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Review article

Gene-environment interactions and the enteric nervous system: Neural plasticity and Hirschsprung disease prevention

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ABSTRACT

Intestinal function is primarily controlled by an intrinsic nervous system of the bowel called the enteric nervous system (ENS). The cells of the ENS are neural crest derivatives that migrate into and through the bowel during early stages of organogenesis before differentiating into a wide variety of neurons and glia. Although genetic factors critically underlie ENS development, it is now clear that many non-genetic factors may influence the number of enteric neurons, types of enteric neurons, and ratio of neurons to glia. These non-genetic influences include dietary nutrients and medicines that may impact ENS structure and function before or after birth. This review summarizes current data about gene–environment interactions that affect ENS development and suggests that these factors may contribute to human intestinal motility disorders like Hirschsprung disease or irritable bowel syndrome.

1. Introduction

The enteric nervous system (ENS) is an integrated network of neurons and glia within the bowel wall that controls most aspects of bowel function (Furness, 2012; Wood, 2008). The complex ENS circuitry permits the bowel to operate largely autonomously so we can eat and enjoy the finer things in life, without having to think about mixing food with digestive enzymes, facilitating contact with the epithelium to enhance nutrient uptake, coordinating motility to avoid excessive bowel dilation, moving luminal contents toward the end of the bowel for elimination, regulating epithelial secretion and proliferation, or altering regional blood flow in response to metabolic needs. To perform these tasks the bowel contains about as many neurons as the spinal cord and diverse neuron subtypes produce and respond to the full spectrum of neurotransmitters found in the central nervous system. In addition to neurons, there are several types of enteric glia (Gulbransen and Sharkey, 2012; Sharkey et al., 2004) with diverse morphology and function. This beautiful nervous system needs to respond to a wide array of dietary patterns to facilitate nutrient intake and growth, and to avoid dehydration. Although significant accommodation to varied diets may occur without changing the fundamental structure of the ENS, if non-genetic factors impacted the types of neurons and glia produced or other aspects of ENS development and maintenance, it would permit broader adaptation to diverse nutrient and fluid intake. Indeed, recent data suggest that many non-genetic factors influence ENS development as well as mature structure and function. This has important implications for birth defects affecting the human enteric nervous system and for acquired intestinal motility disorders.

2. ENS development: The more complex the machine, the more ways it can go wrong!

The ENS forms from enteric neural crest-derived precursor cells (ENCDC) that originate primarily in the vagal region of the neural tube with minor contributions from sacral and upper thoracic ENCDC (Avetisyan et al., 2015a; Goldstein et al., 2013; Lake and Heuckeroth, 2013; Newgreen et al., 2013; Sasselli et al., 2012). Vagal ENCDC exit the neural tube at about embryonic day 8.5 (E8.5) in mice and at about week three of human gestation. These vagal ENCDC then migrate to the bowel and colonize the bowel in a rostral to caudal progression migrating through gut mesenchyme to reach the end of the bowel by E13.5 in mice and week eight of gestation in humans. During migration ENCDC proliferate vigorously, and then exit the cell cycle, differentiate into neurons or

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Table 1
Simplified list of genes that impact ENS development.

Gene	Role in ENS development	Protein function/Comments
RET	Supports ENS precursor survival, proliferation, migration, neuronal differentiation, neurite growth and axon patterning	– Transmembrane tyrosine kinase receptor – Most commonly inactivated gene in people with HSCR.
GDNF	RET activating ligand	– Neurotrophic factor – Rarely mutated in people with HSCR
EDNRB	Prevents premature differentiation of ENCDC Facilitates colon colonization by ENCDC	– G-protein coupled receptor – Mutated in 5% of people with HSCR – Also causes hearing loss and pigmentation defects (Waarderburg-Shah, WS4))
EDN3	EDNRB activating ligand	– Peptide – Rarely mutated in people with HSCR
SOX10	Required for bowel colonization by ENCDC Activates RET expression	– Transcription factor – Mutations cause HSCR plus hearing loss and pigmentation defects (WS4)
PHOX2B	Required for bowel colonization by ENCDC Activates RET expression	Transcription factor Mutations cause HSCR plus congenital central hypoventilation (Haddad syndrome)

The complex cellular processes that occur during ENS development are supported by a wide array of cell surface proteins, extracellular ligands, intracellular signaling molecules, transcriptonal regulators, and non-genetic factors. These pathways have recently been reviewed in detail by our group and other investigators (Amiel et al., 2008; Avetisyan et al., 2015a; Goldstein et al., 2013; Lake and Heuckeroth, 2013; Newgreen et al., 2013; Sasselli et al., 2012).

glia, cluster into ganglia, and form an extensive interconnected network that extends all the way along the bowel. This process is controlled by many known genetic factors discussed in more detail in other articles in this Special Issue of Developmental Biology. A few key molecules are briefly described in Table 1. This article highlights how non-genetic factors may affect the ENS before and after birth and explains links to human intestinal motility disorders.

3. Hirschsprung disease (HSCR)

HSCR is a disorder where the ENS is missing from the end of the bowel. The region without enteric neurons is called "aganglionic bowel". In the absence of all enteric neurons, the bowel tonically contracts causing functional obstruction (Amiel et al., 2008; Heuckeroth, 2013; Skinner, 1996). Symptoms of HSCR include abdominal distension, vomiting, severe constipation, growth failure, a predisposition to bowel inflammation (enterocolitis) and early death. HSCR occurs in about 1:5000 children as a result of the failure of bowel colonization by ENCDC during fetal development. Because vagal ENCDC have a very long migratory route and most children with HSCR (80%) have only a "short-segment" of bowel with missing enteric neurons (i.e., rectum and sigmoid colon aganglionosis), a marginal increase (e.g. 10%) in fetal bowel colonization by vagal ENCDC would have prevented HSCR from occurring in many of these children. This may explain why the male to female ratio for short segment human HSCR is 4:1 while the male to female ratio for long segment HSCR is 2:1 (Badner et al., 1990). In long segment HSCR, small increases in bowel colonization by ENCDC will shorten the aganglionic zone, but would not prevent HSCR. In short segment HSCR, a 10% increase in bowel colonization by ENCDC could have prevented HSCR for many affected children. These data suggest that in human females, ENCDC colonize fetal bowel slightly more efficiently than in males, consistent with observations in mouse models (Bergeron et al., 2015; Cantrell et al., 2004; McCallion et al., 2003; Vohra et al., 2007b). The minimal improvement in bowel colonization by ENCDC needed to prevent HSCR in most affected children also suggests that many genetic and non-genetic factors could influence HSCR risk even if they only marginally affect bowel colonization efficiency.

4. Bowel colonization by ENCDC is driven by cell proliferation

ENCDC must form a network of neurons and glia along the entire length of the bowel suggesting that the entire migratory route is hospitable for ENCDC. Full bowel colonization by ENCDC, however, appears to be driven by competition for available space and trophic factors as ENCDC proliferate, instead of by trophic factor gradients (Gianino et al., 2003; Landman et al., 2007; Newgreen et al., 2013; Wang et al., 2010). Although gradients of some trophic factors like glial cell line-derived neurotrophic factor (GDNF) exist in fetal bowel and might drive migration (Natarajan et al., 2002), individual ENCDC can actually migrate through the bowel in either direction (and do so during normal development) (Burns et al., 2002; Druckenbrod and Epstein, 2007; Young et al., 2014). Because proliferation drives bowel colonization, anything that reduces the number of ENCDC or the proliferation of these cells during development should predispose to HSCR. Consistent with this hypothesis, removing parts of the vagal neural tube to reduce neural crest-derived cells that enter the bowel can cause HSCR-like disease (Barlow et al., 2008). Reducing cell proliferation with mycophenolic acid, a drug that blocks the rate limiting step in *de novo* guanine nucleotide synthesis, also caused distal bowel aganglionosis in mice and reduced ENCDC colonization of distal bowel in fish (Lake et al., 2013). Consistent with this hypothesis, inactivating mutations in the GDNF receptor RET (a transmembrane tyrosine kinase) are the most commonly identified cause of human HSCR (Amiel et al., 2008), and RET signaling is needed for ENCDC survival and proliferation (Schuchardt et al., 1994). Inactivating mutations in the G-protein coupled receptor EDNRB also cause HSCR (Puffenberger et al., 1994) and may permit early differentiation of ENCDC within the colon (Barlow et al., 2003), reducing proliferation that normally drives bowel colonization by ENCDC.

One prediction from these observations is that reduced activity of any of the signaling molecules that are needed to drive ENCDC proliferation should increase HSCR risk, especially in the context of other genetic changes that predispose to HSCR. Furthermore, fetal malnutrition and other causes of intrauterine growth retardation might increase HSCR occurrence if they occurred during the period of ENCDC migration, but this hypothesis has not yet been tested. Importantly, formation of some structures during development (like the ENS) depends on cell proliferation, so a global reduction in fetal cell proliferation may result in not only a smaller baby, but also a higher risk of structural birth defects. This hypothesis is

supported by higher rates of major congenital anomalies in children born after placental abruption (OR 3.81, 95% confidence interval (CI) 1.34–2.37 for gastrointestinal anomalies and 1.92, 95% CI 1.6–2.52 for all anomalies) (Riihimaki et al., 2013).

5. HSCR genetics

HSCR is one of the best understood complex human genetic diseases (Alves et al., 2013; Amiel et al., 2008; Avetisyan et al., 2015a; Lake et al., 2013; McKeown et al., 2013; Panza et al., 2012). As might be predicted by the complex cellular mechanisms needed to form the ENS (i.e., proliferation, migration, controlled differentiation), many gene defects can increase HSCR risk. This includes mutations that reduce activity of cell surface receptors (RET, EDNRB), extracellular ligands (GDNF, NRTN, EDN3), and transcription factors (SOX10, PHOX2B, ZFHX1B), as well as specific chromosomal anomalies (Down syndrome). Work in model systems suggests that many additional signaling molecules (Pik3, MEK, PLCγ, PKCζ, GSK3), enzymes (RALDH2, EDN3), extracellular matrix proteins (laminin, fibronectin, collagen VI), integrins (ITGB1), synaptic vesicle proteins (Synaptobrevin, SNAP25), morphogens (BMP2/BMP4, Shh, Ihh), and small molecules (retinoic acid, serotonin) influence ENS development. Undoubtedly there is more to learn since the known molecules are inadequate to explain how the ENS forms. Non-genetic factors may influence ENS development by modifying the expression levels or activity of these molecules already known to guide ENS development. This means that there are many targets through which non-genetic factors could affect the developing ENS.

6. Gene–environment interactions and the ENS

Human development is largely driven by genetics, but gene products do not work in isolation (Fig. 1). Intrauterine growth requires energy, building blocks for protein, ions that act as enzyme cofactors or are essential for membrane electrical properties and vitamins that play diverse roles in intermediary metabolism. Oxygen is essential for efficient energy production from the electron transport chain in mitochondria, but reactive oxygen species reduce bowel colonization by ENCDC in some settings (e.g. TCOF1 mutation) (Barlow et al., 2012) and low intrauterine oxygen levels relative to post-natal levels appear to be optimal for ENS stem cell proliferation (Hegewald et al., 2011). Many of the signaling molecules needed for ENS development are also common drug targets and some vitamins (vitamin A, folate), nutrients and small metabolites (butyrate) can influence gene expression. Given the large number of potential targets and non-genetic factors that might impact proteins critical for ENS morphogenesis, it is not surprising that non-genetic factors impact ENS development. This is especially important since known genetic changes that predispose to HSCR are partially penetrant (Alves et al., 2013). This means that a child with HSCR typically has more than one predisposing genetic change or a combination of genetic and non-genetic risk factors led to the disease. If non-genetic factors can be identified and eliminated, some cases of HSCR might be prevented.

7. Non-HSCR motility disorders

In contrast to HSCR, the underlying problems that cause other types of bowel motility disorders are relatively poorly understood. These disorders include achalasia (Castell, 2013), gastroparesis (Camilleri et al., 2013), chronic intestinal pseudoobstruction syndrome (CIPO) (Di Lorenzo and Youssef, 2010; Schappi et al., 2013), slow transit constipation, and irritable bowel syndrome (IBS) (Camilleri, 2013; Heuckeroth, 2014; Knowles et al., 2010; Panza et al., 2012; Wood, 2013) (Table 2).

Also, in contrast to HSCR where the ENS is completely missing from distal bowel, other intestinal motility disorders may be caused by altered numbers of enteric neurons and glia, by changes in the types of neurons present, by disruptions in neuronal circuitry or by altered neuronal function. Damage to the ENS may be induced by destructive effects of systemic illness (e.g. diabetic gastroparesis) (Thazhath et al., 2013), infection (achalasia and colon dysmotility in Chagas disease (Machado et al., 2012), varicella zoster (Chen et al., 2011; Holland-Cunz et al., 2006)), toxins (e.g.,

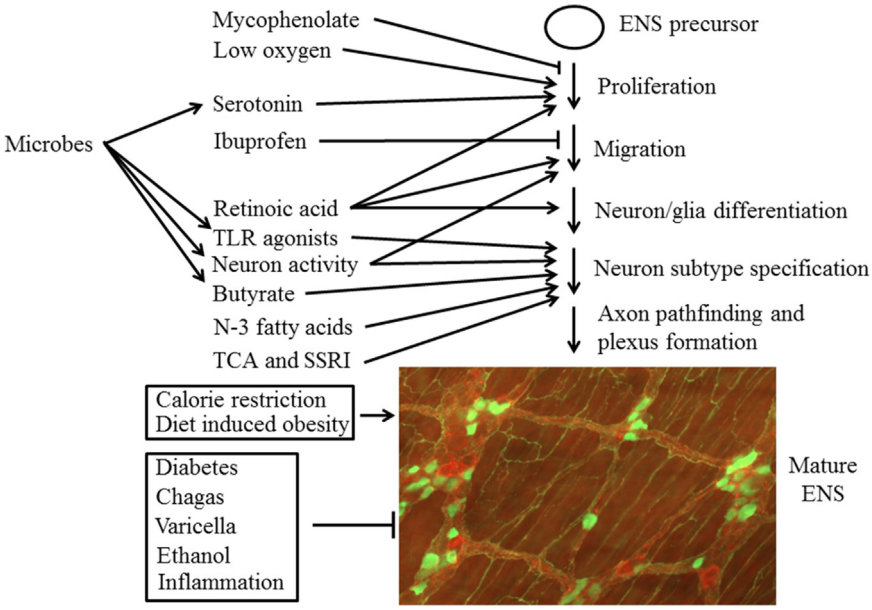

Fig. 1. This schematic shows many of the non-genetic factors that affect ENS development or the mature ENS. The mature ENS is an intricate structure as represented in the image that shows the adult mouse myenteric plexus stained with antibodies to calretinin (green) and substance P (red) (courtesy of Marina Avetisyan). Arrows indicate aspects of ENS development that are "modulated" (i.e., changed) by a specific factor, or indicate that the factor acts on the mature ENS. The symbol "———|" indicates that these factors inhibit a specific process or damage the mature ENS.

Table 2
Human intestinal motility disorders where ENS defects my cause symptoms (simplified).

Hirschsprung disease (HSCR)	Absence of enteric neurons in distal bowel causes tonic contraction and functional obstruction leading to abdominal distension, vomiting, constipation, growth failure and early death
Achalasia	Defective esophageal peristalsis and poor relaxation of the lower esophageal sphincter causes swallowing problems
Gastroparesis	Defective stomach emptying or accommodation in association with visceral hypersensitivity causes nausea, abdominal pain, vomiting and weight loss
Chronic intestinal pseudoobstruction (CIPO)	Abnormal intestinal contractility as a result of defects in the ENS, intestinal smooth muscle or pacemaker cells (Interstitial cells of Cajal) causes abdominal distension, vomiting, and the inability to survive solely on enteral feeding
Slow transit constipation	Constipation due to slow movement of luminal content through the colon
Irritable bowel syndrome (IBS)	Abnormal bowel motility and visceral hypersensitivity causes diarrhea and/or constipation and abdominal pain. IBS affects about 10-15% of the adult population and can be uncomfortable, but does not affect nutrition or longevity

ethanol) (Krecsmarik et al., 2006), or local inflammation (causing inflammatory bowel disease associated dysmotility (Mawe, 2015; Mawe et al., 2009; Vasina et al., 2006) and post-infectious irritable bowel syndrome (Spiller and Garsed, 2009)). In part, post-inflammatory changes in the ENS may be mediated by cytokines that induce GDNF and NGF synthesis in enteric glia or alter glial phenotypes (von Boyen et al., 2006a; von Boyen et al., 2004, 2006b). Enteric glia modulate neuronal function to regulate motility, and can affect neuron survival and bowel epithelial barrier function, among other roles (Brown et al., 2016; Ochoa-Cortes et al., 2016; Sharkey, 2015). Furthermore, inflammation can destroy the ENS, change neurochemical content or alter enteric neuron and glial function through a variety of complex mechanisms (Brierley and Linden, 2014; MacEachern et al., 2015; Moynes et al., 2014; Poole et al., 2015). Remarkably, early life emotional distress (e.g., maternal separation model) and adult stress (e.g. water immersion in rodents) can also cause long-term changes in enteric glia and neurons, as well as changes in colon and gastric function in ways that may be relevant for gastroparesis and IBS (Bian et al., 2011; Fujikawa et al., 2015; Li et al., 2015; Moloney et al., 2015; Tominaga et al., 2016). Non-genetic factors may also protect the ENS from injury. For example, quercetin, a flavonol antioxidant found in fruits, vegetables and grains, reduced enteric neuron and glia loss in a diabetic rat model (Lopes et al., 2012). Clearly we need to find additional non-genetic factors that reduce ENS injury if we hope to prevent serious intestinal motility disorders.

8. Evidence in model systems that non-genetic factors affect HSCR risk

Although data that non-genetic factors cause human HSCR is limited, population based surveys are underpowered to uncover these links (i.e., even if you had good data about 100,000 pregnancies, only about 20 children with HSCR would be expected) and little work has been done in this area. To find evidence that non-genetic factors alter HSCR risk, a dedicated case-control study focused on early pregnancy events, maternal health, nutrition and exposures would be needed. One epidemiologic study suggested that maternal coffee consumption and first trimester fever increased HSCR risk in children with Down syndrome (Torfs and Christianson, 1999), but this has not been replicated. The results are plausible, at least for coffee, since caffeine increases cAMP by blocking phosphodiesterase (Salzman et al., 1972) and reduced cAMP-dependent Protein kinase A (PKA) activity is an important aspect of EDNRB signaling (Barlow et al., 2003; Fuchs et al., 2001). Studies of first trimester maternal fever and HSCR have yielded inconsistent results (Larsson et al., 1989; Torfs and Christianson, 1999), but there is little data about the duration or intensity of fever during pregnancy and the period of ENCDC migration lasts five weeks in humans (a long time for a febrile illness).

In the absence of robust human data, model systems powerfully support the hypothesis that non-genetic factors may alter HSCR risk. A drug screen in zebrafish using only a single drug concentration identified nine medicines that reduce ENCDC colonization of fish bowel including artesunate, lovastatin and mycophenolic acid (Lake et al., 2013). Artesunate is a commonly used antimalarial whose mechanisms is poorly understood (Haynes et al., 2013). Lovastatin blocks 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting step in de novo cholesterol synthesis (Alberts, 1988). Mycophenolate reduces cellular GTP levels (and reduces other guanine nucleotides) as detailed above. The reason that lovastatin reduces ENCDC colonization of zebrafish bowel is not known, but hedgehog proteins are cholesterol modified morphogens that affect the ENS (Fu et al., 2004; Nagy et al., 2015; Porter et al., 1996; Sukegawa et al., 2000), and lipid rafts are cholesterol rich membrane domains that appear to be important for efficient RET signaling (Pierchala et al., 2006; Tansey et al., 2000). Since only a single drug concentration was used for the zebrafish screen described above, it is reasonable to expect that other medicines would be found to affect ENCDC colonization of developing bowel if additional drug concentrations were tested (i.e., since the concentration tested may not be near the active concentration for many tested drugs). In support of this hypothesis, when drugs used by $>0.5\%$ of U.S. women during early pregnancy were tested at a range of concentrations on zebrafish, ibuprofen was found to slow ENCDC migration by altering actin cytoskeletal dynamics (Schill et al., 2016). Interestingly, inactivation of the cyclooxygenase enzymes that make prostaglandins (the primary therapeutic targets of ibuprofen) did not slow ENCDC colonization of fetal bowel in mice, suggesting that “off target” effects of ibuprofen lead to ENCDC bowel colonization defects. The concentration of ibuprofen needed to slow ENCD migration and the magnitude the effect differs among species tested (zebrafish, chick and mouse), emphasizing the need for data in human populations about maternal medicine use and HSCR occurrence.

Maternal nutrition is also likely to influence HSCR risk. The most powerful data to support this hypothesis is that relatively mild vitamin A deficiency causes HSCR-like disease in mice (Fu et al., 2010). Vitamin A is the precursor for retinoic acid (RA), a small molecule that alters transcription by binding to and regulating the RAR and RXR transcription factors that control many aspects of development (D'Ambrosio et al., 2011; di Masi et al., 2015). Interestingly, RALDH1, RALDH2, and RALDH3, the retinaldehyde dehydrogenases that make RA, also appear to affect ENS development (Wright-Jin et al., 2013), with RALDH2 activity absolutely essential for formation of the ENS (Niederreither et al., 2003). Furthermore, retinol binding protein 4 (RBP4) deficiency causes HSCR-like disease in mice that are also heterozygous for *Ret*, a major HSCR risk allele (Fu et al., 2010). The mechanism for RA-deficiency induced HSCR-like disease in mice is not completely understood since there appear to be retinoid effects on the abundance of multiple proteins at various stages. Early in development as ENCDC migrate from the neural tube to the bowel, RA signaling is essential to induce *Ret* expression within ENCDC (Simkin et al., 2013) and at later stages RA supports neuronal

precursor proliferation and neuronal differentiation (Sato and Heuckeroth, 2008). In contrast, when the wavefront of migrating ENCDC has reached the mid-colon, RA reduces PTEN protein levels in ENCDC at the leading edge of the migration wavefront (Fu et al., 2010). This is important because PTEN reverses the action of PI-3 kinase, a major RET regulated protein required for ENCDC survival, proliferation and migration. Indeed, a balance of PTEN/PI-3 kinase activity is likely to be important for ENS development since cell-autonomous PTEN deficiency within ENCDC causes bowel hyperganglionosis and a CIPO phenotype (i.e., intestinal distension and weight loss) that results in early death (Puig et al., 2009). This phenotype is reminiscent of the hyperganglionosis and dysmotility that occurs when mutations inactivate SPROUTY2 (Taketomi et al., 2005), a protein that reduces RET activity. These data suggest that vitamin A deficiency, one of the most common micronutrient deficiencies in many parts of the world (West, 2002), may be a preventable cause of HSCR. Unfortunately, even though large studies of maternal vitamin A supplementation have been performed (as summarized in a recent Cochran review of 153,500 women), there is no data about HSCR frequency as a function of vitamin A supplementation (McCauley et al., 2015). Furthermore, because HSCR is often not apparent at birth and diagnosis requires sophisticated medical tests, ascertainment of HSCR cases is unlikely to be good in nutrient poor populations. Based on known biochemistry, it seems likely that other micronutrient deficiencies also predispose to ENS defects. For example, mycophenolate causes HSCR-like aganglionosis in mice because it blocks *de novo* guanine nucleotide synthesis via IMPDH inhibition. *De novo* guanine synthesis also requires folate, niacin, vitamin B6 and vitamin B12 dependent enzymes. Recent data also suggest that biotin may enhance migration of ENS precursors (Fattahi et al., 2016). Biotin is a nutrient required by a family of carboxylases that have important roles in fatty acid metabolism, amino acid metabolism, carbohydrate metabolism, polyketide synthesis, and urea utilization among other cellular processes.

9. Evidence that non-genetic factors may alter ENS structure without causing HSCR

ENS function depends on a balance of specific neuron subtypes (e.g. excitatory motor neurons, inhibitory motor neurons, intrinsic primary afferent neurons (IPANs, sensory), interneurons, etc.). These neuronal subtypes differ in morphology, neurotransmitters produced, receptors, axon number, axon trajectory and function (Furness, 2012; Hao and Young, 2009). The factors that guide neuronal subtype identity in the ENS remain poorly understood, but the ratio of neuronal subtypes is influenced by the timing of cell cycle exit (Avetisyan et al., 2015a; Bergner et al., 2014; Chalazonitis et al., 2008; D'Autreaux et al., 2011; Pham et al., 1991; Wang et al., 2010) and many factors impact the decision to exit the cell cycle (e.g., GDNF, EDN3, SHH, BMPs, RA) (Lake and Heuckeroth, 2013). One intriguing observation is that serotonin (5-HT) produced by early differentiating enteric neurons acts as a trophic factor for surrounding ENCDC and that altered serotonin levels influences neuron subtype ratios (Li et al., 2011). For this reasons, mice with a mutation in the 5-HT producing enzyme tryptophan hydroxylase 2 (TPH2) have fewer "late born" GABA and DAT (dopamine active transporter) immunoreactive neurons than WT animals. The norepinephrine reuptake transporter (NET) is also required for development of serotonergic neurons and reduced NET activity affects ENS development (Li et al., 2010). Interestingly, in postnatal bowel, serotonin also promotes new neurogenesis and ENS repair, at least partially via 5-HT4 receptor (Gershon, 2012; Matsuyoshi et al., 2010; Takaki et al., 2014). Consistent with these observations, human data based on 35,400 pregnancies suggest that first trimester exposure to tricyclic antidepressants (TCAs) and second or third trimester exposure to selective serotonin reuptake inhibitors (SSRIs) increases the use of laxatives in children after birth up to 10-fold for combined drug exposures (Nijenhuis et al., 2012). This epidemiologic observation may be explained by the ability of TCAs to block NET and of SSRIs to block serotonin reuptake from the synapse leading to increased norepinephrine and 5-HT signaling respectively.

Neuronal subtype specification also appears to be influenced by neuronal activity. Blocking neural activity not only slows migration of ENCDC through fetal bowel (Vohra et al., 2006), but also selectively reduces the number of NO producing neurons (Hao et al., 2010). In contrast, ENS precursor depolarization increases tyrosine hydroxylase and vasoactive intestinal peptide (VIP) producing neurons in culture without increasing NO producing cells (Chevalier et al., 2008). While the precise mechanisms underlying these observations are not known, many neuromodulatory medicines (antidepressants, antipsychotics, anti-epileptics, anti-cholinergics, anti-hypertensives) cross the placenta and could therefore affect enteric neuron subtype specification by altering neuron activity, leading to changes in post-natal bowel motility.

After birth, diet has complex effects on the ENS that may be due direct effects on enteric neuron activity or to changes in gut microbes that secondarily affect the ENS. In support of direct effects on the ENS, many nutrients activate enteric neurons (e.g., glucose, fatty acids, amino acids) and may induce long term changes in neurotransmitter expression (Neunlist and Schemann, 2014). As an example, the short chain fatty acids butyrate can alter the ratio of myenteric choline acetyltransferase (ChAT) and nNOS immunoreactive neurons (Soret et al., 2010; Suply et al., 2012). This might result from changes in neuron activity that can affect cell fate since mucosally projecting myenteric neurons undergo transient depolarization and late hyperpolarization in response to butyrate. This occurs via calcium release from intracellular stores that then acts on calcium dependent potassium channels (Hamodeh et al., 2004; Haschke et al., 2002; Neunlist et al., 1999). Alternatively, butyrate could alter gene expression within developing enteric neurons by inhibiting histone deacetylases (Soret et al., 2010; Steliou et al., 2012). In addition to the short chain fatty acid butyrate, feeding long chain N-3 polyunsaturated fatty acids to pigs during gestation and while nursing also altered enteric neuron subtype ratios, increasing ChAT and decreasing VIP immunoreactive neurons in the jejunal submucosal plexus of their piglets (De Quelen et al., 2011). The mechanism underlying this observation is not understood, but N-3 unsaturated fatty acids are known to compete with N-6 unsaturated fatty acids, inhibiting arachidonic acid metabolism and reducing inflammation (Yates and Calder, 2014). As noted above, inflammation can alter enteric neuron subtype ratios. Finally, calorie restriction reduced aging related neuron loss in the ileal ENS of rats (Thrasivoulou et al., 2006), while diet induced obesity reduced age associated antral nitrergic neuron loss (Baudry et al., 2012) possibly via altered trophic factor expression in the bowel wall that results from these dietary manipulations (Korsak et al., 2012; Saavedra et al., 2008). The impact of diet on the ENS is difficulty to separate from the role of gut microbes after birth. For example, butyrate may be ingested, or can be synthesized in the colon by anaerobic bacteria that ferment dietary fiber (Soret et al., 2010). Collectively these data suggest that neuron subtype ratios and therefore cell fate in the ENS is regulated in part by dietary nutrients. This adaptation may facilitate the bowel's ability to digest a wide range of nutrients.

Strong support for microbial effects on the ENS comes from studies in germ free mice, since these animals have altered motility and changes in ENS structure including fewer myenteric neurons, an increased proportion of NO neurons, and fewer calbindin+ myenteric neurons compared to specific pathogen free

or conventionally colonized animals (Collins et al., 2014; Dey et al., 2015; McVey Neufeld et al., 2015). The mechanisms underlying these observations are likely to be complicated. In addition to bacterial metabolites, microbial structural components may affect the ENS directly and indirectly via Toll-like receptors, a subset of pattern recognition receptors that bind to and are activated by microbial molecules. TLR activation stimulates intracellular signaling cascades that may result in the release of cytokines, chemokines and neurotrophic factors (Frosali et al., 2015). For example, TLRs 1-9 are expressed by intestinal smooth muscle, a prominent source of neurotrophic factors. Stimulation of TLR2, TLR4, TLR5 or TLR9 increased levels of GDNF, NGF, BDNF and LIF by intestinal muscle cells (Brun et al., 2015) and GDNF prominently supports the developing and mature ENS (Lake and Heuckeroth, 2013; Rodrigues et al., 2011). In contrast, TLR2-/- mice have reduced GDNF expression, altered intestinal motility, smaller ganglia and fewer neurons than wild type animals (Brun et al., 2013). NGF may also enhance neurite growth from enteric neurons (Dothel et al., 2015; Esteban et al., 1998) while BDNF supports enteric glia (Levanti et al., 2009). TLR2 can be activated by a wide range of bacterial, fungal and viral components and is expressed by enteric neurons and glia in addition to smooth muscle. Similarly, TLR3, TLR4, and TLR7 are expressed in the human ENS (Barajon et al., 2009). TLR4 recognizes lipopolysaccharide (LPS), a major gram negative bacteria cell wall component and TLR4-/- mice have reduced nitrergic neuron number in the colon myenteric plexus and delayed gastrointestinal motility (Anitha et al., 2012). This fits well with the observation that LPS enhances proliferation of ENS neural progenitor cells in culture and delays precursor differentiation (Schuster et al., 2014). Bacteria also release diverse neurotransmitters (e.g., GAGA, serotonin, acetylcholine) (Wall et al., 2014) and changes in ENS structure may reflect altered neuronal activity as discussed above. Similar mechanistic observations may underlie the increased galanin and calcitonin gene-related peptide (CGRP) immunoreactive submucosal neurons observed in piglets fed the probiotic Pediococcus acidilactici (di Giancamillo et al., 2010) or the reduced calbindin+ myenteric neurons in pig jejunum after feeding with Saccharomyces boulardii (Kamm et al., 2004).

Special mention should be made of breast milk, which provides not only a full spectrum of nutrients, but also many bioactive compound including neurotrophic factors (GDNF, BDNF, NT3, CNTF) and cytokines (tumor necrosis factor (TNF)-α, Interferon (IFN)-γ, RANTES, monocyte chemotactic protein (MCP)−1, MIP-1-a, IL-1, IL-6, IL-8, IL-10, ENA78, GRO-a, Leptin, IL-7 and IL-17) that could support enteric neurons and glia (Collado et al., 2015; Fichter et al., 2011). It is not clear if these compounds can get across the gut epithelium in the absence of injury, but at least during early postnatal stages the mucosal barrier partially permits transport of many macromolecules (Drozdowski et al., 2010). Interestingly, breast milk reduces occurrence of a deadly bowel disease of premature infants called necrotizing enterocolitis (NEC) (Good et al., 2014). Bowel injury during NEC should further increase translocation of neurotrophic factors and many other breast milk components (e.g., nitrite, L-arginine, glutamine, lactoferrin, or epithelial growth factors) across the epithelium where they may act in concert to prevent further injury and modulate bowel inflammation (Avetisyan et al., 2015b; Gershon, 2012; Savidge et al., 2007; Sigalet et al., 2007). The role of the ENS in NEC is underinvestigated, but NEC clearly causes injury to the ENS and transplantation of enteric neural crest-derived stem cells prevents death in an experimental NEC model (Zhou et al., 2013).

Collectively these data show that dietary components may alter the ratio of neuron subtypes in the bowel, a plausible way to permit adaptation to varied diets and intestinal microbes. These changes may be mediated by altered neuronal activity, dietary nutrients, their metabolites, or microbial products that regulate transcription, modulate signal transduction, or regulate neurotrophic factor production. Much more work is needed to define mechanisms of subtype specification in the ENS, and to establish how neuromodulatory medicines, food and microbes affect ENS development.

10. Even “simple” observations have complex implications for ENS development when non-genetic factors impact protein abundance (“a tale of GDNF”)

GDNF is an essential trophic factor for ENCDC during fetal development because GDNF activates the RET transmembrane tyrosine kinase via the co-receptor GFRα1 (Cacalano et al., 1998; Durbec et al., 1996; Enomoto et al., 1998; Heuckeroth et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Trupp et al., 1996). RET supports ENCDC survival, migration and proliferation during bowel colonization, but also enhances neuronal differentiation and neurite growth once cells stop dividing (Schafer and Mestres, 1999; Schuchardt et al., 1994; Vohra et al., 2007a). Because different enteric neuron subtypes exit the cell cycle at different stages of development (Bergner et al., 2014; Pham et al., 1991), the timing, location and intensity of GDNF production in the bowel affects enteric neuron subtype ratios. The location of GDNF production also affects patterning for NO producing neurites (Wang et al., 2010). Inactivating mutations in GDNF, RET or their co-receptor GFRα1 cause total intestinal aganglionosis (no neurons in the small bowel or colon) in mice. Furthermore, mice with GDNF heterozygosity have reduced enteric neuron number and abnormal bowel motility (Gianino et al., 2003). As might be expected, RET heterozygosity is a common cause of human HSCR (about 30% of all cases) producing about a 50% HSCR risk (i.e., partial penetrance) (Alves et al., 2013; Amiel et al., 2008), while homozygous RET inactivating mutations cause human total intestinal aganglionosis (Shimotake et al., 2001) similar to the murine phenotype. These observations powerfully suggest that altered RET activity as a result of changes in GDNF production should lead to changes in ENS structure and function during development and in adulthood, especially since GDNF appears to have a direct role in the peristaltic response (Grider et al., 2010).

Regulation of GDNF expression is remarkably complex (Fig. 2) (Saavedra et al., 2008) suggesting that some changes in ENS structure and function before and after birth might be induced by changes in GDNF in response to non-genetic signals. Regulators of GDNF expression in the bowel are incompletely understood, but in other tissues GDNF levels are influenced by many transcription factors (SIX1, SIX2, PAX3, EYA1, SALL1, HOX11, FOXC1, FOXC2, NF-κB, CREB), signaling proteins (MAPK, PKC, PKA, Ca^{++}, PP2A, SPROUTY1), neurotransmitters (glutamate, dopamine, adenosine, serotonin), extracellular ligands (GDF11, SLIT2, BMP4, EDN1, FGF2), pro-inflammatory cytokines (IL-1β, IL-6, TNFα, TNFβ, IL-10), bacterial products (LPS), lifestyle choices (calorie restriction, exercise), pharmacologic agents (dopamine receptor agonists, monoamine oxidase inhibitors, glutamate receptor antagonists, antidepressants, antipsychotics, mood stabilizers, anti-epileptics, antidementia drugs, immunophilin ligands), hormones (melatonin, estrogen), vitamin D3, and by some traditional medicines (Rehmannia glutinosa, Ibogaine, royal jelly). These GDNF regulatory mechanisms coupled with GDNF effects on ENS structure and function suggest that many non-genetic factors may affect the ENS simply by modifying GDNF levels in fetal or post-natal bowel.

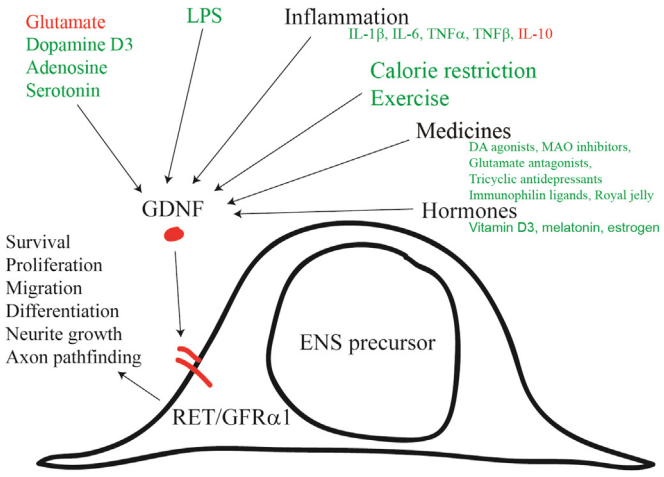

Fig. 2. GDNF/GFRα1/RET signaling impacts many aspects of ENS development and has important roles in the adult ENS. For this reason, the timing, location and intensity of GDNF signaling can profoundly alter ENS structure and function. Remarkably, many non-genetic factors impact GDNF abundance, although most of these effects have not been well studied in the fetal or adult bowel. Based primarily on work in other systems, green text indicates factors that increase GDNF mRNA or protein levels. Red text indicates that the factor reduces GDNF mRNA or protein levels.

11. Summary

The ENS is an elegant nervous system in the bowel wall that controls most aspects of bowel function. Because the developmental pathways needed to form the ENS are complicated, it is not surprising that many molecular mechanisms critically influence ENS precursor colonization of fetal bowel and the differentiation of enteric neurons and glia. While the underlying genetics provides the infrastructure for successful fetal morphogenesis, maternal health, placental function, maternal nutrition, and maternal medicines impact development changing the risk of structural birth defects. Hirschsprung disease seems particularly likely to be impacted by gene–environment interactions because in most children with HSCR the bowel is almost fully colonized by ENCDC, and even a small increase in bowel colonization could mean the difference between health and life threatening disease. These observations provide new hope that some cases of HSCR may be preventable and that we can take advantage of observed non-genetic mediators to enhance bowel function in children and adults with a wide range of bowel motility disorders.

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