathetic neurons (Farinas, 1999). In addition to its role in sympathetic neuron survival (Crowley et al., 1994; Levi-Montalcini and Booker, 1960), NGF plays a role in the projection of sympathetic axons to and within the gastrointestinal tract (Glebova and Ginty, 2004). In the developing gastrointestinal tract, reporter mice for NGF promoter activity revealed NGF-expressing cells in the epithelial layer and in enteric ganglia (Kawaja et al., 2011). As sympathetic nerve terminals form close appositions with various subtypes of myenteric neurons (Tan et al., 2010), NGF from myenteric neurons may play a role in the development of the precise connectivity between sympathetic neurons and target enteric neurons.

3. Formation of the submucosal ganglia

ENS precursors derived from vagal NCC migrate caudally within the gut mesenchyme and initially settle at the site of the future myenteric ganglia (MG). Several days after each gut region is populated, developing myenteric neurons and extrinsic nerves begin to project to submucosa and mucosa. Simultaneously, a subpopulation of cells in the myenteric region migrates radially (inward) to form the submucosal ganglia (SMG) (Kapur et al., 1992; Pham et al., 1991).

Netrins in the gut wall not only attract DCC-expressing vagal sensory axons into the gut (Ratcliffe et al., 2006) but also migrating ENS precursors in vitro (Jiang et al., 2003). Importantly, *Dcc*-deficient mice lack SMG (Jiang et al., 2003). In *Dcc*-deficient mice, immunoreactivity for the pan-neuronal marker, PGP9.5, cannot be detected in the submucosal layer, suggesting that the radial migration of SMG precursors towards the submucosal layer and the projection of axons to the submucosal region require netrin/DCC signaling. It is unclear, however, whether the absence of SMG in *Dcc*-deficient mice reflects a direct action of netrin on SMG precursors, or is a secondary effect of defects in netrin-mediated axon projections toward the submucosal region. Further studies using cell-specific *Dcc* knockout mouse are needed to discriminate between these possibilities.

Glial cell line-derived neurotrophic factor (GDNF) signaling is also required for the radial migration of ENS precursors. Conditional ablation of the GDNF receptor, *Gfrα1*, or *Ret*, at E15.5 leads to severe deficits in submucosal neurons, but does not affect axonal projections toward the submucosal layer (Uesaka et al., 2013). The radial migration of SMG precursors begins after E14.5, and coincides with the inward migration of SCPs that are associated with the ingrowing extrinsic nerves (Uesaka et al., 2015) (see below). One possibility is that migrating SCPs are required to attract a subpopulation of cells in the MG to migrate into the submucosal region. Neonatal Schwann cells express *Gdnf* mRNA (Henderson et al., 1994), and so presumably SCPs express GDNF. It will be interesting to examine whether some cells in the MG are attracted by GDNF-expressing SCPs into the submucosal region.

The mechanisms underlying radial migration of ENS precursors to the submucosal region in the colon are largely unknown, and elucidating the mechanism involved is complicated by some species differences in the formation of SMG in the colon. In the avian colorectum, the submucosal region has been reported to be colonized simultaneously with the myenteric region (Conner et al., 2003), or slightly prior to the colonization of the myenteric region (Burns and Le Douarin, 1998). In contrast, in the large intestine of mice, ENS precursors and extrinsic nerve-derived SCPs remain in the myenteric layers until around birth, and the submucosal region is colonized postnatally (McKeown et al., 2001; unpublished data). Similarly, in the human large intestine, the SMG forms approximately 5 weeks after the MG formation (Wallace and Burns, 2005; Rauch et al., 2006). Further studies are needed to understand the mechanism of the SMG formation in the large intestine.

4. Enteric neurogenesis from Schwann cell lineage cells

The peripheral nervous system includes the nerve fibers that extend to all parts of the body. A large number of NCC-derived cells migrate along the nerves. Parasympathetic ganglia in the head and thorax have been shown to arise from nerve-associated cells that migrate along the fibers of cranial nerves to reach their final destinations in the periphery (Coppola et al., 2010; Dyachuk et al., 2014; Espinosa-Medina et al., 2014). Cells associated with the cranial nerves begin to express the transcription factor, Phox2b (autonomic neuron marker), between E10 and E11, and have dual competence to generate Schwann cells and neurons. After around E13, Phox2b expression level becomes low or undetectable in nerve-associated cells during the transition from bi-fated precursors to committed SCPs (Espinosa-Medina et al., 2014). Committed SCPs along nerves have been shown to give rise to Schwann cells, fibroblasts, and melanocytes (Adameyko et al., 2009; Joseph et al., 2004). Moreover, a recent, genetic lineage tracing study revealed that a subset of SCPs also gives rise to enteric neurons, including a small proportion ($< 5\%$) of submucosal neurons in the small intestine and around 20% of neurons in the large intestine (Uesaka et al., 2015). Since at least some SCPs are likely to arise from trunk level NCCs, all major axial levels of NCCs (cranial-vagal, trunk, and sacral) contribute to the ENS.

Sacral NCC-derived cells that enter the hindgut consist of two distinct populations. One population, which enters the hindgut after E13.5 but before E15.5, expresses dopamine β-hydroxylase (DβH) and Phox2b (Anderson et al., 2006; Uesaka et al., 2015). A second population, which enters the hindgut along the pelvic nerves after E16.5, expresses glial cell markers, but not Phox2b, and are therefore phenotypically SCPs (Uesaka et al., 2015). Both populations (early non-SCPs and SCPs) can give rise to neurons in the large intestine.

4.1. Common signaling molecules regulating ENS and Schwann cell development

Hirschsprung disease (HSCR) is a congenital disease in which neurons are missing from variable lengths of the distal bowel. Some of the signaling molecules that regulate ENS development and have been associated with HSCR, for example, endothelin and neuregulin (McKeown et al., 2013), are also required for the development of Schwann cells (Jessen and Mirsky, 2002). Here we discuss the possibility that developmental deficits in early non-SCP-derived and SCP-derived ENS precursors in combination can lead to HSCR.

HSCR patients carrying heterozygous mutations in *endothelin receptor type B (EDNRB)*, its ligand *endothelin 3* (*ET3*), and

endothelin-converting enzyme (*ECE*) comprise ~5% of HSCR cases (Amiel et al., 1996; Auricchio et al., 1996; Chakravarti, 1996; Kusafuka et al., 1996). Ablation of *Ednrb* in mice results in colonic aganglionosis and a lack of trunk melanocytes (Hosoda et al., 1994). Ednrb-mediated signaling is required for the migration of ENS precursors and melanocyte precursors (Lee et al., 2003). In addition, a study using *Ednrb*-null rats showed that ET3 acts as a negative regulator of Schwann cell differentiation (Brennan et al., 2000). ET3 also inhibits the neuronal differentiation of ENS precursors in vitro (Bondurand et al., 2006; Hearn et al., 1998; Nagy and Goldstein, 2006; Wu et al., 1999). Furthermore, Schwann cells and pigment cells are able to interconvert in the presence of ET3 in vitro (Dupin et al., 2000, 2003; Nataf and Le Douarin, 2000). ET3 is expressed most highly by the mesenchyme of embryonic cecum (Barlow et al., 2003; Leibl et al., 1999; Nagy and Goldstein, 2006; Woodward et al., 2000). One possibility is that ET3 signaling may act to inhibit the neuronal differentiation of early non-SCP-derived and SCP-derived ENS precursors in the large intestine so that neurons can be generated postnatally. It will be interesting to examine whether vagal NCC- or/and SCP-specific ablation of *Ednrb* reduces enteric neurogenesis in the colon.

Neuregulin 1 (*NRG1*) is a HSCR susceptibility locus that was identified through genome-wide association studies in Asian subjects, and has been implicated as a modifier of *RET*-dependent HSCR risk (Garcia-Barcelo et al., 2009; Gunadi et al., 2014; Phusantisampan et al., 2012). In NCC-derived cells, Nrg1 binds to and activates ErbB2/ErbB3 heterodimers (Garratt et al., 2000a). ErbB3 is expressed in ENS precursors at E13.5 (Paratore et al., 2002), and its expression is restricted to enteric glia and a subset of neurons in the developing and mature ENS (Chalazonitis et al., 2011). Several studies have examined the role of Nrg1 in ENS development, but the conclusions are inconsistent. Under culture conditions, glial growth factor 2 (Ggf2, a Nrg1 isoform) and Nrg1 inhibit GDNF-induced neuronal differentiation, while enhancing glial differentiation (Chalazonitis et al., 2011; Gui et al., 2013). However, no defects are apparent in early ENS formation in conventional *Erbb2* knockout mice or in *Erbb2* and *Erbb3* mutant zebrafish (Britsch et al., 1998; Honjo et al., 2008). In contrast, enteric ganglia were reported to be reduced in number in E13.5 mice lacking *Erbb3* (Erickson et al., 1997). In *Erbb2/Nestin::Cre* mice, the number of enteric neurons and glial cells in the colon is not significantly changed at birth, but there was postnatal loss of neurons which was proposed to be due to a loss of a survival signal from epithelial cells (Crone et al., 2003).

Conditional ablation of *Erbb2* leads to severe lack of Schwann cells at P3.5 (Garratt et al., 2000b). If SCPs entering the gut are reduced, SCP-derived neurogenesis is also likely to be decreased. However, Nrg1 signaling is required not only for SCP survival but also for the maintenance of SC identity as loss of ErbB3 enhances transition from SCPs to melanocytes (Adameyko et al., 2009).

In summary, it is still uncertain whether the Nrg1-ErbB2/ErbB3 signaling pathway plays a crucial role in ENS development, and it will be interesting to examine the role of Nrg1 signaling in SCP-derived neurogenesis in the colon.

4.2. Neurons in the mucosal region

SCP-derived neurogenesis may not be restricted to MG and SMG. Rare small cluster of neurons have been reported within the intestinal mucosa in pig and human (Balemba et al., 1998, 2002; Fang et al., 1993; Metzger et al., 2009). Neurosphere-like bodies can be isolated from endoscopic biopsies of the mucosa in children and adults, suggesting that enteric neural stem cells are also present in the mucosal region (Becker et al., 2012; Metzger et al., 2009). Intramucosal neurons, glial cells and neurosphere-forming cells are even present in the mucosa of the aganglionic colon from HSCR patients (Badizadegan et al., 2014; Wilkinson et al., 2015). Furthermore, “neuroglial cells” , which express both neuronal and glial markers, have been reported in the aganglionic colon of HSCR patients (Badizadegan et al., 2014). In the ENS, cytoplasmic localization of S100β is considered a specific marker for glial cells (Ferri et al., 1982), and mucosal neuroglial cells coexpress S100 β and neuronal markers, class III β-tubulin (TuJ1) or calretinin. Although the origins of mucosal neurosphere-forming cells, neurons, neuroglial and glial cells are unknown, SCP-derived cells associated with the extrinsic fibers are a potential source. Further studies of the ontogeny of neural cells in the mucosa, including aganglionic colon, are needed to resolve the question.

5. Neuronal differentiation in the ENS

5.1. Timing of cell cycle exit and neuronal differentiation

The times at which neuronal precursors in the MG of mice exit the cell cycle have been analyzed by pulse-labeling with H^3-thymidine, BrdU (5-bromo-2′-deoxyuridine), or EdU (5-ethynyl-2′-deoxyuridine) (Bergner et al., 2014; Chalazonitis et al., 2008; Pham et al., 1991). These analyses have revealed the birthdate of neurons that undergo final cell division at the time of the labeling. Based on the results, enteric neurogenesis begins at around E10.5, and almost all myenteric neurons are born in the small intestine by P10. Nonetheless, neurosphere-forming cells can be isolated from the adult gut, showing the presence of cells retaining neurogenic potential (Kruger et al., 2002; Metzger et al., 2009). Although cumulative BrdU labeling failed to detect newborn neurons in adult mice (Joseph et al., 2011), a genetic tracing study reported that 1.6% of neurons are generated from cells expressing Sox10 at P30 and that Sox10-expressing cells can give rise to neurons in adult mice in response to injury (Laranjeira et al., 2011). These data collectively suggest that enteric neurogenesis can occur from Sox10-expressing cells without cell division in the adult ENS, which is different from the CNS where adult neurogenesis occurs from dividing progenitors. As activation of 5-hydroxytryptamine 4 (5-HT_4) receptors induces enteric adult neurogenesis in the normal and injured ENS (Belkind-Gerson et al., 2015; Liu et al., 2009; Matsuyoshi et al., 2010), future studies are required to determine whether adult neurogenesis occurs by the differentiation of glial cells expressing 5-HT_4 receptors.

The ENS contains many distinct subtypes of enteric neurons that differ in neurotransmitters, electrophysiological properties, axon projections (targets) and functions (Furness, 2000). Myenteric neuron subtypes include intrinsic sensory neurons, excitatory and inhibitory motor neurons, and multiple types of descending and ascending interneurons. Nitric oxide synthase 1 (NOS1) neurons comprise multiple subtypes of neurons, but the majority are inhibitory motor neurons (Qu et al., 2008). Calretinin is also expressed by multiple subtypes of myenteric neurons including excitatory motor neurons and intrinsic sensory neurons. Different neuron subtypes exit the cell cycle at different ages: The peak birthdates of serotonin, calbindin, NOS1, and calretinin neurons occur at E10.5–11.5, E14.5, E14.5–15.5, and P0, respectively. Within calretinin neurons, late-born neurons (the majority) are excitatory motor neurons, while a smaller sub-population of calretinin neurons born at E13.5 are putative sensory neurons. Each MG contains both early-born and late-born neurons.

5.2. Role of GDNF in neuronal differentiation

GDNF plays many roles in ENS development (Heanue and Pachnis, 2007), one of which is an essential role in neuronal differentiation. GDNF induces neuronal differentiation of enteric

neural crest-derived cells in vitro (Chalazonitis et al., 1998; Focke et al., 2001; Hearn et al., 1998; Taraviras et al., 1999; Worley et al., 2000), and in vivo, GDNF signaling levels are highly correlated with the differentiation status of ENS precursors (Uesaka et al., 2013). Furthermore, insufficient activation of GDNF signaling appears to be important for the persistence of precursors within the MG (Uesaka et al., 2013). GDNF might also play a role in enteric neuron subtype specification, survival or differentiation as there is a decrease in the proportion of myenteric neurons that express NOS1 in *Gdnf*$^{+/-}$ mice (Wang et al., 2010) and in mice lacking *Ret* (Anderson et al., 2006; Uesaka and Enomoto, 2010; Yan et al., 2004), while increased levels of GDNF as the result of transgenic expression of GDNF in enteric glia results in an increased proportion of NOS1 neurons (Wang et al., 2010).

5.3. Mechanisms regulating axonal projections

Different subtypes of neurons project their axons in different directions, so understanding the mechanisms controlling the spatial organization of axons in the developing ENS is a crucial step for understanding how the circuitry within the ENS is established.

Neurturin (NRTN), a member of the GDNF family of ligands, is expressed in the circular muscle layer, and its receptor, GFRα2, is expressed in the MG and SMG in the newborn intestine (Golden et al., 1999; Rossi et al., 2003). *Nrtn*- or *Gfrα2*-deficient mice do not display any changes in the number of enteric neurons, but exhibit a reduction in cell size and in neuronal fiber density, especially in the number of substance P-expressing excitatory axons in the circular muscle layer (Gianino et al., 2003; Heuckeroth et al., 1999; Rossi et al., 2003, 1999). Therefore, NRTN signaling seems to be required for the projection of excitatory motor neurons to the circular muscles and/or the growth and branching of excitatory axons within the circular muscle.

The planar cell polarity (PCP) pathway components, the Wnt receptor Frizzled 3 and the cadherin EGF LAG seven-pass G-type receptor 3 (Celsr3), are expressed in the developing ENS, and the PCP pathway has been shown to be essential for the directionality of axonal projections from early stages of enteric neurogenesis (Sasselli et al., 2013). PCP mutant mice exhibit motility defects that are likely to be due to defects in the formation of motility-controlling circuits (Sasselli et al., 2013).

Sonic hedgehog (Shh), is expressed in the epithelium of the embryonic gut (Marigo et al., 1996; Narita et al., 1998; Ramalho-Santos et al., 2000; Roberts et al., 1995). Ablation of *Shh* in mice leads to abnormal neuronal projections into the mucosal region at E18.5 (Ramalho-Santos et al., 2000). However, these abnormalities may arise as a secondary patterning defect due to reduced mesenchyme/smooth muscle mass (Mao et al., 2010; Sukegawa et al., 2000) or to reduced levels of BMP4 in the mesenchyme (Ramalho-Santos et al., 2000), as ectopic expression of BMP4 influences enteric progenitor positioning and proliferation (Sukegawa et al., 2000). Selective inactivation of Hh receptors in ENS precursors is needed to elucidate the direct roles of Hh signaling. Hh signaling is mediated by two transmembrane proteins: smoothened (Smo) that acts as a transducer of Hh signal, and the Hh-binding receptor patched homolog 1 (Ptch1) that suppresses Smo (Marigo et al., 1996; Stone et al., 1996). Hh ligands also interact with CAM-related/down-regulated by oncogenes (Cdo; Zhang et al., 2006), Brother of Cdo (Boc; Tenzen et al., 2006), Hh interacting protein (Hip1; Chuang and McMahon, 1999), Patch2, and Growth arrest-specific 1 (Gas1; Allen et al., 2007; Martinelli and Fan, 2007). ENS cells exhibit little to no expression of Cdo and Boc, and *Cdo* or *Boc* single-knockout and Cdo/Boc double knockout mice do not display any noticeable defects in the location of axons in the late embryonic gut (Jin et al., 2015). Gas1 is expressed in gut smooth muscle layers, ENS precursors, and neurons (Biau et al., 2013). Like *Shh* mutants, E18.5 *Gas1*-deficient mice have more ENS precursors and neurites in the mucosa than control mice (Biau et al., 2013; Jin et al., 2015). Specific ablation of *Gas1* in NCCs leads to abnormal axon projections into the mucosa, but does not cause the mislocalization of ENS precursors (Jin et al., 2015). Thus, inactivation of Shh-Gas1 signaling affects ENS precursor positioning in a non-cell-autonomous manner, while Shh signaling via Gas1 directly regulates the growth of axons into the mucosa. In the colon, Gas1 expression level is low, and *Shh* or *Gas1* mutants do not display axon projection defects at E18.5 (Jin et al., 2015). However, myenteric axons and extrinsic axons do not project to the colonic mucosa until after E18.5 (Uesaka et al., 2015). There is a need to elucidate the role of Shh-Gas1 signaling during postnatal ENS development.

Semaphorin 3A (Sema3A), which is secreted from target tissue, generally acts as an inhibitory or repulsive cue for axonal outgrowth and cell migration (Tran et al., 2007). *Sema3a*-deficient mice display abnormal axon projections from DRGs and disruptions to sympathetic chain ganglia (Behar et al., 1996; Taniguchi et al., 1997). Sema3A is transiently expressed in the outer layers of the distal hindgut in E11.5 to E14.5 mice, and in Sema3A-deficient mice, pelvic nerves and sacral NCCs prematurely invade the distal hindgut (Anderson et al., 2007). Sema3A-mediated inhibition of extrinsic nerves and sacral NCCs appears to prevent them from entering the hindgut before the arrival of vagal ENS precursors into the distal hindgut. However, the roles of Sema3A in regulating axonal projections of other types of intrinsic and extrinsic neurons have yet to be explored in detail.

6. Enteric glial differentiation

In most species and gut regions, glial cells outnumber neurons in the ENS (Hoff et al., 2008). A variety of morphological and functional types of enteric glia have been identified. Hanani and Reichenbach (1994) described intraganglionic type I or protoplasmic enteric glia, which are astrocyte-like glial cells. These authors also described type II or fibrous enteric glia at the edges of the interganglionic connectives (Hanani and Reichenbach, 1994). In the subepithelial space, type III or mucosal enteric glia have been reported (Gulbransen and Sharkey, 2012; Savidge et al., 2007). Glial cells in the intestinal mucosa have been suggested to engage in multidirectional interactions with the epithelium, blood vessels, nerves, and immune system (Bush et al., 1998; Cornet et al., 2001). In addition, enteric glia have been reported to release soluble factors to regulate inflammation, promote mucosal wound healing, and protect against pathogen invasion (Flamant et al., 2011; Savidge et al., 2007; Steinkamp et al., 2003; Van Landeghem et al., 2011). Type III-like glial cells have been also identified in the MG and SMG (Boesmans et al., 2015). Intraganglionic glial cells communicate with, and support, neurons (Boesmans et al., 2013; Gulbransen et al., 2010; Gulbransen and Sharkey, 2009). In the muscle layers, type IV or intramuscular enteric glia are present that have a characteristic bipolar morphology and are located close to nerve fibers (Boesmans et al., 2015; Gulbransen and Sharkey, 2012). Our knowledge of the role of intramuscular glia is extremely limited, but they might serve a similar function to glial cells at the skeletal neuromuscular junctions.

The relationship between the origin and morphology of enteric glia is complex. Genetic lineage tracing have revealed that extrinsic nerve-derived (probably vagal and trunk SCP-derived) Schwann cells settle in the deep muscle, submucosal, and mucosal region (Uesaka et al., 2015). Surprisingly, SCP-derived cells are rarely detectable in the MG of the small intestine (Uesaka et al., 2015), and hence it is likely that all glia in MG in this region arise

from vagal NCCs. It is unknown why SCPs do not populate MG in the small intestine, even though extrinsic nerves innervate the MG. In contrast to the small intestine, most SCPs derived from trunk and sacral NCCs in the large intestine remain in the MG and differentiate into glial cells and neurons, and then a subpopulation of SCPs invades into the submucosal region postnatally (Uesaka et al., 2015). Morphologically distinct classes of astrocyte-like enteric glia exist (types I–III) in MG (Boesmans et al., 2015), but it remains to be determined whether SCP-derived enteric glia give rise to specific glial subtypes in the colon. It has been previously demonstrated that enteric glial cells and myelinating Schwann cells can be directed to transdifferentiate into one another by appropriate environmental cues (Dulac and Le Douarin, 1991; Sextier-Sainte-Claire Deville et al., 1994). Although SCP-derived glial cells are present in the ENS, RNA sequencing analysis shows that enteric glial cells are transcriptionally distinct from other glia in the nervous system (Rao et al., 2015).

Some of the factors required for the development of enteric glia have been identified. Sox10 is required for the development of all peripheral glia, including enteric glia (Britsch et al., 2001; Paratore et al., 2001). A study in which Notch signaling was perturbed in all NCC concluded that Notch signaling promotes gliogenesis (Taylor et al., 2007), but a later study concluded that the primary role of Notch is to maintain enteric neural progenitors (Okamura and Saga, 2008). Signaling by glial growth factor 2 (GGF2) might also play a role in the differentiation of enteric glia as GGF2 signaling via ErbB3 inhibits GDNF-induced neurogenesis and stimulates enteric gliogenesis in vitro (Chalazonitis et al., 2011). The glial cell network in the mucosa does not develop until postnatal stages and requires signals from gut microbiota that have yet to be identified (Kabouridis et al., 2015).

7. Concluding remarks

In the past 20 years, there have been astonishing advances in our understanding of ENS development, especially concerning the major genes and signaling pathways that regulate the migration, proliferation, survival, and differentiation of ENS precursors. However, important developmental processes including those that maintain a progenitor sub-population in reserve for late-born neurons, the determination of neuronal identity, axon guidance, and target-specific innervation within the gut remain unexplained. Furthermore, although several guidance molecules that control extrinsic nerve innervation in the GI tract have been characterized, the molecular mechanisms that orchestrate extrinsic and intrinsic gut innervation remain largely unknown. New genetically engineered animal models should be explored, and more systematic and molecular profiling and phenotype analysis will allow us to understand long term and systematic development of the ENS and to elucidate mechanisms underlying synergistic coordination between intrinsic and extrinsic components in the ENS. This research might also help to develop novel therapeutic strategies for patients with gastrointestinal disorders.

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