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Development of Enteric Neurons and Muscularis Macrophages

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by

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ABSTRACT OF THE DISSERTATION

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Professor Robert O. Heuckeroth, Chair

Professor Aaron DiAntonio, Co-Chair

The enteric nervous system (ENS) is a complex interconnected network of neurons and glia in the bowel wall that regulates intestinal motility, blood flow, and epithelial function. The ENS also controls aspects of inflammatory signaling within the bowel. To perform these tasks, there are at least 20 types of enteric neuron and four types of enteric glia. Although much is known about early events in ENS development, signals governing the development of specific neuronal subtypes and communication with neighboring cell types within the bowel remain poorly understood. One fundamental hypothesis is that diverse trophic factors support distinct neuronal populations in the bowel.

Based on our observations that the hepatocyte growth factor (HGF) receptor *Met* is expressed in all ENS precursors and in a subset of adult enteric neurons, we investigated the role of HGF and MET in the ENS. We found that mice lacking functional MET receptor in the ENS (*Met* cKO mice) exhibit defects in a subset of CGRP-expressing sensory neurons in the myenteric plexus. These sensory cells, known as intrinsic primary afferent neurons (IPANs), are responsible for transmitting signals such as bowel stretch and villus deformation from the bowel

lumen to other myenteric neurons. *Met* cKO mice have altered CGRP-IR neurite patterning with a corresponding failure to trigger peristalsis in response to villus stroking, but a normal response to bowel stretch. This work suggests that MET is important for the development and function of a subset of IPANs the bowel.

HGF has also been known for more than a decade to protect the bowel from injury in colitis models. It had been assumed that HGF's protective effects were mediated by MET present on bowel epithelial cells. Using *Met* cKO mice that are missing MET in the ENS, but have normal MET in gut epithelium, we showed that neuronal HGF signaling contributes to the protective effects of HGF in the bowel that have been previously reported. *Met* cKO mice have significantly more bowel injury following treatment with dextran sodium sulfate (DSS) to induce colitis. Furthermore, these animals show reduced proliferation of epithelial cells in the context of injury. Together these findings suggest that that HGF/MET signaling is important for development and function of a subset IPANs and that these cells regulate intestinal motility and epithelial cell proliferation in response to bowel injury.

Our studies of *Met* cKO mice highlighted how defects in neurite patterning of enteric neurons can profoundly affect bowel function. Surprisingly little is known about factors that govern appropriate formation of neuronal connections in the bowel. To identify other cues important for enteric neuron axon patterning, we used broad microarray gene expression profiling. Gene expression in embryonic day 17.5 (E17.5) ENS cells was compared to gene expression in surrounding cells to identify genes encoding cell surface receptors or adhesion molecules enriched in the ENS that have known roles in axon pathfinding. We found that the semaphorin receptor Plexin-A4 was highly enriched in the ENS and confirmed this by *in situ hybridization* (ISH) at E17.5. Immunohistochemistry using a commercial antibody to Plexin-A4

suggested that Plexin-A4 was found in a subset of calretinin-IR neurons. However, staining of Plexin A4 knockout (Plexin A4 KO) bowel revealed this antibody to be non-specific. Despite testing two other Plexin-A4 antibodies, we were unable to determine which enteric neurons produce Plexin A4. We analyzed the ENS of Plexin A4 knockout (Plexin A4 KO) mice but could not identify any gross defects in neurite patterning. Additionally, functional analysis of Plexin A4 KO mice did not reveal any defects in the peristaltic reflex of these animals. As is true for other parts of the nervous system, it is likely that Plexin A4 acts redundantly with Plexin A2 (also enriched in the ENS) and any effects on axon pathfinding in the ENS would only be revealed in Plexin A4/Plexin A2 double knockout mice.

Remarkably, the non-specific Plexin A4 antibody we had been using also labeled a population of poorly characterized muscularis macrophages within the bowel muscularis externa, and we decided to study these cells. Bowel macrophages integrate a variety of environmental stimuli to assume either a pro-inflammatory or tissue-protective phenotype. A growing body of evidence suggests that neuronal cholinergic and noradrenergic signaling dampens the inflammatory phenotype of muscularis macrophages found in close contact with enteric neurons. Additionally, it's been suggested that enteric neurons produce CSF1, the main survival factor for muscularis macrophages. This would imply that these macrophages would be abnormal when the ENS is missing. Surprisingly, we found that muscularis macrophage colonization of the bowel precedes colonization by enteric neurons and that the main source of CSF1 during development is non-neuronal. Furthermore, *Ret* knockout mice that are completely missing enteric neurons in the small bowel and colon contain normal numbers of well patterned macrophages. Additionally, these macrophages do not differ in their expression of activating cell surface markers, or in their *ex-vivo* response to lipopolysaccharide (LPS) stimulation. These studies help clarify the role of

ENS in the homeostasis and activation of muscularis macrophages, suggesting that, at least developmentally, enteric neurons are dispensable for muscularis macrophage survival and do not alter the baseline inflammatory status of muscularis macrophages.

Chapter 1: Introduction

The enteric nervous system is the largest and most complex division of the peripheral and autonomic nervous system in vertebrates (Lake & Heuckeroth 2013; Obermayr et al. 2013). It contains various types of neurons and glia in numbers comparable to that found in the spinal cord, and an array of neurotransmitters similar to that seen in the central nervous system (CNS) (Obermayr et al. 2013). Such a complex array of cells and signals is necessary to mediate the many bowel functions controlled by the ENS. These functions include regulating motility, secretion, and blood flow. In addition, the ENS has also been recently implicated as a major regulator of immune signaling in the bowel. This occurs in part through communication with muscularis macrophages (Matteoli et al. 2014; Muller et al. 2014; Gabanyi et al. 2016). The work in this dissertation deals with several distinct aspects of enteric neuron development, and the impact that enteric neurons have on developing muscularis macrophages.

In Chapter 2, I review the stages of ENS development, beginning with delamination of neural crest cells and ending with functional neural circuit formation in the bowel. I also discuss various pathologies that arise as a result of disturbances in ENS development.

In Chapter 3, I define the role of hepatocyte growth factor (HGF) and its receptor MET in the development and function of a set of sensory neurons in the bowel. I demonstrate that mice lacking functional MET in the ENS have defects in MET-positive neurite formation, the peristaltic reflex, and epithelial repair in response to injury.

In Chapter 4, I describe our efforts to use gene expression profiling to identify axon guidance molecules with potential roles in wiring the ENS. Using this approach we determined that the classic semaphorin guidance cue receptor, *Plexin A4* was highly enriched in the ENS. I

also describe our findings that the ENS of *Plexin A4* KO mice appears grossly normal, and that these mice do not have defects in peristalsis.

In Chapter 5, I demonstrate that enteric neurons are dispensable for the development of neonatal muscularis macrophages. I also provide evidence that mechanisms governing the maintenance of muscularis macrophages in the steady state adult bowel differ from those regulating other macrophage populations in the bowel.

In Chapter 6, I discuss the implications of my work and propose future directions.

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Chapter 2: Building a Second Brain in the Bowel

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2.1 Abstract

The enteric nervous system (ENS) is sometimes called "The Second Brain" because of the diversity of neuronal cell types and complex integrated circuits that permit the ENS to autonomously regulate many processes in the bowel. Mechanisms supporting ENS development are intricate with numerous proteins, small molecules, and nutrients that impact ENS morphogenesis and mature function. Damage to the ENS or developmental defects cause vomiting, abdominal pain, constipation, growth failure and early death. Here we review molecular mechanisms and cellular processes that govern ENS development, identify areas where more investigation is needed, and discuss clinical implications of new basic research.

2.2 Introduction

Survival requires elegantly integrated mechanisms that control bowel motility, secretion, and blood flow to permit fluid and nutrient absorption and support waste elimination. If control of bowel function required conscious thought, there might be little else that we could do in life. Fortunately, the enteric nervous system (ENS) controls most aspects of bowel function (1, 2). The ENS is a complex network of neurons and glia that resides in the myenteric and submucosal plexus of the bowel. The myenteric plexus, located between longitudinal and circular muscle,

primarily controls muscle contraction and relaxation. The submucosal plexus, found between circular muscle and bowel mucosa, regulates fluid secretion and absorption, modulates blood flow, and responds to stimuli from epithelium and lumen to support bowel function.

In most people, the ENS works well, making it easy to forget that the bowel needs its own nervous system. However, ENS defects may underlie common problems like irritable bowel syndrome (IBS) (3) and less common problems like Hirschsprung disease (HSCR) (4, 5), chronic intestinal pseudoobstruction syndrome (CIPO) (6), or gastroparesis (7). HSCR is a life-threatening birth defect where the ENS is completely missing from distal bowel. In CIPO or gastroparesis, the ENS or other bowel cells are defective causing dysmotility, pain and difficulty maintaining enteral nutrition. In IBS, altered bowel motility and sensory responses cause pain accompanied by diarrhea or constipation, but health is not otherwise affected. ENS defects also occur in Parkinson's (8), diabetes (9), or inflammatory bowel disease (IBD) (10) and recent data suggest that ENS damage might play an early etiologic role in IBD (11, 12) and Parkinson's (13-15). Here we focus on cellular and molecular mechanisms controlling ENS development, highlighting areas that require further investigation and potential clinical implications of new discoveries.

2.3 ENS morphogenesis

The ENS forms from enteric neural crest-derived cells (ENCDC) that delaminate primarily from vagal neural tube with smaller contributions from sacral and upper thoracic neural tube (2, 16-18). Vagal ENCDC migrate through paraxial mesoderm before entering bowel and then migrate in a rostral to caudal direction to colonize the entire fetal bowel (Figure 2.1A). Vigorous ENCDC proliferation during migration is important for full bowel colonization (19, 20). ENCDC differentiate into at least 20 neuronal subtypes or enteric glia (21), form ganglia, extend neurites, and establish and refine functional neural circuits to control the bowel

(22) (Figure 2.1B). A subset of ENCDC undergoes radial migration either inward to form the submucosal plexus or out of the bowel to form ganglia in the pancreas. These complex processes require transcription factors, cell surface adhesion molecules, receptors, extracellular ligands, cytoskeletal rearrangements and diverse intracellular signaling molecules summarized in excellent recent reviews (1, 2, 16, 23-25).

2.4 Retinoids, RET, and bowel colonization

Migrating ENCDC are surrounded by regulatory molecules that guide development. One early critical interaction occurs as ENCDC migrate through paraxial mesoderm before invading foregut (E8.5 in mice, E2.5-3 in quail) (17, 26). During this transition, ENCDC begin to express RET in response to local retinoic acid (RA) synthesis by paraxial mesoderm (17) (Figure 2.2A). This is important because RET supports ENCDC survival, proliferation and migration (2, 27-33), and homozygous *Ret* inactivation prevents ENCDC from colonizing bowel distal to the stomach (34, 35). Exogenous RA can substitute for an otherwise essential paraxial mesoderm interaction in quail to induce RET (17). Mice deficient in retinaldehyde dehydrogenase 2 (RALDH2), an enzyme that makes RA, also fail to express *Ret* and have total intestinal aganglionosis (36).

RET is the signaling receptor for glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (37), ligands that bind preferentially to the co-receptors GFRA1, GFRA2, GFRA3, and GFRA4 respectively (38). GFRA1 and RET are co-expressed by migrating ENCDC, but GFRA1 is also produced by surrounding gut mesenchyme along with GDNF (39, 40). Like RET, GDNF and GFRA1 are required for ENCDC survival, proliferation and migration. Homozygous mutations in *Ret*, *Gdnf* or *Gfra1*

Figure 2.1

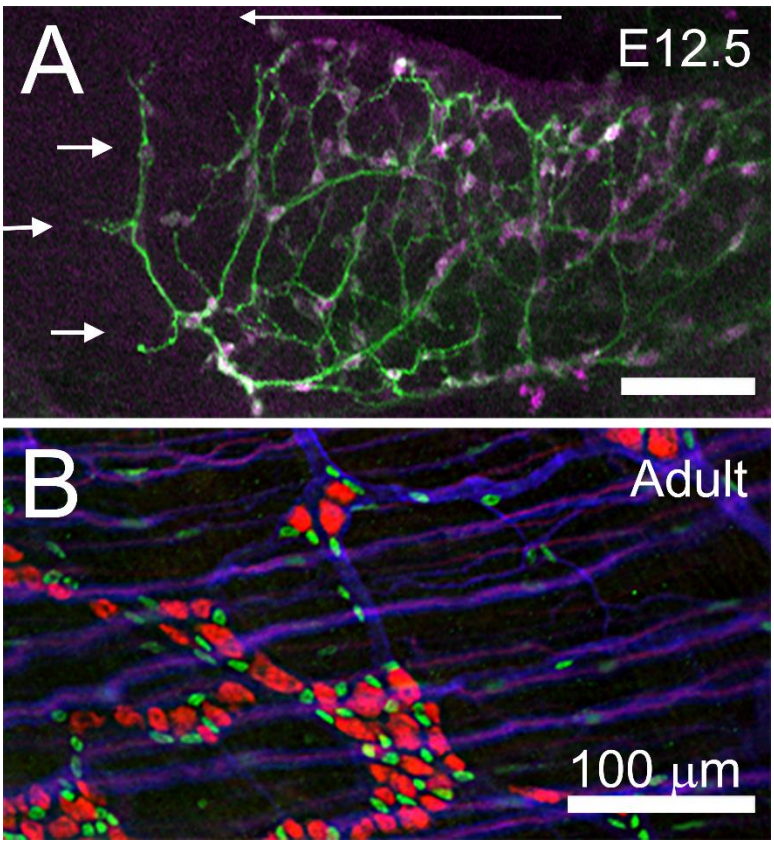

Figure 2.1. ENS Morphogenesis.

(A) Vagal ENCDC migrate in a rostral to caudal direction through fetal bowel (long white arrow). At E12.5 ENS precursors have migrated half way through fetal colon. ANNA-1 antibody binds HuC/D antigen and identifies enteric neurons (magenta cells) while TuJ1 binds neuron specific beta III tubulin and labels neurites (green). ENCDC migrate in chains though the bowel, but during the period of migration some precursors differentiate into neurons and extend neurites including at the migration wavefront (white arrows). (B) Adult small bowel myenteric plexus shown by ANNA-1 antibody (red neurons), SOX10 antibody (green enteric glia), and TuJ1 antibody (blue) demonstrates clusters of neurons and glia in mature ganglia as well as many neurites within and between ganglia. Scale bars = 100 microns.

Figure 2.2

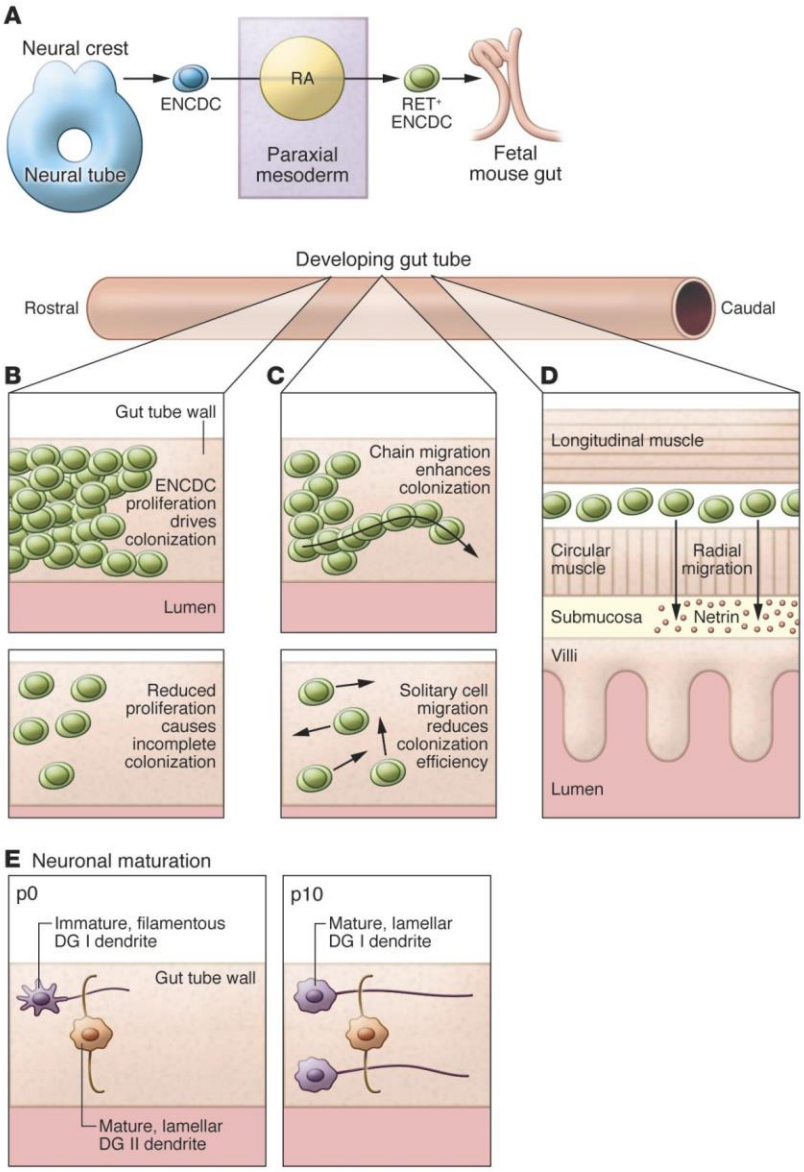

Figure 2.2 ENS Development.
(A) Murine vagal neural crest cells destined for the ENS delaminate from the neural tube at E8.5. These ENCDC are exposed to RA as they migrate by paraxial mesoderm on their way to the foregut at E9. (B) Once ENCDCs are in developing bowel, efficient caudal migration relies on vigorous ENCDC proliferation (top panel) as disorders that reduce ENCDC proliferation (bottom panel) commonly cause incomplete bowel colonization. (C) Efficient ENCDC migration is facilitated by contact between migrating cells. Chain migration of ENCDCs is quicker and more directed than migration of isolated ENCDC. Disorders that alter ENCDC cell adhesion also delay bowel colonization and may cause HSCR. (D) After ENCDCs have populated the whole developing bowel (E13.5 in mice) in the region of the future myenteric plexus, a subset of ENCDC migrates inward radially to form the submucosal plexus. Radial migration is regulated by RET-GDNF signaling axis and by Netrin/DCC chemoattraction. (E) nNOS-IR DG I neurons send caudal projections in the longitudinal axis, whereas CGRP-IR DG II neurons project circumferentially. Both DG I and DG II neurons are present at P0. However, only DGII neurons exhibit mature lamellar dendrites at this age, whereas most DG I dendrites are still filamentous. The proportion of DG I lamellar dendrites increases from P0-P10. DG II projections do not grow in length from P0-P10, whereas DG I projections do, though their growth rate does not match that of the bowel. There is significant maturation of the ENS after birth, at least in rodents.

cause indistinguishable ENS phenotypes in mice (34, 39, 41-44). *RET* mutations are also very common in people with HSCR (2, 16, 23, 45, 46).

2.5 Cell proliferation and bowel colonization

One long-standing mystery is why ENCDC colonize the entire fetal bowel since individual ENCDC appear to thrive anywhere along the bowel. The absence of essential intrinsic guidance cues for rostrocaudal migration is likely since ENCDC migrate in either direction through aganglionic bowel (47-49) and bypass a cecal zone with high concentrations of the chemoattractant GDNF (47). Theoretical modeling suggests that rostrocaudal bowel colonization is driven by ENCDC proliferation that causes competition for space and trophic factor (19) (Figure 2.2B). In vitro and in ovo data support this idea since reduced ENCDC proliferation or cell number may cause incomplete bowel colonization. For example, removal of vagal neural tube segments where ENCDC originate causes distal intestinal aganglionosis (50, 51). *RET* mutations that reduce ENCDC proliferation or enhance cell death also cause bowel aganglionosis (27, 28), as does treatment with mycophenolic acid, an inhibitor of guanine nucleotide synthesis that reduces ENCDC proliferation, but does not prevent ENCDC from migrating at appropriate doses (20). Finally, mutations in endothelin receptor B (EDNRB) or its ligand endothelin-3 (EDN3) cause premature differentiation and cell cycle exit (52-54) leading to HSCR in humans and distal bowel aganglionosis in mice (55, 56). Interestingly, *Ednrb* and *Edn3* deficient ENCDC can colonize distal bowel normally if accompanied by adequate numbers of WT ENCDC, proving they are able to migrate into distal bowel (57, 58). One corollary to the hypothesis that competition for "space" drives ENCDC migration is that ENCDC must be restricted to specific layers of the bowel. In part, sonic hedgehog from gut epithelium restricts ENCDC to the outer bowel wall during migration (59), but the mechanism underlying this observation is not understood.

2.6 Chain Migration and Cell Adhesion

Bowel colonization by ENCDC is enhanced by "chain migration", a process where ENCDC preferentially contact each other while migrating (60, 61) (Figure 2.2C). The importance of ENCDC cell-cell contact is suggested by human L1 cell adhesion molecule

(*L1CAM*) mutations that increase risk of HSCR, hydrocephalus and corpus callosum defects (62-64). *L1cam* mutations also increase "unattached" ENCDC and reduce bowel colonization in mice (62). Ex vivo imaging shows that ENCDCs in chains migrate more quickly through bowel and with more directional persistence than isolated ENCDC (48). We do not, however, understand why contact between ENCDC enhances bowel colonization. One hypothesis is that cells at the front of the migrating ENCDC chain "pull" trailing ENCDC via L1CAM-mediated adhesion. This seems unlikely since migrating ENCDC constantly change position moving over each other (47, 48). Another possibility is that ENCDC enhance bowel colonization by altering extracellular matrix (ECM) to enhance migration (65) or by degrading ECM to create spaces to migrate through. Consistent with this latter hypothesis, matrix metalloproteinase-2 (MMP2) inhibition slows ENCDC migration (66). Preferential ENCDC migration along neurites (48, 67) might also help cells navigate gut mesenchyme to preexisting gaps in the ECM. These hypotheses could explain why chain migration is more efficient than "isolated" ENCDC migration. Alternatively, isolated ENCDC may simply be "happy" to stay where they are since there is no competition for space or trophic factors, an idea espoused in the previous section. In either case, defining mechanisms supporting chain migration may provide new insight into human bowel motility disorders.

2.7 Intracellular signaling and Migration

Complex intracellular signaling controls cytoskeletal rearrangements and focal adhesions essential for ENCDC migration. Mechanisms must be dynamically regulated because cells at the ENCDC migration wavefront behave differently from cells behind the "wavefront". Recent elegant Förster resonance energy transfer (FRET) studies confirm that PKA, RAC1, and CDC42 are differentially activated in migrating ENCDC depending on proximity to the wavefront (68). These proteins regulate the mode of cell migration (69, 70), and the efficiency of bowel colonization. Interestingly, both PKA inhibition and exogenous cAMP analogs slow ENCDC migration in organ culture (68, 71), but critical PKA targets in this context are not known. This suggests that localized PKA activation or moderate activation is needed to support migration. One possible PKA target is RET, which is phosphorylated and activated to enhance migration (71). RET in turn activates many molecules including RAC1 (72-74), which orchestrates

cytoskeletal rearrangements to induce lamellipodia in cells migrating across two-dimensional substrates (75). RAC inhibition slows ENCDC migration in vitro and in ex vivo organ culture (68, 73, 76). Elevated RHOA, which often acts in opposition to RAC, also disrupts ENCDC chain migration and causes hypoganglionosis (77). Curiously, inhibition of ROCK, an effector of RHOA, enhances ENCDC migration in vitro on collagen, but reduces ENCDC migration through bowel in organ culture (76). These apparently conflicting data suggest that in vitro results may not mimic mechanisms needed for migration through the bowel in vivo because different migration modes are optimal for different contexts. Evaluating mechanisms of ENCDC migration in "natural" three dimensional environments like bowel wall may therefore provide new insight into how medicines, toxins, genetics, or disease affect the developing ENS.

Additional complexity arises because signaling molecules may have different roles in different developmental contexts. For example, RA from paraxial mesoderm induces RET in neural crest cells (NCCs) migrating from the neural tube to the foregut (17). In contrast, when ENCDC have almost fully colonized the embryonic mouse bowel (E12.5), RET levels do not appear altered by retinoic acid receptor (RAR) antagonists (74, 78). Instead, in distal bowel RA reduces phosphatase and tensin homolog (PTEN) in ENCDC at the migration wavefront to support migration. PTEN dephosphorylates phosphatidylinositol-3 phosphate (PIP3), a lipid that activates AKT and other proteins that, together with downstream effectors, orchestrate cytoskeletal rearrangements needed for migration, and promotes survival and proliferation (74). Interestingly, PTEN levels increase in ENCDC behind the migration wavefront despite the presence of RA and this increase in PTEN protein supports neuronal differentiation. RA also decreases SMURF1 ubiquitin ligase expression in axon tips of enteric neurons to reduce neurite growth (78). This appears to be a unique adaptation of developing enteric neurons that may facilitate migration. Thus, several RA regulated genes play critical roles in ENS development, but effects of RA are context dependent. Care is needed when evaluating protein function to consider the developmental context since the role of many proteins (e.g., RET, BMP4, Notch, Hand2, Shh) changes depending on developmental stage, concentration, and cellular target (2, 16, 23-25).

2.8 ENS morphogenesis does not end when ENCDC reach "The End"

During rostrocaudal bowel colonization in mice, ENCDC migrate in the location of the future myenteric plexus, an area rich in GDNF (79). Beginning at E14.5, some ENCDC migrate inward ("radial migration") to form the submucosal plexus, while at earlier stages other ENCDC migrate into the pancreas to form ganglia near the Islets of Langerhans (23, 79, 80) (Figure 2.2D). Radial migration inward to the submucosal plexus and outward toward the pancreas is regulated by the chemoattractant netrin, which is secreted by fetal gut epithelium and pancreas, and binds the receptor Deleted in Colon Cancer (DCC), expressed by a subset of ENCDC (79). Cells that move to the submucosal plexus also have less RET signaling than adjacent ENCDC (80). During radial migration, peri-muscular gut mesenchyme down-regulates *Gdnf* and mesenchyme closer to the lumen up-regulates *Gdnf* (80). This change in GDNF localization provides additional stimulus for ENCDC with low RET activity to migrate inward. How cells modulate levels of RET and how neighboring cells communicate to ensure that only a subset of cells leave the myenteric plexus is unknown.

As rostrocaudal migration nears completion, ENCDC aggregate to become ganglia. Differential adhesion is important (81) and mediated at least in part by neural cell adhesion molecule 1 (NCAM1) (82, 83). Polysialic acid (PSA) addition to NCAM1 in response to mesenchyme-derived bone morphogenetic protein 4 (BMP4) further promotes aggregation and reduces ENCDC migration. PSA-NCAM1 is not abundant in ENCDC until gangliogenesis begins and blocking PSA addition reduces ganglia formation (83). Some migratory ability is necessary for ganglia organization and may explain why mice lacking □1 integrin, a receptor that binds ECM, have disorganized ganglia (84, 85). A more severe defect occurs in mice with conditional inactivation of the transcription factor *Hand2* in ENCDC where myenteric ganglia fail to form completely (86). *Hand2* mutations reduce NCAM1, among many other defects. For example, HAND2 is needed for terminal differentiation of enteric neurons (12, 87, 88) and this more global effect on neurogenesis may underlie the effect of *Hand2* mutations on ganglion formation and bowel motility. Additional work is needed to identify HAND2 targets and to define mechanisms controlling enteric ganglia formation.

2.9 Neuronal subtype specification

Enteric ganglia contain at least 20 distinct neuronal subtypes that differ in function, transmitters, neurite patterning and electrophysiology (21). Undoubtedly diverse trophic factors, morphogens, and transcriptional regulators influence enteric neuron subtype specification (summarized in Table 2.1), supporting lineage commitment and selective cell proliferation. However, mechanisms determining enteric neuron subtype are barely understood and a genetic blueprint for specifying a single type of enteric neuron does not yet exist. Birth-dating studies show that progenitors for neuronal subtypes exit the cell cycle at different times throughout development (89-91). The observation that factors influencing ENCDC proliferation (e.g., GDNF) or differentiation (BMPs) may alter the ratio of enteric neuron subtypes within the bowel in a way that depends on "birth date" suggests genetically programmed lineage commitment tied to the timing of cell cycle exit (28, 32, 92, 93). Cell fate, however, is determined by a combination of extrinsic signals from a cell's microenvironment and the intrinsic transcriptional programming that renders the cell competent to selectively respond to some extracellular signals. It is assumed that over time pluripotency of enteric neural precursors becomes increasingly restricted, resulting in greater lineage commitment. Reconstructing lineage relationships among enteric neuron subtypes, however, or defining events that restrict cell lineage is challenging with currently available data. Figure 2.3 provides one model for enteric neuron subtype lineage relationships reflecting recent data about transiently catecholaminergic (TC) cells. TC ENS precursors arise early in development (89) and absolutely require the transcription factor ASCL1/MASH1 (94). Serotonergic neurons, but not other subtypes, also require ASCL1/MASH1 and were thought to arise exclusively from TC precursors. Fate mapping studies of tyrosine hydroxylase expressing cells now suggest that TC precursors give rise to only 30% of serotonergic neurons, but also can become calbindin, calretinin, and neurofilament-M expressing neurons (95). Importantly, TC-precursors give rise to less than 3% of myenteric neurons and 13% of submucosal neurons in the mouse distal small intestine. It is unclear if TC precursor-derived serotonergic neurons differ from non-TC-derived serotonergic neurons. The complexity of these data highlights how little we understand enteric neuron subtype specification.

Table 2.1. Neuronal subtype specification. Changes in expression levels for these proteins have been shown to alter the abundance of specific subtypes of enteric neurons in vivo.

Molecule	Experimental Manipulation	Effect on Enteric Neuron Subtypes	References
GDNF (glial cell derived neurotrophic factor)	Over-expression in late development (E17) under glial *GFAP* promoter	Increased nNOS-IR neuron number No change in ChAT neuron number	(32)
RET	1. *Ret-/-*	1. More dramatic loss of NOS neurons than other neurons in esophagus	(93)
	2. Conditional loss of *Ret* plus rescue by *Bcl-xL* expression	2. Striking loss of NOS neurons in colon	(28)
BMP	Over expression of *BMP4* under neuronal NSE promoter	1. Increased TrkC-IR, NT3-IR, nNOS-IR neurons	(90)
NT3 (neurotrophin 3)	Loss of *NT3* or the NT3 receptor, *TrkC*	1. Decreased neuron number. 2. Preferential decrease in submucosal CGRP-IR neurons	(132)
NT3	Over expression of *NT3* under dopaminergic *DBH* promoter	Increase in neuronal number	(132)
EDN3 (endothelin 3)	Loss of the *EDN3* or receptor, *EDNRB*	1. Increased proportion of nNOS-IR and VIP-IR neurons 2. Decreased proportion of ChAT-IR neurons 3. Region specific changes in proportion of Substance P-IR, NPY-IR, galanin-IR, PACAP-IR and VIP-IR neurons	(133, 134)
Ascl1/Mash1	Loss of *Ascl1/Mash1*	1. Decreased neuron number 2. Reduced esophageal neuron number 3. Loss of transiently catecholaminergic neurons 4. Loss of 5HT-IR neurons	(94, 135)

Hand2	Loss of *Hand2*	Loss of or reduced nNOS/VIP-IR, reduced ChAT-IR, calretinin-IR, DAT-IR neurons	(86, 88)
5HT (5- hydroxytryptophan/serotonin)	Loss of neuronal 5-HT synthesizing enzyme *TPH2*	Decreased GABA-IR, DAT-IR neurons	(97)
NET (norepinephrine transporter)	Loss of *NET*	Decreased 5HT-IR, calretinin-IR neurons	(98)

Figure 2.3

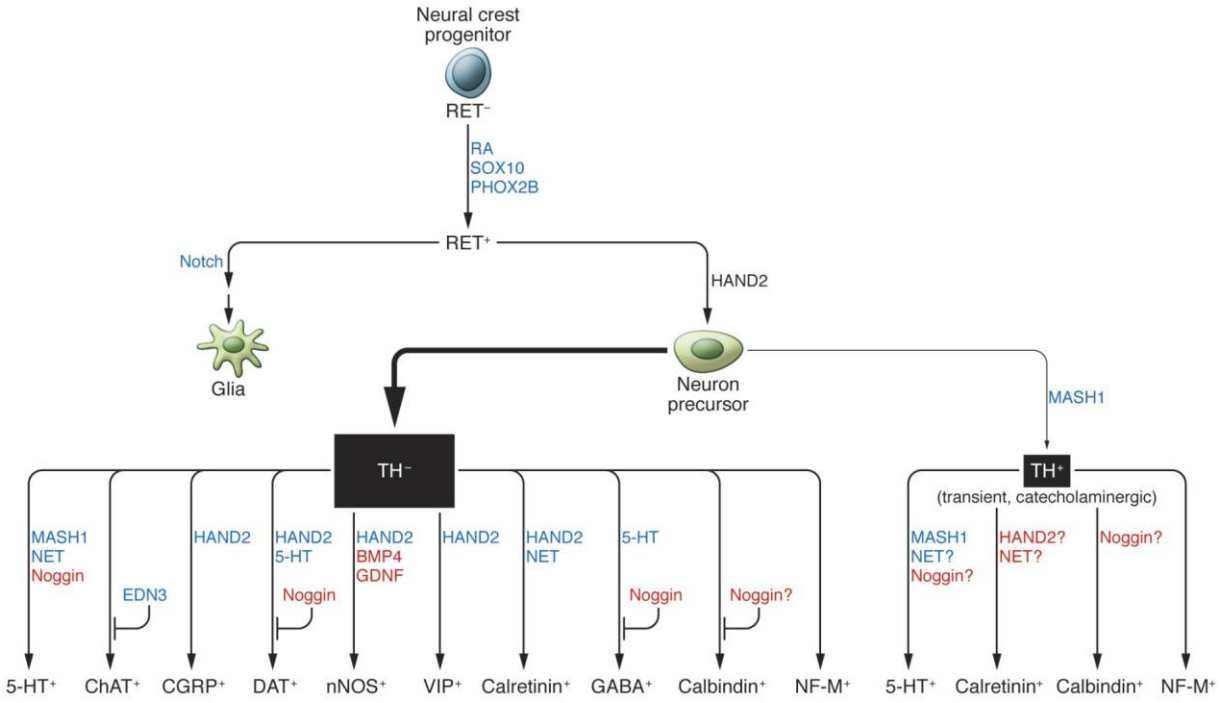

Figure 2.3 ENS precursor lineage relationships and neuronal subtype specification. Lineage relationships among enteric neuron subtypes remain poorly understood. This figure summarizes in vivo observations. Transcription factors are in italics. Gain of function data are indicated in red. Loss of function data are indicated in blue. Most myenteric neurons arise from TH negative precursors, as indicated by the relative thickness of arrows.

One additional critical question is how ratios of enteric neuron subtypes are controlled to produce integrated circuits and ganglia with diverse neuron types. If proliferating ENCDC are lineage restricted early, establishment of ganglia with multiple neuron subtypes would require segregation and organization of committed precursors (96). An alternative mechanism that could support this outcome would be if signals from one neuron affected the phenotype of other neurons. In vivo data support this hypothesis since loss of neuronal serotonin causes selective decreases in "late-born" dopaminergic and GABAergic neurons (97). Additionally, the norepinephrine reuptake transporter (NET) supports development of serotonergic and calretinin-immunoreactive (IR) neurons (98). Neural activity also influences differentiation since hindgut explants treated with tetrodotoxin or tetanus toxin have fewer nitrergic neurons, but normal total neuron number (99). In contrast, depolarization of ENS precursors in culture increases tyrosine hydroxylase- and vasoactive intestinal peptide (VIP)-expressing neurons, but not nitrergic neurons (100). Signals from the ECM might also direct subtype differentiation. Culture of ENS precursors on collagen IV leads to more nNOS-IR neurons than growth on laminin or heparin sulfate (101). Mechanisms underlying this observation are not known.

Remarkably, enteric neuron subtype plasticity appears to continue after birth, at least in rodents, where close proximity of the developing ENS to bowel lumen facilitates diet and intestinal microbe-induced changes in neuronal cell fate (102). In rats, the percentage of ChAT-IR neurons in colon myenteric plexus increases from P14 to P36, whereas the percentage of nNOS-IR neurons increases from P1 to P5, and then remains unchanged to P36 (103) . These ratios can be altered by intraluminal lipids. Rats given daily (P7-P17) colon enemas with the short-chain fatty acid butyrate have a larger percentage of myenteric and submucosal nNOS-IR and myenteric ChAT-IR neurons than controls at P21 (104). Butyrate treated rats also had increased nitrergic and cholinergic neurotransmission and slowed colonic transit. Piglets whose mothers were fed n-3 polyunsaturated fatty acids during pregnancy and lactation also had increased ChAT-IR and decreased VIP-IR neurons in the jejunal submucosal plexus (105). These data suggest that maternal dietary factors can affect ENS subtype ratios in offspring during pregnancy or via altered breast milk composition. Thus, neuronal subtype specification appears to depend on a combination of intrinsic genetic programming and environmental factors. The duration of plasticity is not known, but may underlie some human intestinal motility disorders and if better understood might suggest novel treatment strategies.

2.10 Axon pathfinding and synaptogenesis: A tangled web of neurites

Enteric neurons must extend processes to communicate with diverse cell types including other enteric neurons, interstitial cells of Cajal, smooth muscle, endothelial cells, mucosal epithelia, and intestinal glands (21). It is not clear if subtype identity is established before enteric neurons innervate targets or what guides axons. This work is challenging because single enteric ganglia contain heterogeneous neuron subtypes and fasciculated fiber tracts have diverse axon types. Nonetheless, axons of different enteric neuron subtypes clearly have stereotypic projection patterns. For example, intracellular dye filling after electrophysiologic recording from guinea pig intrinsic primary afferent neurons (IPANs) shows projections circumferentially around bowel and then into mucosa (106). Studies using myotomy and myectomy followed by immunohistochemistry demonstrate that excitatory motor neuron axons project rostrally, while inhibitory motor neurons project caudally within the longitudinal axis of the myenteric plexus before entering circular or longitudinal muscle (106, 107). Elegant models of enteric neuron circuitry now exist based on decades of work (1, 22, 108-110), but mechanisms needed to establish these connections remain obscure.

A few mouse mutations provide insight into axon guidance mechanisms. Mutations in the RET ligand neurturin or its preferred co-receptor GFR□2 reduce substance P-IR, but not VIP-IR, fibers innervating bowel circular muscle (111, 112). This phenotype could occur because of reduced neurite growth or branching, or might reflect problems with axon targeting to circular muscle. More convincing evidence that RET ligands direct neurite growth comes from mice that ectopically express GDNF from enteric glia. In these animals nitrergic, but not serotonergic or cholinergic fibers, redistribute towards the source of GDNF, suggesting a chemoattractive effect of GDNF on nitrergic axons (113). It is not known if GDNF-dependent chemoattraction is direct or if GDNF induces production of new guidance receptors, rendering nitrergic neurons competent to respond to new attractant cues. Non-neurotrophic factors important for regulating ENS connectivity were also recently identified. Elegant analysis showed that mice with disruption of planar cell polarity genes *Fzd3* and *Celsr3* have serious defects in axon pathfinding, but these were only seen with DiI or by expressing fluorescent proteins in small numbers of

enteric neurons. These data highlight the utility of single cell labeling techniques for identifying axon pathfinding defects in the ENS (114). Importantly, *Celsr3*$^{-/-}$ mice have severe dysmotility, increased whole gut transit time, and disorganized colonic migrating motor complexes, but major neuronal subtypes are present and enteric neuron number is normal, suggesting that difficult to recognize innervation patterning defects may seriously affect bowel motility.

After pathfinding, enteric neurons must form appropriate synaptic connections within the ENS. Although spontaneous neural activity and synaptic markers are present at E11.5 (115), electrophysiologic maturity is not reached until after birth suggesting ongoing refinement of neural networks. Based on morphology and electrophysiology, mature enteric neurons are classified as Dogiel Type I (DG I) or Dogiel Type II (DG II) (116) (Figure 2.2E). DG I neurons have a single axon, fast excitatory post-synaptic potentials, somas with lamellar dendrites, and include excitatory and inhibitory motor neurons (22). DG II neurons have multiple axon-like processes, prominent after-hyperpolarizing potentials, smooth somas without lamellar dendrites, and include IPANs. Although both DG I and DG II neurons are present by P0 in mice, most DG I neurons possess filamentous instead of lamellar dendritic projections. The proportion of neurons with lamellar dendrites increases until adulthood (117). Furthermore, while the length of DG II circumferential projections remains constant despite dramatic increases in bowel circumference from P0 to adulthood, DG I longitudinal axons continue to grow, though slower than the bowel. In contrast, DG I neurons have mature electrophysiologic properties by P10, while DG II neuron electrophysiologic properties differ from adults at P10. These studies suggest that synaptic development, refinement, and remodeling occur postnatally in growing bowel and support the hypothesis that environmental disturbances in early life (infection, psychological stress, etc.) could profoundly influence adult bowel motility (118) by altering enteric neuron anatomy or subtype specification.

2.11 Clinical implications

Mechanisms controlling ENS development and function are complicated. Hundreds of genes control precursor survival, proliferation, migration, differentiation, subtype specification, polarity, neurite growth, axon pathfinding and synaptogenesis. Tremendous basic research

progress provides new insight, but advances have had little impact on care provided for people with motility disorders. Actionable ideas follow naturally from current data.

1. Vitamin A deficiency, a common global problem (119), might be a preventable cause of some birth defects including Hirschsprung disease. Human studies in high risk populations are needed to test this hypothesis. Note: Vitamin A excess also causes birth defects (120) so high doses must be avoided in pregnancy.
2. Some medicines cause Hirschsprung-like disease in mice (20). Human epidemiologic studies identifying non-genetic risks for Hirschsprung disease may lead to new prevention strategies.
3. Current clinical evaluation of the ENS is often limited to "present" or "absent" even when manometry suggests enteric neuropathy underlies life threatening dysmotility. We need to evaluate neuronal subtypes and track neurites in biopsies from people with bowel motility disorders. New 3D-visualization approaches make this possible (121, 122). We also need age and region specific control data to know what ENS anatomy should be considered normal (123).
4. We need to incorporate "next-generation" genetic analysis into clinical testing for people with severe motility disorders. This will permit development of novel individually targeted therapies.
5. Post-natal gut-derived neural stem cells can now be harvested from humans, cultured, and transplanted into animal models where they partially restore function or improve outcome (124-130). This provides new hope that autotransplantation and gene editing can be used to treat bowel motility disorders and perhaps other serious medical problems. More work is needed to define how to grow and direct differentiation of these stem cells, although recent studies suggest that the gut environment might induce differentiation of many types of functioning enteric neurons and that these stem cells can integrate into existing ENS circuitry (131).

We remain optimistic that as mechanisms controlling bowel motility are better defined, new approaches to treatment, prevention, diagnosis and cure will be developed to help people with serious bowel motility disorders.

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Chapter 3: HGF and Met support mouse enteric nervous system development, the peristaltic response, and intestinal epithelial proliferation in response to injury.

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3.1 Abstract

Factors providing trophic support to diverse enteric neuron subtypes remain poorly understood. We tested the hypothesis that hepatocyte growth factor (HGF) and the HGF receptor MET might support some types of enteric neurons. HGF and MET are expressed in fetal and adult enteric nervous system (ENS). In vitro, HGF increased enteric neuron differentiation and neurite length, but only if vanishingly small amounts (1 pg/mL) of glial cell line-derived neurotrophic factor (GDNF) were included in culture media. HGF effects were blocked by phosphatidylinositol-3 kinase (PI3K) inhibitor and by MET blocking antibody. Both of these inhibitors and MEK inhibition reduced neurite length. In adult mice, MET was restricted to a subset of calcitonin gene related peptide (CGRP) immunoreactive (IR) myenteric plexus neurons thought to be intrinsic primary afferent neurons (IPANs). Conditional MET kinase domain inactivation (Metfl/fl; Wnt1Cre+) caused a dramatic loss of myenteric plexus MET-IR neurites and DiI labeling suggested reduced MET-IR neurite length. In vitro, Metfl/fl;

Wnt1Cre+ mouse bowel had markedly reduced peristalsis in response to mucosal deformation, but normal response to radial muscle stretch. However, whole bowel transit, small bowel transit, and colonic bead expulsion, were normal in Metfl/fl; Wnt1Cre+ mice. Finally, Metfl/fl; Wnt1Cre+ mice had more bowel injury and reduced epithelial cell proliferation compared to WT animals after dextran sodium sulfate (DSS) treatment. These results suggest that HGF/MET signaling is important for development and function of a subset IPANs and that these cells regulate intestinal motility and epithelial cell proliferation in response to bowel injury.

3.2 Introduction

Survival depends on controlled intestinal motility to mix food with digestive enzymes, bring nutrients into contact with gut epithelium, eliminate waste, and facilitate fluid reabsorption. This requires neuronal networks that sense stretch, villus distortion, and luminal content composition, and then alter motility to suit constantly changing conditions. Fortunately, this occurs without conscious thought because the bowel has an intrinsic nervous system called the enteric nervous system (ENS) that controls most aspects of intestinal function (Bornstein et al., 2004; Grundy and Schemann, 2005; Wood, 2008; Furness, 2012; Goldstein et al., 2012; Sasselli et al., 2012). The ENS contains at least 20 neuron subtypes that differ in function, neurotransmitters, axonal projections and electrophysiology (Furness, 2006b). Some signals governing ENS development and maintenance are known (Obermayr et al., 2012; Sasselli et al., 2012; Lake and Heuckeroth, 2013), but it is unclear how diverse neuronal populations are established or what factors support most enteric neurons after birth. Trophic factors that affect ENS development, maintenance and function include (GDNF) (Moore et al., 1996; Sanchez et

al., 1996; Treanor et al., 1996; Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998), neurturin (Heuckeroth et al., 1998; Heuckeroth et al., 1999), nerve growth factor (NGF) (Mulholland et al., 1994), brain derived neurotrophic factor (BDNF) (Grider et al., 1997b; Boesmans et al., 2008), ciliary neurotrophic factor (CNTF) (Grider et al., 1997a; Chalazonitis et al., 2001; Schafer et al., 2003), and neurotrophin-3 (NT3) (Chalazonitis et al., 1994a; Chalazonitis et al., 1998; Chalazonitis et al., 2001). We hypothesized, that hepatocyte growth factor (HGF) and its receptor MET might also be important because HGF supports spinal motor neurons (Ebens et al., 1996a), dorsal root ganglion (DRG) subtypes (Maina et al., 1997), retinal ganglion cells (Tonges et al., 2011), and hippocampal neurons (Lim and Walikonis, 2008). Our prior studies also suggested HGF expression in the ENS (Vohra et al., 2006). Finally, we were intrigued by the protective effect of HGF in rodent colitis models (Tahara et al., 2003; Mukoyama et al., 2005; Numata et al., 2005; Oh et al., 2005; Hanawa et al., 2006; Kanbe et al., 2006), and hypothesized this might be mediated through MET expressing enteric neurons.

We now demonstrate MET immunoreactivity in most ENS precursors and in a subset of adult calcitonin gene related peptide (CGRP) expressing myenteric neurons thought to be intrinsic primary afferent neurons (IPANs, i.e., sensory neurons) (Furness et al., 2004a; Furness et al., 2004b). *In vitro*, HGF/MET signaling influences ENS precursor neurite growth and neuronal differentiation, but conditional *Met* null mutations driven by *Wnt1Cre* (i.e., $Met^{fl/fl}$; *Wnt1Cre+*; called "*Met cKO*") did not cause major ENS developmental defects. *Met cKO* mice had a normal density of MET-IR myenteric neurons, but fewer or shorter MET-IR neurites. *Met cKO* mice also had a specific defect in the peristaltic response elicited by mechanical deformation of intestinal villi, however, *in vivo* tests of bowel motility were unaltered. Finally, *Met cKO* mice had increased susceptibility to dextran sodium sulfate (DSS) induced mucosal

damage, suggesting that CGRP expressing enteric neurons protect the bowel from injury and that HGF's ability to protect the bowel might depend on signaling within the ENS.

3.3 Materials and Methods

3.3.1 Animals.

c-Met$^{fl/WT}$ mice (129SV/C57BL/6 background) (Huh et al., 2004) were generously provided by Dr. Snorri S. Thorgeirsson (NCI, NIH, Bethesda, MD). *Wnt1Cre* mice (*STOCK Tg(Wnt1-Cre)11Rth Tg(Wnt1-GAL4)11Rth/J, stock # 003829,* C57BL/6; Swiss albino mixed background) and *R26R-EYFP* reporter mice (*B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J, Stock # 006148, C57BL/6J*) were from The Jackson Lab. *RET-EGFP* mice were previously described (Jain et al., 2006). *Etv5*$^{M/M}$ and *Etv4*$^{-/-}$;*Etv5*$^{lacZ/WT}$ mice were kindly provided by Dr. Kenneth Murphy (Washington University School of Medicine in St. Louis, MO) and Dr. Silvia Arber (University of Basel, Switzerland). CF-1 mice were from Charles River. The morning of vaginal plug was considered embryonic day 0.5 (E0.5). The use and care of mice were accredited and approved by the Washington University Animal Care Committee and by The Children's Hospital of Philadelphia Research Institute Institutional Animal Care and Use Committee.

3.3.2 Antibodies and reagents.

Primary antibodies for mouse analysis: p75NTR antibody (rabbit, 1:1000, #G323A, Promega), Choline acetyltransferases (Chat, goat, 1:10, #AB144P, Millipore), calretinin (rabbit, 1:2500, #AB5054, Chemicon), HGF (goat, 1:100, #sc-1357, Santa Cruz), HuC/D (mouse, 1:200, #A21272, Invitrogen), GFP (chicken, 1:1000, #GFP-1020, Aves Labs), S100B (rabbit, 1:800,

DAKO), PGP9.5 (guinea pig, 1:100, #GP14104, Neuromics), TuJ1 (rabbit, #PRB-435P, 1:10 000, Covance) Tuj1 (mouse, #MMS-410P, 1:100, Covance), RET (goat, 1:200, #GT15002, 1:100, Neuromics), MET (goat, AF527, 1:100, R&D Systems), CGRP (rabbit, #C8198, 1:100, Sigma), phosphohistone 3 (pH3) (rabbit AB#06-570, 1:800, Upstate), nNOS (rabbit, #AB 5380, 1:1000, Chemicon), substance P (rabbit, 1:1000, Inestar), VIP (rabbit, 1:1000, Peninsula), NF145 (rabbit, #ab1987, 1:100, Millipore). Primary antibodies for human gut tissue: PGP9.5 (rabbit, #7863-0504, 1:100, Serotec), c-MET (goat, #AF276, 1:100, R&D Systems). Secondary antibodies: donkey anti-goat Alexa 594 (1:400, Molecular Probes), donkey anti-rabbit Alexa 488 (1:400, Molecular Probes), donkey anti-mouse Alexa 647 (1:400, Molecular Probes). Tissue culture reagents included GDNF (Creedon et al., 1997), HGF (mouse, #2207-HG, R&D Systems), Neurobasal media (Life Technologies), B27 (Life Technologies), Dulbecco's Modified Eagle Medium (DMEM), glutamine (Fisher), penicillin and streptomycin (Fisher). Inhibitors: PD98059 (MEK1 inhibitor, #EI360-0005, Enzo life) and LY294002 (PI3K inhibitor, #ST420-0005 Enzo Life Sciences).

3.3.3 Quantitative ENS analysis.

Whole mount myenteric plexus analysis was performed using 8-12 weeks old mice (n=3-6) as described (Wang et al., 2010). Briefly, gut was opened along the mesenteric border, pinned to Sylgard®, fixed (4% paraformaldehyde (PFA), 30 minutes, 25^{o}C), and then dissected to separate muscle layers from submucosa. After immunohistochemistry or NADPH diaphorase staining, quantitative analysis was performed. For CGRP antibody staining, peeled gut muscle layers were cultured with colchicine (0.1 mg/mL, C9754, Sigma), DMEM, glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 ug/mL) for 24 hours before fixation. Neuronal

density was quantified by counting cells within twenty randomly selected 20x-fields per mouse. At least 3 mice of each genotype were analyzed.

3.3.4 Immunohistochemistry and image processing.

After fixation, cells, organs or peeled gut muscle layers were kept in TBST (100 mM Tris, 150 mM NaCl, 0.5% Triton X-100) for 30 minutes at 37°C, blocked with 5% donkey serum/TBST (30 minutes, 37°C) and then incubated with primary antibody (overnight, 4°C). Images were obtained with an Olympus BX60 microscope, Axiocam and AxioVision software (Zeiss) or with Zeiss Axio Imager.A2, AxioCam MRm Rev.3 Camera and ZEN software. Image processing included only cropping and uniform adjustments of brightness, contrast and saturation.

3.3.5 Human gut.

Paraformaldehyde-fixed, paraffin-embedded human colon was obtained from the Washington University Digestive Disease Research Core Center (DDRCC) after approval from the Institutional Review Board at Washington University School of Medicine. Five micron sections were deparaffinized and rehydrated for immunohistochemistry.

3.3.6 DiI Labeling combined with immunohistochemistry.

Adult mouse bowel was dissected, fixed, and peeled as for quantitative whole mount analysis. Muscle layers from distal small intestine were cut into 3 cm long pieces and pinned out on a Sylgard® dish. A dissecting pin dipped in NeuroTrace® DiI Tissue-Labeling Paste (#N-22880, Life Technologies) was inserted into the middle of each tissue piece. Pierced samples were kept in 4% PFA at 37ºC for three weeks. Immunohistochemistry for MET was performed as described above, except that instead of Triton X-100, 1000 ug/mL digitonin (#D141 Sigma) was used to permeabilize tissue while preserving DiI staining (Matsubayashi et al., 2008). For cell counting, tissue pieces were evaluated using a 5 X 7 grid of 20X fields centered on the pin

insertion site. The grid was additionally subdivided into 3 zones of varying distances from the pin (Figure 3.5) and cells within each 20X field were counted.

3.3.7 Dissociated cell culture.

E12.5 CF-1 ENS precursor cells from dissociated bowel were immunoselected with p75NTR antibody (1:1000) and maintained in culture as previously described (Sato and Heuckeroth, 2008) except that GDNF was not included in media for cell dissociation or immunoselection. Briefly, whole bowel was treated with collagenase (0.5 mg/mL) and dispase (0.5 mg/mL), triturated and filtered through a 40 um cell strainer before incubation with $p75^{NTR}$ antibody (Chemicon) and goat anti-rabbit coupled paramagnetic beads (Miltenyi Biotec, GmbH). After separation of p75NTR expressing cells using a MACS Separation column (Miltenyi Biotec, GmbH), immunoselected cells were plated at 6000 cells/well on poly-D-lysine and laminin coated 8-well chamber slides. Cells were cultured in Neurobasal media supplemented with B27 (2%), glutamine (2mM), penicillin (100IU/mL), and streptomycin (100 ug/mL) for 48 hours before fixation with 4% paraformaldehyde and analysis by immunohistochemistry.

3.3.8 Slice culture.

E12.5 CF-1 gut slice cultures were performed as described (Fu et al., 2006) with minor modifications. Briefly, 300-500 um long small bowel slices were cultured on fibronectin-coated plastic chamber slides (Nunc™ Lab-Tek™, Thermo Scientic) in DMEM, B27 (2%), glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 ug/mL). Immediately after plating, slices were treated with PBS (vehicle), HGF, or GDNF at the indicated concentration for 24 hours before fixation (4% PFA, 15 minutes, 25^{o}C) and processing for immunohistochemistry. For analysis, the distance from the edge of the explant to the most distant TuJ1+ neurites or RET+ cells was determined in at least 3 and up to 8 regions per explant.

3.3.9 In vitro peristaltic response.

The colon of adult mice was opened along mesenteric attachments to form flat sheets and pinned mucosal side up in a three-chambered organ bath as previously described (Grider and Jin, 1994; Grider et al., 2010). Force-displacement transducers were attached to the circular muscle to record ascending contraction in the orad peripheral compartment and descending relaxation in the caudad compartment. A sensory stimulus that initiates the peristaltic reflex was applied to the bowel in the central chamber. We used a hook-and-pulley system to produce graded (2-8 grams) radial stretch of the circular muscle layer to test the stretch-activated sensory pathway. Mechanical deformation of villi was tested using graded mucosal stroking with a fine brush to stimulate the mucosal-activated pathway.

3.3.10 Whole Gastrointestinal Transit Assay.

Adult mice were fed by intragastric gavage with 300 µL of 6% carmine red dye solution (#C1022 Sigma) dissolved in distilled water containing 0.5% methylcellulose (#274429 Sigma). Mice then were placed into individual cages without bedding. A white sheet of paper covered the cage bottom to facilitate detection of carmine in fecal pellets. Following gavage, cage bottoms were checked for dyed fecal pellets at 10 minute intervals. Each mouse was tested three times with at least three days between tests.

3.3.11 Colon Motility Assay.

After adult mice were anesthetized with isoflurane, a fire polished glass rod (3 mm in diameter, custom made by University of Pennsylvania Glass Shop) was used to insert a glass bead (3 mm in diameter, #Z143928, Sigma) into the rectum, 2 cm from the anal verge. The glass rod and beads were lubricated with sterile corn oil (#C8267 Sigma) prior to insertion. The time required

to eject the bead was measured as an estimate of colonic motility. Each mouse was tested three times with at least one day between trials.

3.3.12 Small Intestine Transit Assay.

Adult mice were fasted overnight and then fed by intragastic gavage with 100 uL of 10 mg/mL fluorescein isothiocyanate-dextran (FITC-dextran, average molecular weight 70,000, #46945 Sigma) dissolved in distilled water containing 2% methylcellulose. Animals were euthanized 90 minutes later and the stomach, small intestine, cecum, and colon were collected in 1X PBS. Small intestine was divided into 10 segments, cecum into 2 segments, and colon into 3 segments. Each segment was opened along the mesenteric border without losing luminal content and placed into an individual 1mL Eppendorf tube containing 500 uL 1X PBS. Tubes were vortexed 15 seconds and then centrifuged (2000 x g, 10 minutes) to obtain FITC-dextran containing supernatant. FITC fluorescence was measured in 100 uL aliquots of supernatant in a 96-well plate using a FilterMax F5 (Molecular Devices) plate reader. Small intestine transit was evaluated by determining the geometric center of the FITC-dextran in the bowel. The geometric center (Miller et al., 1981) was calculated as follows: Total geometric center = $\sum$(fluorescence in each segment x segment number)/(total fluorescence recovered) and ranges from 1 (minimum motility) to 16 (maximum motility).

3.3.13 Dextran sodium sulfate (DSS) injury.

Colitis was induced with DSS (2.5% in drinking water) as previously described (Pull et al., 2005). Control littermate mice were placed in separate cages at the time of the experiment and received water without DSS. Anatomic analysis of the colon was done on day 14 after starting DSS or water with the exception of bromodeoxyuridine (BrdU) experiments which were completed on day seven. For BrdU studies mice received intraperitoneal injections (10 mg/mL,

100 ug/g body weight) and were analyzed one hour later. Colons were pinned flat mucosal side up and fixed (4% PFA, 30 minutes, 25^{o}C) before gross morphologic analysis. Longitudinal 5 um sections of paraffin-embedded distal and proximal colon were stained with hematoxylin and eosin for additional analysis.

3.3.14 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Total RNA isolated using TRI Reagent (Sigma, St Louis, MO) and purified using RNeasy Mini kit (Qiagen Inc., Valencia, CA) was reverse-transcribed using SuperScript II Reverse Transcriptase for PCR cDNA synthesis (Invitrogen, Carlsbad, CA). qRT-PCR was performed in duplicate using SYBR green PCR Master mix (Applied Biosystems, Foster City, CA) and an iCycler iQ (Bio-Rad, Hercules, CA). Primers are in Table 3.1

3.3.15 Statistical analysis.

SigmaPlot 11 (Systat Software) was used for statistical analyses. All studies included at least 3 biological replicates. Measurements were made by observers blinded to conditions used for studies. Student's t test or one-way ANOVA with post hoc multiple comparisons tests (Dunn or Holm-Sidak) were used for statistical analysis. Log-Rank testing was performed for analysis of the Kaplan-Meier survival curves. Data are plotted as mean +/- SEM for all graphs. For all tests, $P < 0.05$ was considered significant.

Table 3.1. Primers for qRT-PCR

Accession Number	Gene Symbol	Unigene Title		Sequences [a]
NM_008084	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	f	AAC TTT GGC ATT GTG GAA GG
			r	GTC TTC TGG GTG GCA GTG AT
NM_008591	Met	Met proto-oncogene	f	CCA GCA GCT TCA GTT ACC GG
			r	GCG ATG CTG ACA TGC CAC TG
NM_008815	Etv4	Ets variant gene 4	f	CCC AGA TGA TGT CTG CAT TG
			r	GCC TGT CCA AGC AAT GAA AT
NM_023794.2	Etv5	Ets variant gene 5	f	GACCCCAGGCTGTACTTTGA
			r	CAGTCCAGGCGATGAAGTG

[a] 5’ to 3’sequences; f = forward primer; r = reverse-strand primer

3.4 Results

3.4.1 Met and HGF are expressed in a subset of adult enteric neurons.

Immunohistochemical analysis of adult mouse small bowel demonstrated that MET is present in 34 +/- 6% of HuC/D+ myenteric neurons (Figure 3.1A) and that all MET+ cells express the pan-neuronal marker HuC/D+ (Table 3.2). As expected, MET immunoreactivity was not detected in S100B+ enteric glia (Figure 3.1B). MET immunoreactive myenteric neurons were also detected in cross sections of human colon, where MET was co-expressed with the neuronal marker PGP9.5 (Figure 3.1, C, D). To determine which enteric neuron types express MET, remaining studies used adult mouse small bowel whole mount preparations to facilitate analysis of many cells at once. Interestingly, RET and MET were detected in mutually exclusive subsets of myenteric neurons as confirmed by immunohistochemistry (Figure 3.1E) and by using a *RET-EGFP* reporter mouse thought to faithfully reproduce normal *Ret* expression patterns (Figure 3.1F). 100% of MET+ neurons were calcitonin gene related protein (CGRP) immunoreactive and 49.9+/-2.0% of CGRP+ neurons were MET+ (Figure 3.1G-I). MET/calretinin staining demonstrated MET immunoreactivity in 16 +/- 5% of calretinin+ cells and that 8+/- 2% of MET+ cells are calretinin+ (Figure 3.1J-L). Consistent with this observation, since calretinin and choline acetyltransferases (CHAT) are largely expressed in the same cell population, we also detected MET immunoreactivity in 12 +/- 0.1% of CHAT immunoreactive neurons and found that 8 +/- 0.1% of MET+ neurons were CHAT+ (Figure 3.1M-O). There was no overlap between MET and NADPH diaphorase stained nitric oxide (NO) producing neurons (Figure 3.1P-R). Thus, MET appears to be primarily expressed in a

Table 3.2: Immunohistochemical localization of MET and HGF in the mouse small bowel myenteric plexus.		
Method	**% of MET+ cells that are + for the "second marker" tested**	**% of "second marker" + cells that are MET+**
MET + HuC/D	100% of MET+ are HuC/D+	34+/-6% of HuC/D+ are MET+
MET + S100B	0% of MET+ are S100B+	0% of S100B+ are MET+
MET + CGRP	100% of MET+ are CGRP+	49.9+/-2% of CGRP+ are MET+
MET + RET MET + *RET-EGFP*	0% of MET+ are RET+	0% of RET+ or EGFP+ are MET+
MET + HGF	43+/-11% of MET+ are HGF+	100% of HGF+ are MET+
MET + CHAT	7.6+/-0.1% of MET+ are CHAT+	12.3+/-0.1% of CHAT+ are MET+
MET + Calretinin	8.3+/-2.3% of MET+ are Calretinin+	16.4+/-5.2% of Calretinin+ are MET+
MET + NADPHd	0% of MET are NADPHd+	0% of NADPHd+ are MET+

subset of adult CGRP expressing cells that are likely to be intrinsic primary afferent neurons (IPANs). To determine where the MET ligand HGF was expressed in adult mouse bowel muscle layers, HGF/MET double label immunohistochemistry was performed. Surprisingly, HGF was detected in 43 +/- 11% of MET+ neurons and 100% of the HGF+ neurons had MET immunoreactivity (Figure 3.1S-U). Finally, MET and HGF were also detected in cross sections of E14.5 fetal bowel (Figure 3.1V-X). HGF immunoreactivity (Figure 3.1V) was prominent in the mesenchymal cells that surround the developing ENS (seen with TuJ1 in Figure 3.1W) and also was detected at lower levels in developing gut submucosa. MET immunoreactivity was present in the region of the developing ENS, but prominent signal was also detected in gut epithelium and in the mesenchymal cells surrounding the ENS as well as in developing enteric neurons (Figure 3.1X). This fetal immunoreactivity for HGF and MET suggested that these proteins might have roles during development as well as in the adult ENS.

3.4.2 HGF and Met signaling support fetal ENS neurogenesis and neurite growth in vitro.

To determine if HGF/Met signaling could affect fetal ENS development, we cultured ENS precursors from E12.5 bowel after immunoselection with p75NTR antibody. In these dissociated cell cultures, we initially tried adding HGF at a range of concentrations (0-100 ng/mL) to Neurobasal media, with B-27 supplement and L-glutamine, but in the absence of added GDNF, ENS precursors grew poorly or died and there was no evidence that HGF had any effect. When we tried including GDNF at commonly used concentrations (e.g. 50 or 100 ng/mL) in the media, the trophic effects of GDNF were so strong, that no additional effect of HGF could be discerned. Recognizing that the concentrations of GDNF used commonly in culture are higher

Figure 3.1

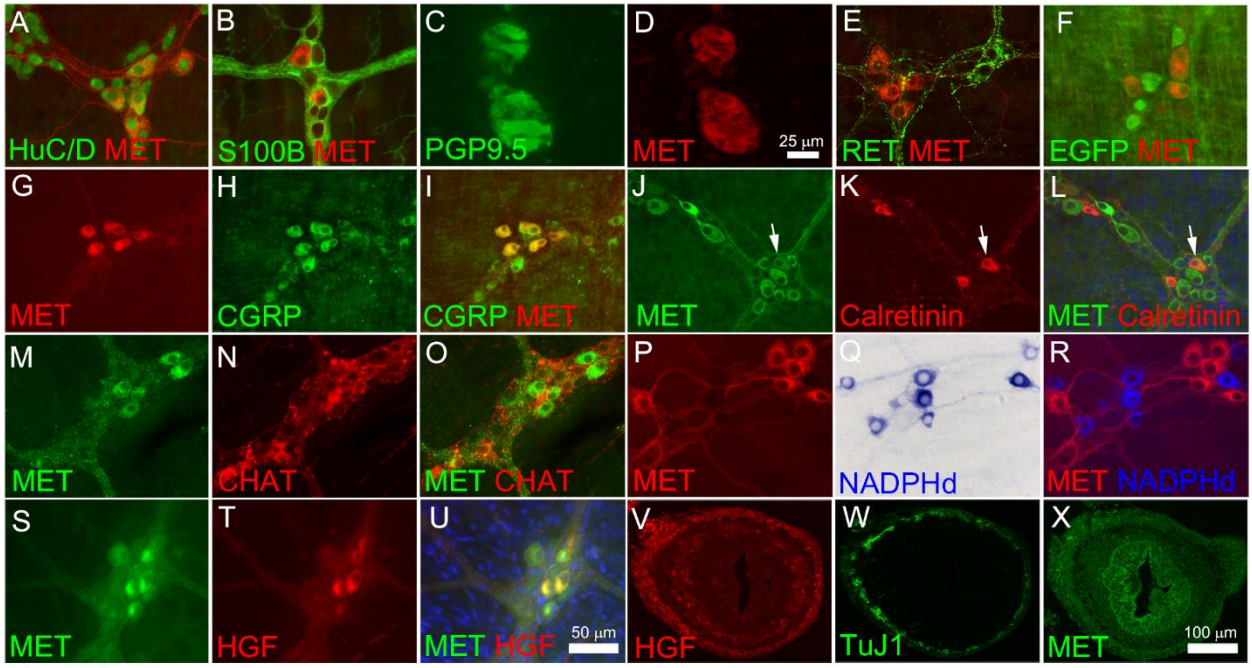

Figure 1

Figure 3.1. MET immunoreactivity was detected in a subset of myenteric plexus CGRP+ intrinsic primary afferent neurons (IPANs).

(A) MET-immunoreactivity was detected in 34 +/- 6% of HuC/D+ myenteric neurons of the adult mouse small bowel. All MET+ cells expressed the pan-neuronal marker HuC/D. (B) MET staining was absent from S100B+ enteric glia. (C, D) MET was detected in human myenteric neurons using colon cross sections. (E) MET and RET are detected in mutually exclusive sets of neurons as confirmed by immunohistochemistry and (F) by using a RET-EGFP reporter mouse. (G-I) 100% of MET+ neurons were CGRP+ and 49% of CGRP+ neurons were MET+. (J-L) MET was also found in 16+/-5% of calretinin+ cells and 8+/- 2% of MET+ cells are calretinin+. (M-O) 12 +/- 0.1% of CHAT+ cells were MET+ and 8 +/- 0.1% of MET+ neurons were CHAT+. (P-R) There was no overlap between MET and NADPH diaphorase stained nitric oxide (NO) producing neurons. (S-U) HGF was detected in 43 +/- 11% of MET+ neurons and 100% of the HGF+ neurons were MET+. (V) In cross sections of E14.5 fetal bowel HGF-IR is found in mesenchymal cells surrounding developing ENS stained with Tuj1 (W). (X) At E14.5, MET+ cells were present in the region of developing ENS as well as in gut epithelium and mesenchymal cells. Scale bar in Figure 1U applies to Figures 1A, B, E-U. Scale bar in Figure 1D applies to C and D. Scale bar in Figure 1X applies to V-X. N = at least 3 replicates/ staining condition.

than the ED50 for GDNF induced ENS precursor proliferation (1.5 ng/mL) (Heuckeroth et al., 1998) and dramatically higher than the Kd for GDNF binding to GFRα1 (30 pg/mL) (Jing et al., 1996), we decided to titrate the GDNF to low levels that might be more physiologic, yet support ENS precursor survival and permit effects of HGF to be observed. Under these conditions, all TuJ1+ enteric neurons were also MET antibody immunoreactive (Figure 3.2A-C). Remarkably, including GDNF at 1 pg/mL (50,000- to 100,000-fold less than is typically used in culture) led to robust and dose dependent effects of HGF (Figure 3.2D-E). ENS precursors were therefore immunoselected with p75NTR and cultured at low density with 1 pg/mL GDNF plus either no added HGF, 1 ng/mL HGF, 20 ng/mL HGF, 50 ng/mL HGF, or 100 ng/mL HGF. In cultures containing only low levels of GDNF, there were very few TuJ1+ cells in culture after 48 hours. Increasing HGF doses led to progressively more TuJ1+ cells in culture, with a 6-fold increase in TuJ1+ cells in cultures containing 100 ng/mL HGF plus 1 pg/mL GDNF compared to cells maintained in GDNF alone (Figure 3.2M). Including HGF in culture media also dramatically increased average neurite length compared to 1 pg/mL GDNF alone (Figure 3.2N). We confirmed that the HGF effects are MET dependent using MET blocking antibody and 50 ng/mL HGF (Figure 3.2E-F, 3.2M-N). Under these conditions, the blocking antibody almost completely prevented HGF effects on TuJ1+ cell number and neurite length.

Given the similarity of HGF and GDNF effects on the number of TuJ1+ cells in culture and on neurite length, we hypothesized that GDNF and HGF depend on the same signaling pathways to support neurogenesis and neurite growth. We previously reported using rat enteric neurons that GDNF-induced increases in neuron number and neurite length depend on phosphatidylinositol 3-kinase (PI3K), but not MEK signaling (Srinivasan et al., 2005). Using the MEK inhibitor PD98059 and the PI3K inhibitor LY294002, we confirmed these findings in mice

Figure 3.2

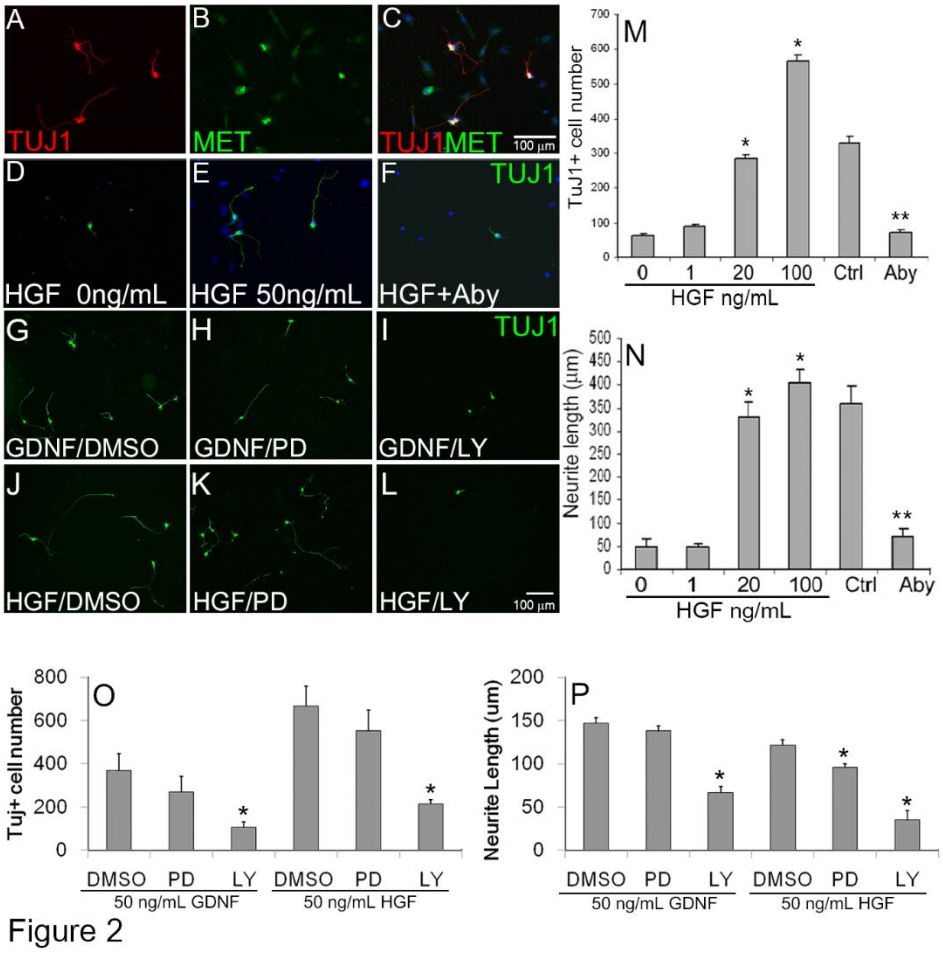

Figure 3.2. HGF promoted neurogenesis and neurite growth in cultured E12.5 ENS precursor cells.

(A-C) E12.5 ENS precursors immunoselected with p75NTR antibody were maintained in culture for 48 hours in the presence of HGF plus 1 pg/mL GDNF before TuJ1 and/or MET immunohistochemistry and DAPI nuclear staining. All Tuj1+ enteric neurons were MET immunoreactive. (D-F, M, N) HGF caused a dose dependent increase in TuJ1 immunoreactive neuron number and neurite length. * $P < 0.05$, ANOVA with Dunn's multiple comparison test. (F, M, N) MET blocking antibody (Aby) reduced TuJ1+ neuron number and neurite length in surviving cells. Ctrl is 50 ng/mL HGF plus 1 pg/mL GDNF. ** $P < 0.01$, Student's t-test. (G-L, O, P) When ENS precursors were grown in GDNF alone, the MEK inhibitor PD98059 (PD) had no effect on neuron number or neurite length, but the PI-3K inhibitor LY294002 (LY) reduced neuron number and neurite length. In contrast, in HGF (50 ng/mL) plus GDNF (1 pg/mL) treated cells, both MEK and PI-3K inhibition reduced neurite length (P), whereas only PI-3K inhibition reduced neuron number (O). * $P < 0.01$, ANOVA with Dunn's multiple comparison test. Scale bar in C applies to images A-F. Scale bar in L applies to images D-L. (N = at least 3 biological replicates/group; 12 individual wells/group).

using p75NTR immunoselected E12.5 ENS precursors in culture (Figure 3.2G-I, O, P). Similar to the results obtained using GDNF alone at 50 ng/mL, the number of TuJ1+ cells present after 48 hours in culture with HGF (50 ng/mL) plus GDNF (1 pg/mL) was reduced by the PI3K inhibitor, but not by the MEK inhibitor. In contrast, neurite length was reduced by both PI3K and MEK inhibitors in HGF containing cultures (Figure 3.2J-L), but only by the PI3K inhibitor in the GDNF (50 ng/mL) cultures (Figure 3.2P). This difference in downstream effectors suggests that HGF/MET induced neurite growth and GDNF/RET induced neurite growth support ENS precursors via partially overlapping signaling pathways.

3.4.3 HGF/Met signaling enhances ENS precursor differentiation into neurons in vitro.

The increase in TuJ1+ cells in dissociated ENS precursor cultures in response to HGF plus 1 pg/mL of GDNF could occur because of increased precursor proliferation, reduced cell death, or enhanced differentiation of RET+/TuJ1- precursors into RET+/TuJ1+ cells. To distinguish between these possibilities, dissociated $p75^{NTR}$ immunoselected cells from E12.5 mouse bowel were cultured in media with GDNF (1 pg/mL) with and without HGF (50 ng/mL) for 48 hours and then stained with TuJ1, RET and phosphohistone 3 (pH3) antibodies (Figure 3.3 A-H). RET is expressed in ENS precursors and differentiated neurons and TuJ1 immunoreactivity is a marker of neuronal differentiation, whereas pH3 identifies mitotic cells. While the total number of RET+ cells was not significantly altered with HGF (Figure 3.3 I), the number of TuJ1+ cells and the proportion of RET+ cells that are TuJ1 immunoreactive was increased by HGF (Figure 3.3 J, K). HGF also decreased the number of RET+TuJ1- cells in culture, and reduced the number of dividing (pH3+/RET+) ENS precursors (Figure 3.3 L). Collectively these data suggest that HGF increased the number of TuJ1+ enteric neurons *in vitro*

Figure 3.3

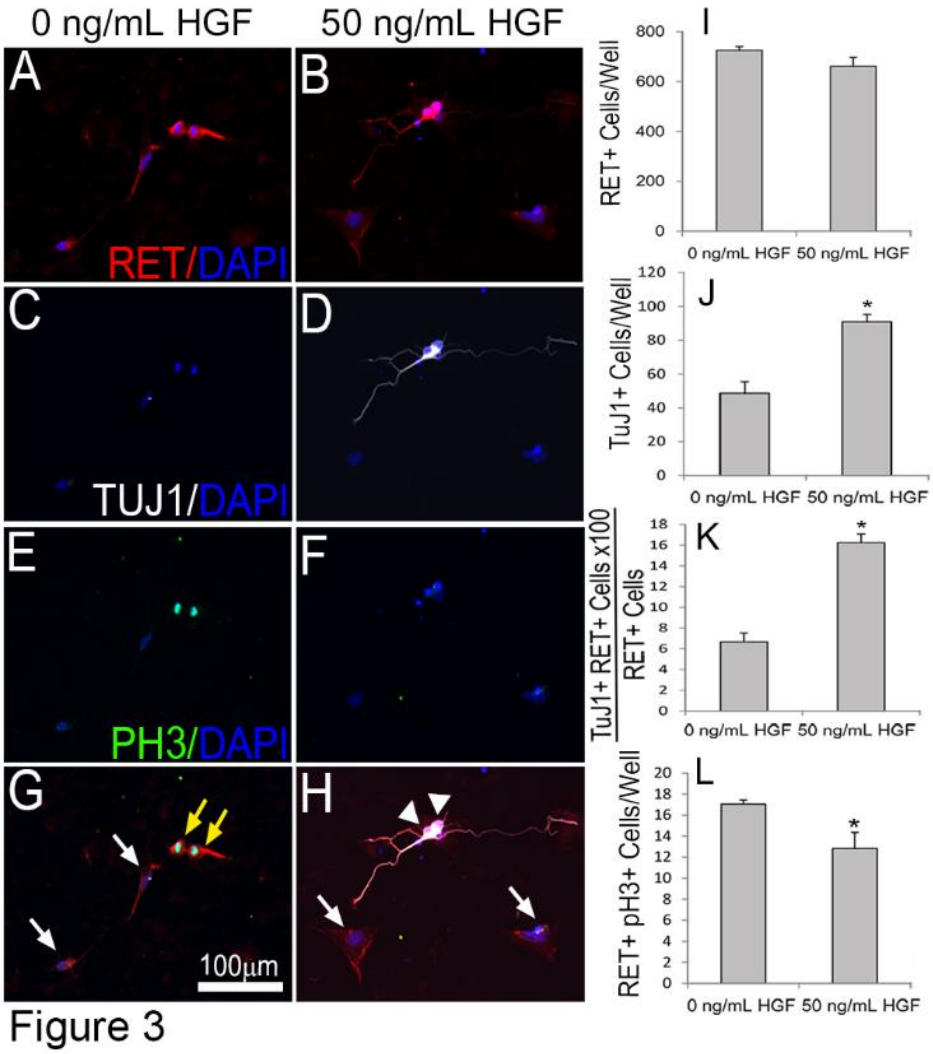

Figure 3.3 HGF/Met signaling enhanced ENS precursor differentiation into neurons in vitro.

(A-H) E12.5 ENS precursors were maintained in culture after p75NTR immunoselection for two days in the presence or absence of 50 ng/mL HGF prior to immunohistochemistry using RET (A,B), Tuj1 (C,D), and phosphohistone 3 (pH3) (E,F) antibodies as well as DAPI nuclear staining (A-H). (G,H) Show merged images. (I) While the total number of RET+ cells was not altered by HGF, the total number of TuJ1+ neurons (J) and the percent of RET+ cells that were TuJ1+ (K) increased with HGF treatment. (L) The number of dividing precursor cells (pH3 and RET double positive) decreased with HGF treatment, suggesting that HGF increased neuronal differentiation and decreased proliferation. White arrows: non-mitotic RET+ pH3- ENS precursors. Yellow arrow: mitotic RET+PH3+ ENS precursors. White arrowhead: Ret+ TuJ1+ neurons. Scale bar in G applies to all images. (N = 3 biological replicates/group; 12 individual wells/group; * $P < 0.01$, Student's t-test).

by enhancing ENS precursor differentiation into neurons instead of through increased precursor proliferation or survival.

3.4.4 HGF/MET signaling did not increase ENS precursor migration *in vitro*.

Since HGF and MET are expressed in fetal bowel when ENS precursors migrate and have well known effects on migration of other neuronal cell types (Giacobini et al., 2007; Garzotto et al., 2008), we hypothesized that HGF/MET signaling might influence ENS precursor migration. To test this hypothesis E12.5 gut slices were cultured on fibronectin coated culture dishes and ENS precursors were allowed to migrate from the slice onto the culture dish for 24 hours. Addition of GDNF (100 ng/mL) to the media markedly increased the distance that ENS precursors migrated onto the culture dish (Figure 3.4). In contrast, HGF (50 or 100 ng/mL) did not increase ENCDC migration onto the culture dish compared to no added factor suggesting that HGF may not be needed for ENCDC migration *in vivo*.

3.4.5 MET inactivation within ENS precursors causes selective defects in *Met* expressing enteric neurons.

Met-/- mice die in utero between E13.5 and E16.5 (Huh et al., 2004). Therefore to investigate the role of HGF/MET signaling in the ENS *in vivo* we bred mice with *LoxP* sites surrounding *Met* exon 16 to *Wnt1Cre* transgenic animals to generate *Met cKO* mice ($Met^{fl/fl}$; *Wnt1Cre*+). CRE dependent recombination inactivates MET by removing the intracellular kinase domain, but should permit production of a truncated protein that includes the extracellular and transmembrane domain. The *Wnt1Cre* transgene is expressed in the developing neural tube and neural crest derivatives including the ENS, but not in other cells within the bowel. Thus *Met cKO* mice should have selective loss of MET activity in ENS precursors within the bowel

without affecting other intestinal cell lineages. *Met cKO* mice survive to adulthood and are born at rates that are not statistically different from expected Mendelian ratios ($P > 0.99$, Chi Square test).

We first tested the hypothesis that *Met* mutations slowed migration of ENS precursors down the fetal bowel, by evaluating ENS structure in *Met cKO* and control littermates (*Met WT* or *CRE* deficient) using TuJ1 and RET antibodies. Consistent with *in vitro* migration studies, control and *Met cKO* mice had ENCDC in the entire small bowel and half the colon at E12.5 and there were no obvious differences in ENS structure (data not shown). We then examined the adult mouse myenteric plexus to test the hypothesis that HGF/MET signaling influences the development of *Met* expressing CGRP+ enteric neurons. Using an antibody to the MET extracellular domain to stain the myenteric plexus, we found that MET+ neuronal cell bodies are easy to identify in *Met cKO* mice and that these cells were normal in size (Figure 3.5A) and abundance (Figure 3.5B). In contrast, MET-immunoreactive inter- and intra-ganglionic neurites are very difficult to see in *Met cKO* mice compared to control animals (Figure 3.5 C-D). To determine if this staining pattern reflects a difference in neurite length for myenteric plexus *Met*-expressing neurons, we performed DiI labeling combined with MET immunohistochemistry. DiI paste on a dissecting pin was inserted into fixed small bowel muscle layers containing the myenteric plexus and tissue was incubated for three weeks. DiI taken up by axons undergoes passive retrograde diffusion through lipid membranes to label cell bodies away from the pin site (Figure 3.5E-J). Co-staining of DiI labeled samples with MET antibody revealed that the number of DiI and MET-double positive cell bodies was dramatically reduced in *Met cKO* mice compared to controls, especially as the distance from the DiI labeling site increased (Figure 5K,

Figure 3.4

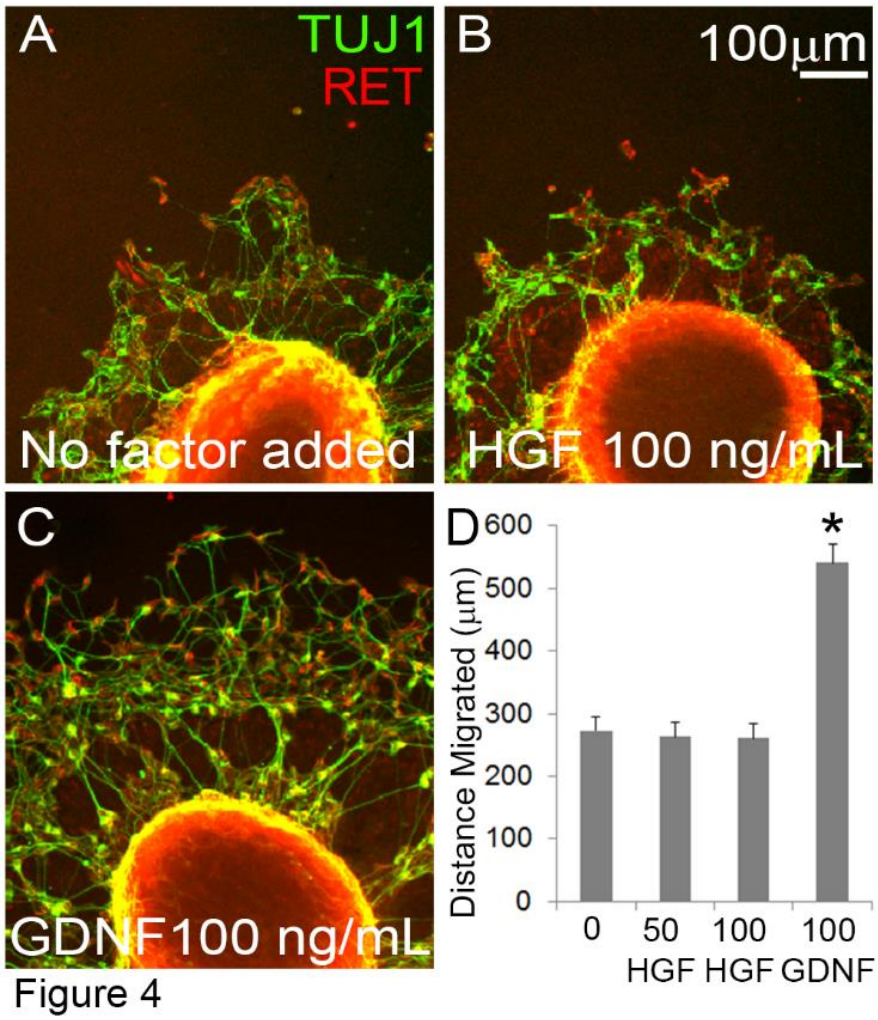

Figure 3.4. HGF/Met signaling did not increase ENS precursor migration in culture.

(A-C) E12.5 gut slices were cultured 24 hours on fibronectin coated dishes with no added factor, HGF or GDNF before staining for RET and TuJ1. (A) ENCDC migrate from the gut slice onto the culture dish even without any added factors. (B) HGF did not increase the distance that ENCDC migrated from the edge of the gut slice. (C) GDNF markedly increased the distance ENCDC migrate from the edge of the gut slice. (D) Quantitative data (No added factor 15 slices; 50 ng/mL HGF 14 slices; 100 ng/mL HGF 19 slices; 100 ng/mL GDNF 30 slices, N = 3 independent experiments). * $P < 0.001$ for GDNF versus no added factor, ANOVA with Dunn's multiple comparison test.

Figure 3.5

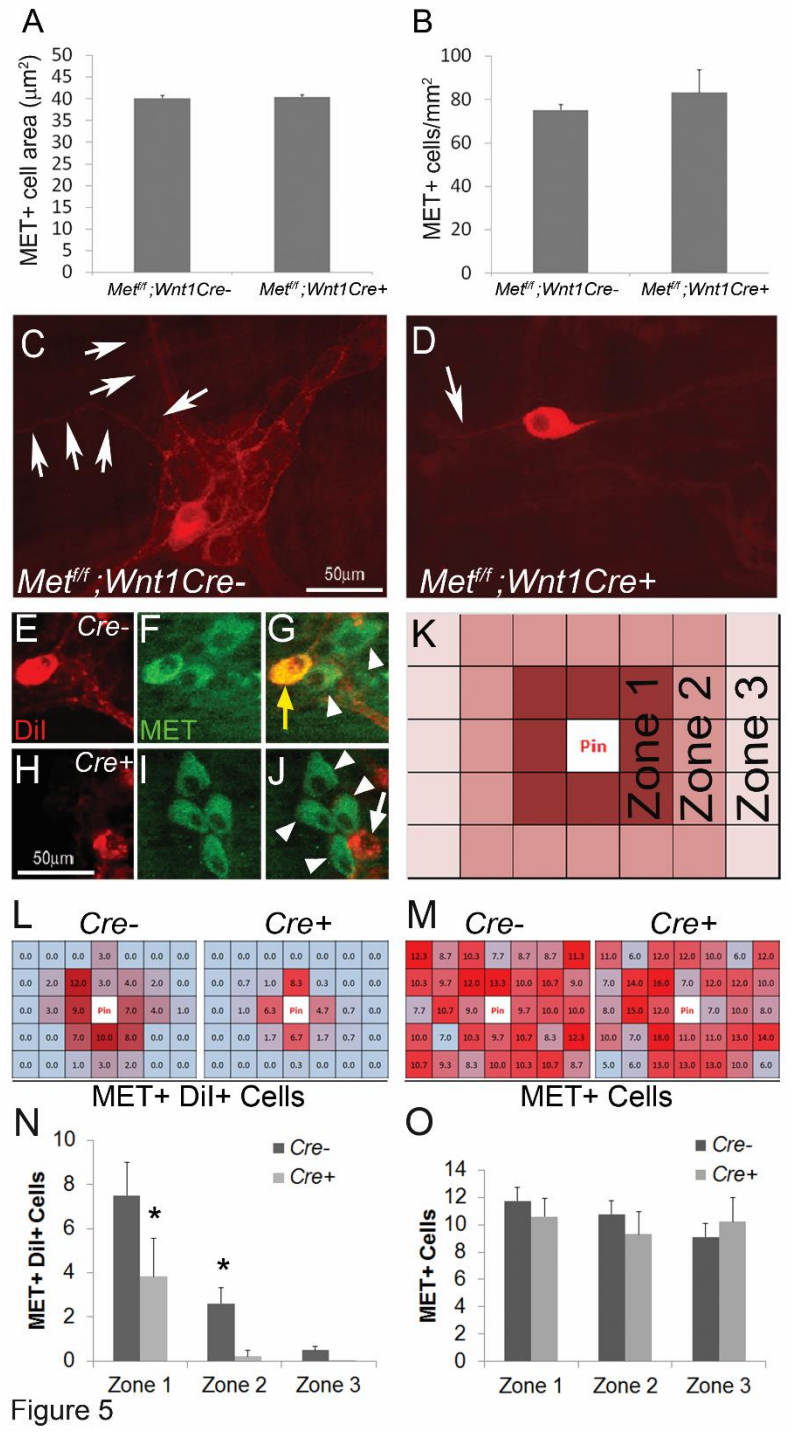

Figure 5

Figure 3.5. MET deletion within ENS precursors caused selective defects in MET expressing enteric neurons in adult mice.

(A, B) The myenteric plexus of mice lacking functional MET receptors in the ENS (*Met*$^{tf/f}$*Wnt1Cre+*) contained MET+ neuronal cell bodies of normal size and abundance. (N = 3 mice of each genotype/condition). (C-D) *Met*$^{tf/f}$*Wnt1Cre+* mice had few inter- and intra-ganglionic MET+ immunoreactive neurites. (N = 6 mice of each genotype/condition) (E-J) Shows double labeling method for myenteric neurons combining DiI labeling and MET immunohistochemistry. Cells shown were two 20X fields away from the DiI application site. Yellow arrow: DiI+ MET+ cell body. White arrow: DiI+ MET- cell body. White arrow heads: DiI- MET+ cell bodies. (N = 4 mice of each genotype; N > 8 distal small bowel Di-labeled regions/genotype). (K) Schematic for analysis of DiI/MET labeled samples: tissue pieces were divided into a 5 X 7 grid of 20X fields centered on the pin insertion site. The grid was additionally subdivided into 3 zones of varying distances from the pin. (L,M) Heat map representations of the average number of MET+ DiI+ cells (L) and total MET+ cells (M) in each 20X field of the grid. Dark red > light red> blue for the number of cells in each region. (O) The number of DiI+ MET+ cell bodies was dramatically reduced in *Met cKO* mice compared to controls in Zones 1 and 2. * $P < 0.02$ (Student's t-test). (P) The number of total MET+ cell bodies did not differ at varying distances from the pin site.

L,O). In contrast, the average number of MET-positive cell bodies was not different at varying distances away from the pin site (Figure 3.5M, P). Furthermore, the number of MET negative neuron cell bodies labeled by DiI did not significantly differ in WT and *Met cKO* mice. These data suggests that neurites in MET immunoreactive myenteric neurons of *Met cKO* mice are shorter than in WT mice.

3.4.6 Functional analysis of gut motility.

To test the hypothesis that HGF/MET signaling is important for intestinal motility, the peristaltic response to stretch or to mechanical stimulation of the mucosal lining was measured in *WT* and *Met cKO* mice using an oxygenated three-compartment organ bath. These studies showed that the ascending and descending components of the peristaltic reflex were strongly blunted in $Met^{fl/fl}$*;Wnt1-Cre+* mice in response to gentle brushing of the mucosa (Figure 3.6A,B,E). In contrast, both components were similar in *WT* and *Met cKO* mice when the peristaltic reflex was elicited by muscle stretch (Figure 3.6C-D). These data suggest that *Met* mutations cause a selective defect in one of the sensory signaling modalities capable of initiating peristalsis (i.e., the reflex elicited by mucosal stimulation). Despite this defined defect in the peristaltic reflex, whole gastrointestinal transit as measured using carmine dye (Figure 3.6F), gastric emptying and small intestine transit measured by FITC-dextran gavage (Figure 3.6G), and colonic motility measured by expulsion of a glass bead from the rectum (Figure 3.6H), were not altered in *Met cKO* mice compared to controls. These data suggest that intact sensory response to villus deformation is not required for normal transit of luminal contents through the bowel.

Figure 3.6

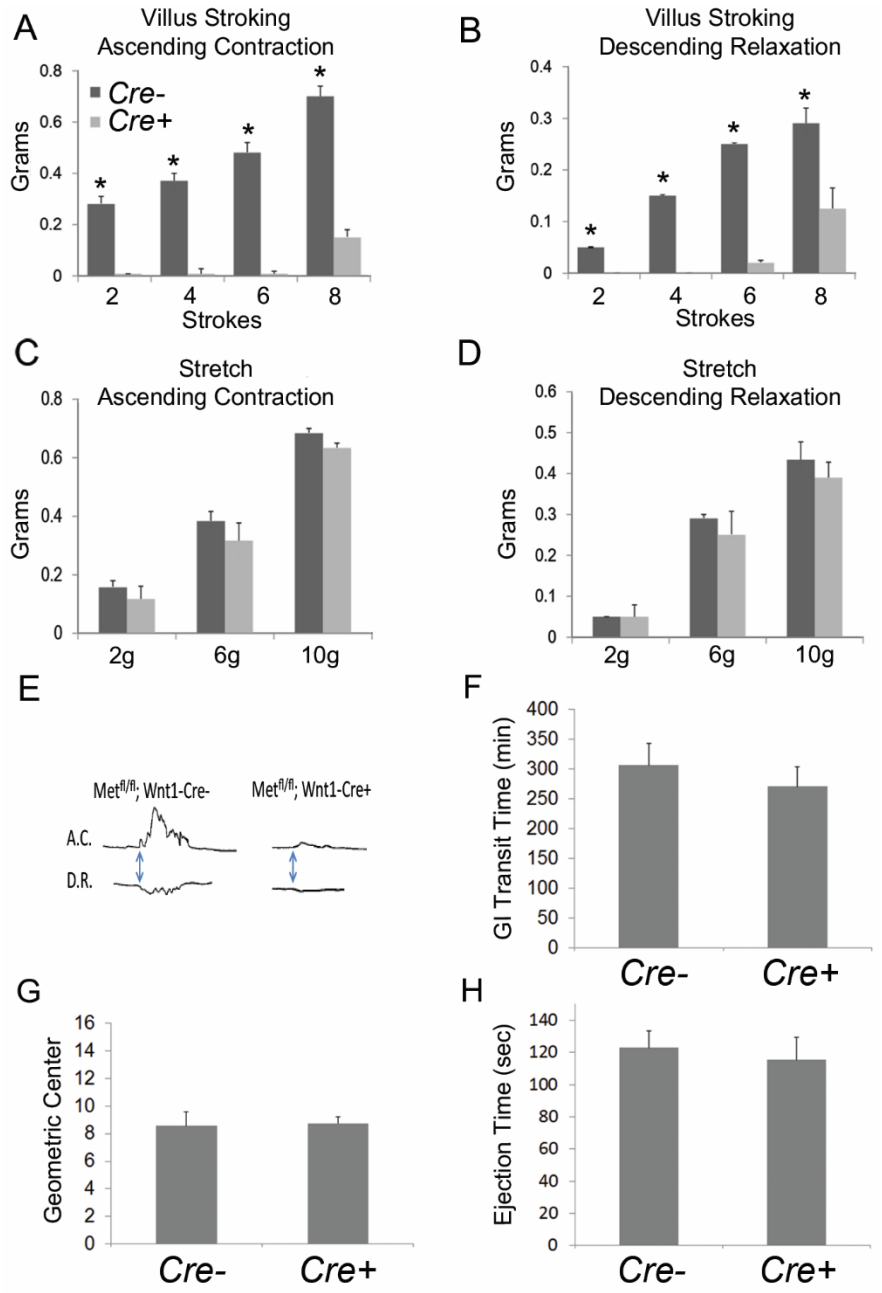

Figure 6

Figure 3.6. MET deletion within ENS precursors resulted in selective defects in the sensory arm of the peristaltic response.

(A, B, E) Mice lacking functional MET receptors in the ENS (*Met$^{fl/fl}$;Wnt1-Cre+*) had an abnormal peristaltic reflex in response to mechanical stimulation of the villi, as evidenced by a severely blunted ascending contraction (A) and descending relaxation (B). (C, D) In contrast, the peristaltic reflex elicited by circular muscle stretch had normal ascending contraction (C) and descending relaxation (D) in *Met$^{fl/fl}$;Wnt1-Cre+* mice, suggesting a selective sensory defect. (N = 3 mice/genotype; * P <0.01, Student's t-test). (F) Whole gastrointestinal transit as measured by the time needed to pass orally gavage fed carmine dye in stool was not altered in *Met$^{fl/fl}$;Wnt1-Cre+* mice (N = 8 *Met cKO* and 7 control mice). (G) Small bowel transit as measured by determining the geometric center of FITC-dextran within the bowel 90 minutes following oral gavage was not altered in *Met$^{fl/fl}$;Wnt1-Cre+* mice. (N = 4 *Met cKO* and 4 control mice). (H) Colonic transit, as measured by the time taken to expel a bead placed 2 cm into the distal colon was not altered in *Met$^{fl/fl}$;Wnt1-Cre+* mice (N = 8 *Met cKO* and 7 control mice).

3.4.7 HGF/Met signaling and bowel injury.

HGF/MET signaling potently reduces bowel injury in response to the toxins DSS and 2,4,6-trinitrobenzene sulfonic acid (TNBS) in rodent models and supports epithelial cell proliferation (Tahara et al., 2003; Mukoyama et al., 2005; Numata et al., 2005; Oh et al., 2005; Hanawa et al., 2006; Kanbe et al., 2006; Setoyama et al., 2011). We hypothesized that these effects might be mediated by the newly discovered MET expressing neurons. To test this hypothesis we treated *Met$^{fl/fl}$; Wnt1Cre+* mice or control littermate animals with 2.5% DSS in drinking water and analyzed the colon after 2 weeks of DSS treatment. *Met$^{fl/fl}$; Wnt1Cre+* mice had significantly more mucosal damage than control animals after 14 days of DSS treatment (Figure 3.7A-F) and higher death rates (Figure 7G). Because increased damage in specific models, including DSS, is linked to diminished epithelial proliferation, we examined intestinal stem and progenitor cell proliferation and found that *Met cKO* mice had reduced proliferation compared to control animals after 7 days of DSS (Figure 3.7 H-J). Collectively these data suggest that HGF/MET signaling protects the intestinal mucosa from DSS induced injury through the activity of MET expressing enteric neurons since the only cells in the bowel that express CRE in this model are in the ENS.

3.4.8 GDNF/RET signaling increased *Met* and *Etv5* mRNA in cultured ENS precursors, but MET protein levels *in vivo* do not depend on *Etv5/Etv4*.

The requirement for very small amounts of GDNF to detect any HGF effects on neurite growth and neuron numbers is striking especially since MET and RET are closely related tyrosine kinase receptors. One possible explanation is that GDNF/RET signaling is needed to induce *Met* expression in cultured ENS precursors as occurs in the kidney and motor neurons via

Figure 3.7

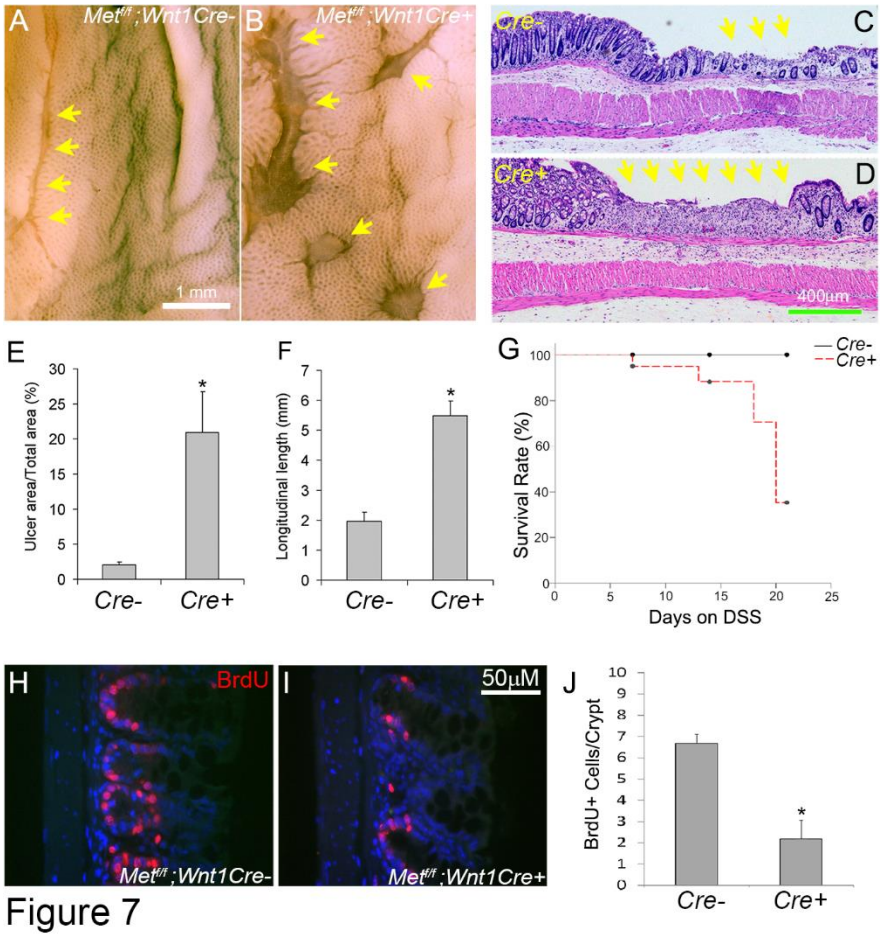

Figure 3.7. MET inactivation in ENS precursors diminished mucosal injury in response to DSS treatment.

(A-F) *Met*$^{fl/fl}$*; Wnt1Cre+* mice and *Met*$^{fl/fl}$*; Wnt1Cre-* control animals were treated with 2.5% DSS in drinking water for 14 days and then examined using a dissecting microscope (A, B) or after paraffin sectioning and hematoxylin and eosin staining (C, D). (E, F) Quantitative analysis of ulcer area in the descending colon and ulcer length in the rectum demonstrated reduced ulcers in *Met*$^{f/f}$*; Wnt1Cre* mice compared to controls. * P <0.01, Student's t-test. (G) Kaplan-Meier analysis demonstrated that DSS treated *Met*$^{fl/fl}$*; Wnt1Cre+* mice had higher death rates than controls. (N = 8 *Met cKO* and 11 control mice). P < 0.05, Log-Rank test. (H-J) BrdU labeling after seven days of DSS treatment showed reduced colonic epithelial cell proliferation within crypts of *Met*$^{f/f}$*; Wnt1Cre* mice compared to control animals. * P <0.01, Student's t-test. Yellow arrows: ulcerated regions. (N = 5 *Met cKO* and 4 control animals/group for ulcer analysis and BrdU labeling).

the *Etv4 (Pea3)* and *Etv5 (Erm)* transcription factors (Haase et al., 2002; Livet et al., 2002; Lu et al., 2009; Kuure et al., 2010). To test this hypothesis we cultured E12.5 immunoselected ENS precursors for 18 hours with or without 1 pg/mL GDNF. Although extended culture without GDNF results in death of ENS precursors, there were many healthy appearing ENS precursors in the GDNF deprived cultures and in cultures containing 1 pg/mL of GDNF when the mRNA was collected after 18 hours in culture. Relative mRNA levels for *Etv4*, *Etv5* and *Met* were analyzed by real time reverse transcriptase PCR (qRT-PCR). We found that compared to cells cultured without GDNF, ENS precursors grown with 1 pg/mL GDNF had an 8-fold increase in *Etv5* mRNA, a 230-fold increase in *Met* mRNA, but no change in *Etv4* mRNA. These data suggest that low levels of GDNF induced *Met* expression, allowing HGF to affect ENS precursor development (Figure 3.8A-B).

To further explore the role of ETV5 signaling on MET expression in vivo, we examined MET expression in the ENS of $Etv5^{M/M}$ animals, as well as in $Etv4^{-/-}; Etv5^{lacZ/WT}$ compound mutants (Lu et al., 2009). $Etv5^{M/M}$ allele is a weak allele that permits survival to adulthood. In contrast, the $Etv5^{lacZ}$ allele used in the $Etv4^{-/-}; Etv5^{lacZ/WT}$ mice causes early fetal lethality prohibiting analysis of the $Etv5^{lacZ/lacZ}$ ENS. We found that the ENS of $Etv5^{M/M}$ and $Etv4^{-/-}; Etv5^{lacZ/WT}$ compound mutants was grossly normal, with MET-IR neuron density comparable to that of WT littermates (Figure 3.8 C-H)). In contrast, in the developing kidney, where GDNF also induces *Met* expression, the $Etv4^{-/-}; Etv5^{lacZ/WT}$ compound mutants fail to express Met in the ureteric bud causing serious defects in renal development (Lu et al., 2009). This suggests that ETV4 and ETV5 are dispensable for MET expression in enteric neurons or that at single allele of *Etv5* is adequate for MET expression in the ENS.

Figure 3.8

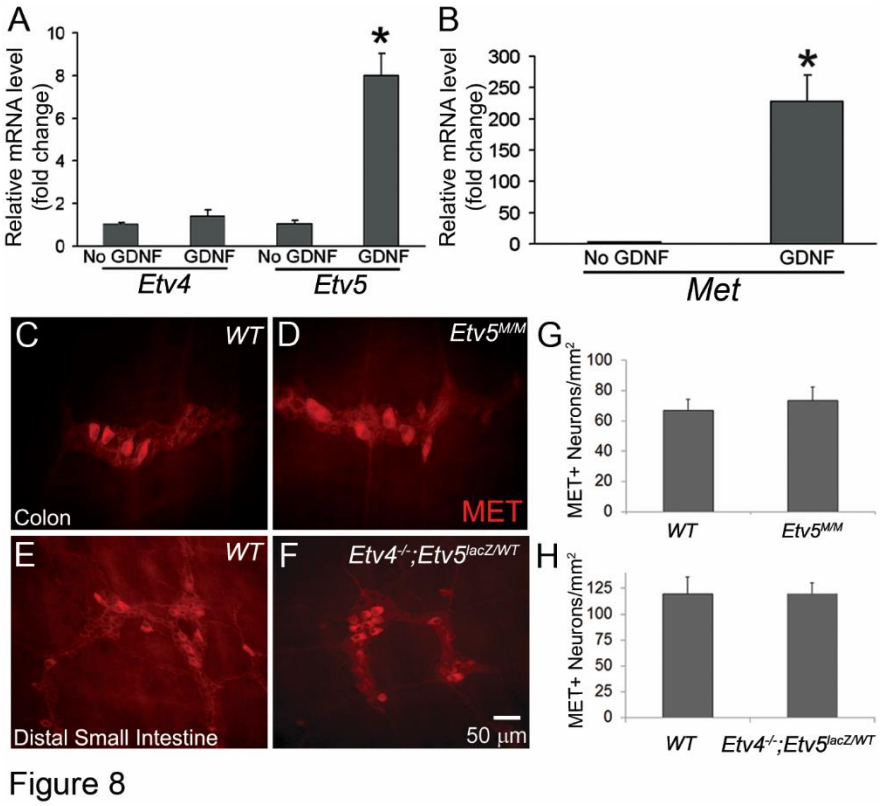

Figure 3.8. GDNF/Ret signaling increased Met and Etv5 mRNA in cultured ENS precursors, but MET protein immunoreactivity appeared normal in mice with Etv4 and Etv5 mutations.

(A) ENS precursors grown with 1 pg/mL GDNF had an 8-fold increase in Etv5 mRNA, but not in Etv4 mRNA when compared to precursors cultured without GDNF. (B) ENS precursors grown with 1pg/mL GDNF also had a 230-fold increase in Met mRNA. (N = 3 biological replicates) (C-H) The myenteric plexus of adult Etv5M/M mice and P14 Etv4-/-; Etv5lacZ/WT compound mutants appeared grossly normal with no differences in MET-IR neuron density. (N = 3 mice for each group). *P < 0.01 (Student's t-test).

3.5 Discussion

HGF enhanced fetal enteric neuron differentiation and neurite growth *in vitro*, but did not affect ENS precursor migration from gut slices or bowel colonization by ENS precursors *in vivo*. In adults, MET immunoreactivity was found in a subset CGRP+ myenteric neurons thought to be IPANs (Qu et al., 2008). MET-IR neuron density was normal in *Met cKO* mice, but MET-IR neurites were short and sparse. *Met cKO* mice also had reduced peristalsis after mucosal deformation and increased mucosal injury after DSS exposure. These data suggest HGF and MET support a subset of CGRP-expressing IPANs that regulate intestinal motility and epithelial function.

3.5.1 HGF, MET, GDNF and RET

MET influences many cellular functions (Trusolino et al., 2010) including neuron survival (spinal motor, sympathetic, sensory and retinal neurons) (Ebens et al., 1996a; Maina et al., 1997; Thompson et al., 2004; Lamballe et al., 2011; Tonges et al., 2011), differentiation (sensory and hippocampal neurons) (Maina et al., 1997; Lim and Walikonis, 2008), axon outgrowth and guidance (spinal motor, GNRH, sensory, and retinal neurons) (Ebens et al., 1996a; Maina et al., 1997; Giacobini et al., 2007; Tonges et al., 2011), precursor migration (cortical and GNRH neurons) (Giacobini et al., 2007; Garzotto et al., 2008), and synaptic plasticity (hippocampal neurons) (Akimoto et al., 2004). Our data reveal previously unsuspected roles for MET and HGF in the ENS, but there is more to learn. One intriguing question is whether HGF in some MET-IR neurons acts as a chemoattractant to support formation of the extensive network of IPAN to IPAN connections (Furness et al., 2004a). HGF is chemoattractive for spinal motor

axons (Ebens et al., 1996b), but has autocrine roles in sympathetic neurons (Maina et al., 1998) and may have similar ENS functions. Another interesting finding was that HGF effects required small amounts of GDNF, which increased *Met* and *Etv5* mRNA (Lu et al., 2009; Costantini, 2010). In kidney *Met* was absent in $Etv4^{-/-}; Etv5^{lacZ/WT}$ mice, but MET was readily detected in myenteric neurons suggesting single *Etv5* alleles may support ENS *Met* expression. Unfortunately, $Etv5^{lacZ/lacZ}$ mice die early (Lu et al., 2009) and milder $Etv5^{M/M}$ mutations did not affect MET-IR myenteric neuron density, so ETV5 function in the ENS remains uncertain. Finally, MET and RET appear in non-overlapping adult myenteric neuron populations, highlighting the need to define mechanisms restricting receptor tyrosine kinases (e.g. RET, NTRK3, and MET) to specific enteric neuron subtypes (Chalazonitis et al., 1994b; Schuchardt et al., 1994; Heuckeroth et al., 1999; Chalazonitis et al., 2001; Uesaka et al., 2007; Uesaka et al., 2008).

3.5.2 HGF/MET and ENS Development

The requirement for 1 pg/mL GDNF to observe HGF effects on ENS precursors is reminiscent of synergistic HGF and NGF effects in DRG. HGF alone did not support DRG neuron survival or axon outgrowth, but HGF enhanced survival, differentiation, and axonogenesis with NGF present (Maina et al., 1997; Maina et al., 1998). In the ENS, low GDNF levels maintain cells as progenitors (Uesaka et al., 2013) and may support survival while enhancing HGF responsiveness. In contrast, high GDNF triggers neuronal differentiation and migration, masking HGF effects. GDNF concentrations *in vivo* are unknown but are probably below the 50-100 ng/mL commonly used *in vitro,* since increased and reduced GDNF alters

enteric neuron number (Gianino et al., 2003; Wang et al., 2010) and the Kd for GDNF binding to GFRα1 is only 30 pg/mL (Jing et al., 1996). These observations suggest that it may be appropriate to evaluate how other factors affect ENS precursors in the presence of low concentrations of GDNF instead of the levels typically used in culture.

3.5.3 HGF/MET and IPAN Subtypes

Functional data suggest IPANs are heterogeneous (Clerc and Furness, 2004; Furness et al., 2004a; Furness, 2006a). For example, stretch opens IPAN gadolinium-insensitive mechanosensitive ion channels (Kunze et al., 1999), whereas mucosal deformation triggers serotonin and ATP release from enteroendocrine cells to activate IPAN 5HT-$_3$/5HT-$_4$ or P2X receptors (Grider and Jin, 1994; Pan and Gershon, 2000; Raybould et al., 2004; Patel, 2014). These IPANs all express CGRP, however only 49% of CGRP+ neurons are MET+. MET signaling is not needed for survival of MET-IR neurons, but *Met cKO* mice have impaired peristalsis *in vitro* after mucosal stroking consistent with a functional defect in a subset of IPANs. In contrast, bowel stretch responsive IPANS are either MET-negative or do not require MET for function. It is not clear if the selective functional defect results from reduced neurite growth in MET+ neurons or from reduced function. In DRG neurons, for example, MET enhances nociceptor peptidergic differentiation (Gascon et al., 2010). Nonetheless, in contrast to *in vitro* results, whole bowel transit, gastric emptying, small bowel transit, and colonic bead expulsion were normal *in vivo* in *Met cKO* mice. These data add to recent studies suggesting mucosal deformation induced peristalsis is not required for normal transit through the bowel, at least when stretch response is intact (Li et al., 2011; Heredia et al., 2013).

3.5.4 HGF, the ENS, and intestinal injury

HGF's ability to reduce bowel injury is fascinating (Tahara et al., 2003; Arthur et al., 2004; Mukoyama et al., 2005; Numata et al., 2005; Oh et al., 2005; Ohda et al., 2005; Hanawa et al., 2006; Kanbe et al., 2006; Gong, 2008; Setoyama et al., 2011). Remarkably, *Met cKO* mice had increased mucosal damage, reduced epithelial stem and progenitor cell proliferation in response to injury, and increased mortality compared to controls after DSS treatment. Although gut epithelial cells express *Met* (Prat et al., 1991), $Met^{fl/fl}$ *Wnt1Cre* mice do not express CRE in epithelium (Danielian et al., 1998), suggesting MET effects on epithelial proliferation after DSS are not cell-autonomous.

Many mechanisms might underlie our observations. One hypothesis is that impaired injury response results from reduced CGRP release from MET+ enteric neurons. CGRP mutations increased colonic damage in DSS treated mice (Thompson et al., 2008). Furthermore, CGRP is a potent vasodilator (Pawlik et al., 2000) and adequate mucosal blood flow may facilitate injured bowel repair. CGRP also supports bowel epithelial proliferation via mast cells and fibroblasts producing transforming growth factor alpha (Hoffmann et al., 2010) and by regulating gene expression in macrophages (Baliu-Pique et al., 2014) that influence the set point of intestinal epithelial proliferation (Baliu-Pique et al., 2014; Sun et al., 2014). MET+ neurons might also support epithelial proliferation via acetylcholine release from CHAT+MET+ neurons, since acetylcholine enhances epithelial growth (Tutton, 1975; Lundgren et al., 2011; Gross et al., 2012).

Consistent with ENS support for bowel epithelium, enteric neurons express the receptor for glucagon-like peptide 2, a potent epithelial mitogen (Bjerknes and Cheng, 2001; Guan et al.,

2006) and neuronal serotonin increases epithelial proliferation (Gross et al., 2012). However, myenteric plexus ablation increases epithelial cell proliferation (Zucoloto et al., 1988; Holle, 1991; Hadzijahic et al., 1993; Holle et al., 2003) and hypomorphic *Ret*+/- mice (Gianino et al., 2003) had increased epithelial proliferation after small bowel resection (Hitch et al., 2012). These data suggest distinct enteric neuron subtypes enhance or inhibit intestinal epithelial proliferation, but it is unclear how these processes are integrated.

We also note that CRE-induced MET mutations are not restricted to the ENS in *Met cKO* mice (Danielian et al., 1998). MET should be disrupted in CGRP expressing DRG neurons (Gascon et al., 2010) that might normally enhance mucosal repair (Takami et al., 2009; Engel et al., 2011; Engel et al., 2012; Lee et al., 2012). MET inactivation in vagal neurons (Freem et al., 2010) could also increase severity of DSS induced injury (Mazelin et al., 1999; Ghia et al., 2006; Ghia et al., 2007; Van Der Zanden et al., 2009) since some vagal nuclei express *Met* (Caton et al., 2000; Wu and Levitt, 2013). Distinguishing between these possibilities is not straightforward, but these data fit with an emerging literature suggesting neuronal activity regulates intestinal epithelial progenitor proliferation and barrier function (Bjerknes and Cheng, 2001; Nezami and Srinivasan, 2010; Hitch et al., 2012; Sharkey and Savidge, 2014).

It is tempting to speculate that neurogenic control underlies high rates of enterocolitis in children with Hirschsprung disease, a birth defect where the ENS is absent from distal bowel (Frykman and Short, 2012; Heuckeroth, 2013). ENS damage in inflammatory bowel disease or necrotizing enterocolitis may also perpetuate bowel inflammation (Margolis and Gershon, 2009; Zhou et al., 2013). Indeed, many ENS transmitters affect bowel inflammation and injury, including CGRP (Eysselein et al., 1992; Wang et al., 2006; Ramachandran et al., 2013), serotonin (Bischoff et al., 2009; Gershon, 2012), neuropeptide Y, vasoactive intestinal peptide

(Chandrasekharan et al., 2013), and substance P (Landau et al., 2007) as do enteric glia (Bush et al., 1998; Savidge et al., 2007), enteric neuron density (Margolis et al., 2011), and toll-like receptor 2 (Brun et al., 2013). Our data reinforce this literature and suggest that new therapeutic strategies to treat or prevent intestinal motility or bowel inflammatory diseases may be targeted to the nervous system instead of the immune system.

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Chapter 4: Axon Pathfinding in the enteric nervous system.

Marina Avetisyan, Saya Bery, Jack Grider, Robert O. Heuckeroth.

4.1 Introduction

Enteric neuron precursors that have completed migration will differentiate into neurons and form integrated circuits that mediate various gut functions. One of the best characterized myenteric plexus circuits regulates peristalsis, the coordinated contraction and relaxation of the bowel necessary for proper propulsion of a food bolus down the GI tract. The peristaltic reflex begins with intrinsic primary afferent cells (IPANS) that sense chemical or mechanical stimuli from the gut lumen or bowel wall and relay this information to excitatory and inhibitory motor neurons via ascending and descending interneurons. Ascending cholinergic interneurons project rostrally onto cholinergic excitatory motor neurons that trigger contraction of the circular smooth muscle in the segment of bowel proximal to the sensory stimulus (Furness et al. 2004). Descending interneurons project caudally onto nitrergic inhibitory motor neurons. Descending interneurons release acetylcholine at the synapse with motor neurons, but also make nitric oxide (NO) which signals retrogradely at the synapse with IPANs to modulate interneuron signaling (ref). Inhibitory motor neurons project up to 10mm caudally (in mice) before turning to innervate circular muscle. Nitrergic (NO producing) motor neurons use VIP as a co-transmitter and mediate relaxation of the muscle distal to the sensory stimulus (Li & Furness 2000; Grider 2003). Both the proper rostro-caudal orientation of axons within the plexus and the innervation of the circular muscle are presumed critical for generating a coordinated peristaltic response. However,

how subsets of neurons orient their projections within the plexus and reach their final targets is largely unknown.

DiI labeling of neuronal projections at E11.5-E12.5 shows that early in development the majority of growing neurites travel longitudinally in the caudal direction, while extension rostrally or circumferentially does not occur until later developmental time points (Fu et al. 2006; Burns et al. 2009). Early neurite orientation in the longitudinal vs. radial axis can be altered by BMP signaling. Treatment with BMP2/4 at E11.5 increased the percentage of fasciculated longitudinally-oriented fibers and decreased circumferentially-oriented fibers in whole gut cultures, whereas treatment with the BMP inhibitor noggin had the opposite effect (Fu et al. 2006).

In addition to broad neurite patterning during early development, there is evidence that axons of specific neuronal subpopulations are guided by specific molecular cues. Part of this evidence comes from mice overexpressing glial cell-line derived neurotrophic factor (GDNF) under the glial fibrillary acidic protein (GFAP) promoter which is first activated at E17 (Magill et al. 2010) These mice have an increased number of nitrergic myenteric neurons due to a GDNF-induced increase in proliferation of nitrergic precursors (Wang et al. 2010). They also display an increased density of nitrergic fibers surrounding glia within ganglia, but no increase in the number of nitrergic fibers innervating the circular muscle (Wang et al. 2010). This suggests that GDNF is inducing growth of new neurites that are selectively directed toward the GDNF-producing glia within ganglia. Importantly, two other types of myenteric neuron examined did not have altered neurite patterning in the GFAP-GDNF transgenic mice, suggesting that excess GDNF specifically directs neurites for NO producing neurons (Wang et al. 2010).

A recent study also suggests that the planar cell polarity proteins Celsr3 and Fzd3 regulate nitrergic axon trajectories. Nitrergic axons in WT mice at E12.5 primarily project caudally in the bowel (Fu et al. 2006). In contrast, *Celsr3* or *Fzd3* knockout mice have many neurites misoriented in the rostral or circumferential direction (Sasselli et al. 2013). Analysis of adult *Celsr3/Fzd3* knockouts also revealed subtle deficits in the thickness and orientation of neuronal nitric oxide synthase (nNOS) expressing interganglionic tracts (Sasselli et al. 2013). Selectivity in axon guidance is again suggested by the observation that the orientation of ChAT neurites is normal in *Celsr3* and *Fzd3* mutant mice. Using mosaic *Celsr3* mutants, these effects were shown to be cell autonomous, as only neurons that were missing *Celsr3* displayed aberrant axonal projection patterns (Sasselli et al. 2013). Additionally, guidance defects occurred without obvious accompanying differences in bowel colonization, precursor proliferation, or differentiation of various neuronal subtypes, suggesting a primary role in axonal pathfinding. Importantly, these subtle alterations in axon trajectories resulted in severe dysmotility, suggesting that precise ENS wiring is required for proper GI function.

Together, these studies demonstrate the existence of distinct molecular mechanisms that "wire the enteric nervous system." However, the actual cues responsible for guiding projections of most neuronal subtypes in the ENS to their final targets are still unknown. I therefore carried out broad gene expression profiling and identified 10 genes with known roles in axon guidance that are enriched in the ENS. One of these was the semaphorin guidance receptor, Plexin A4. I validated Plexin A4 ENS expression at E17.5 using *in situ* hybridization (ISH). I then used a commercial antibody for double label immunohistochemistry (IHC) and localized Plexin A4 to a subset of calretinin-positive neurons. Surprisingly, when I stained the ENS from Plexin A4 knockout (*Plexin A4* KO) mice I discovered that this antibody was non-specific - i.e., the staining

pattern with the "Plexin A4" antibody was similar in *Plexin A4* KO and WT mice, even though the *Plexin A4* KOs do not produce any Plexin A4 protein (Yaron et al. 2005). My work with the non-specific Plexin A4 antibody led directly to my studies of bowel muscularis macrophages (see Chapter 5).

I next assessed ENS morphology and functional motility in *Plexin A4* KO mice but was unable to detect any gross defects. In several peripheral and CNS circuits, Plexin A4 acts redundantly with other semaphorin receptors (Yaron et al. 2005). My array data shows that Plexin A2 is also enriched in the ENS at E17.5. Since Plexin A4 and Plexin A2 are both capable of mediating signals from class 3 semaphorins (Pasterkamp 2012), one reasonable hypothesis is that Plexin A2/A4 double mutants are required to generate an ENS phenotype.

4.2 Materials and Methods

4.2.1 Animals

The use and care of mice were accredited and approved by the Washington University Animal Care Committee and by The Children's Hospital of Philadelphia Research Institute Institutional Animal Care and Use Committee. *Wnt1Cre* mice (Danielian et al., 1998 RRID: IMSR_JAX:003829, C57BL/6; Swiss albino mixed background) and *R26R-EYFP* reporter mice (Srinivas et al., 2001; RRID: IMSR_JAX:006148*; C57BL/6J* background) were from The Jackson Lab. Plexin A4 knockout mice (Yaron et al., 2005, RRID: MGI_3795749) were kindly provided by Dr. Alex Kolodkin (Johns Hopkins University School of Medicine, Baltimore, MD). The morning of vaginal plug was considered embryonic day 0.5 (E0.5).

4.2.2 Antibodies and reagents

TuJ1 (rabbit, #PRB-435P, 1:10 000, Covance), Calretinin (rabbit, 1:2500, #AB5054, Chemicon), Plexin A4 (rabbit, 1:500, #NBP1-85128, Novus Biologicals).

4.2.3 Fluorescence activated cell sorting (FACS) of ENCDC

E17.5 whole bowel from *Wnt1Cre; R26R-EYFP* mice was dissected and dissociated into a single cell suspension as previously described (Sato and Heuckeroth, 2008). Briefly, whole bowel was treated with collagenase (0.5 mg/mL) and dispase (0.5 mg/mL) at 37°C for 15 minutes, triturated and filtered through a 40 μm cell strainer. Dissociated cells were suspended in HBSS and sorted based on EYFP fluorescence using a Beckman Coulter MoFlo sorter (Siteman Flow Cytometry Core, Washington University School of Medicine). EYFP-positive and EYFP-negative fractions were sorted directly into TRIzol® Reagent (Thermo Fisher Scientific #15596-026) and RNA was isolated according to the manufacturer's instructions.

4.2.4 Microarray and analysis

Microarrays were performed using Affymetrix gene chips that contain probes for 28,000 mouse genes (Genome Technology Access Center, Washington University School of Medicine). N=3 chips for EYFP-positive samples, N=4 chips for EYFP-negative samples. Gene expression data was analyzed using the Partek Genomics Suite. Data across multiple arrays was normalized using Robust Multi-array Average (RMA) and ANOVA was used to identify genes with $\geq$ 2-fold higher expression in the EYFP-positive fraction (ENCDC) versus the EYFP-negative fraction (non-ENCDC) at a false discovery rate of 0.05.

4.2.5 Whole mount preparation and immunohistochemistry

Whole mount immunohistochemistry (IHC) was performed as previously described (Wang et al., 2010). Briefly, gut was opened along the mesenteric border, pinned to Sylgard®, fixed (4% paraformaldehyde (PFA), 30 minutes, 25^{o}C), and then dissected to separate muscle layers from

submucosa. Peeled muscle layers were blocked in 5% donkey serum in PBS with 0.5% Triton (PBST) for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C. Samples were washed in PBS before incubating with secondary antibodies. After rinsing with PBS, samples were mounted in VECTASHIELD® mounting medium with DAPI (Vector Laboratories H-1200). After immunohistochemistry or NADPH diaphorase (NADPHD) staining (Neuhuber et al., 1994), quantitative analysis was performed.

4.2.6 Image acquisition and ENS quantification

Stained specimens were imaged using Zeiss Axio Imager.A2 or the Zeiss LSM 710 confocal microscope. Image processing was performed using ImageJ software and was limited to rotation, cropping, uniform brightness and contrast adjustments, and maximum intensity projection of volumes. Neuronal density was quantified by counting cells within twenty randomly selected 20x-fields per mouse. Fiber density was quantified as previously described (Wright-Jin et al. 2013). Briefly, we counted the number of thick fiber bundles and thin individual fibers crossing only the top or left side of each square 20X image acquired. At least 3 mice of each genotype were analyzed. Axonal branching was quantified using AngioTool software (Zudaire et al. 2011).

4.3 Results

4.3.1 Identification of genes with known roles in axon guidance that are enriched in the developing ENS.

To identify genes expressed in the ENS that might control axon pathfinding we used fluorescence activated cell sorting (FACS) to isolate cells from the E17.5 bowel of *Wnt1Cre; R26R-EYFP* mice that express YFP in enteric neurons, glia, and ENS precursors (Fig. 4.1). Broad microarray expression profiling was performed using Affymetrix gene chips. Gene expression levels in YFP-positive enteric neural crest derived cells (ENCDCs) were compared to expression in YFP-negative non-ENCDC surrounding cells of the bowel. Successful enrichment of the ENCDC population by FACS was confirmed by detection of positive control genes encoding various cytoskeletal proteins, transcription factors, and transmembrane receptors that are known to be expressed in the ENS (Table 4.1). Additionally, detection of transcripts specific to enteric neuron subtypes, such as certain neurotransmitter receptors, confirmed sufficient array sensitivity to detect genes enriched only within a small proportion of ENCDCs (Table 4.2). A total of 605 genes were found to be enriched in the ENS at E17.5. Ten of these encoded cell surface receptors, adhesion molecules, or ligands with known roles in axon pathfinding (Table 4.3). Seven out of these ten genes have not been previously described in the ENS. This list of seven genes includes several semaphorin receptors, with Plexin A4 being the most enriched.

4.3.2 *In situ* hybridization (ISH) and imunohistochemistry (IHC) characterization of Plexin A4 in the ENS.

ISH performed on E17.5 bowel sections confirmed ENS-enrichment of *Plexin A4* (Fig. 4.2A). Whole mount bowel IHC using a commercial antibody to Plexin A4 (Novus NBP1-85128) revealed Plexin-A4 immunoreactivity in a subset of enteric neurons at E17.5 (Fig. 4.2 B-

Figure 4.1

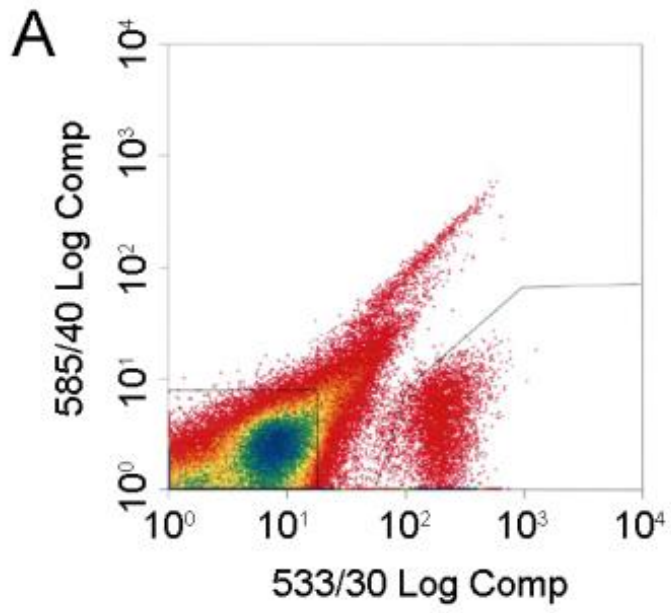

Figure 4.1. FACS of ENCDC from E17.5 *Wnt1Cre;R26REYFP* bowel.

Representative FACS plot demonstrating clean separation of EYFP-positive and EYFP-negative populations from E17.5 *Wnt1Cre;R26REYFP* bowel. X-axis represents YFP-fluorescence level, Y-axis represents auto-fluorescence level.

Table 4.1

Gene	Fold Difference (ENS/Non-ENS)	P-value
Tubb3 tubulin, beta 3	30.8741	2.91E-05
Snap25 synaptosomal-associated protein 25	19.9312	8.73E-06
Fabp7 fatty acid binding protein 7	29.6071	2.02E-05
Ascl1 achaete-scute complex homolog 1	21.5069	1.81E-06
Phox2b paired-like homeobox 2b	18.4885	5.43E-07
Sox10 SRY-box containing gene 10	5.72831	2.84E-05
Ret ret proto-oncogene	10.5607	1.61E-05
Ednrb endothelin receptor type B	5.06597	3.49E-06

Table 4.1. Enrichment of known ENS-specific transcripts. Fold differences and p-values for select positive control genes known to be enriched in the ENS. Data validates efficient FACS sorting of ENCDC and specificity of microarray results.

Table 4.2

Gene	Fold Difference (ENS/Non-ENS)	P-value
Vip vasoactive intestinal polypeptide	15.11	0.000423366
Nos1 nitric oxide synthase 1	7.01395	1.28E-05
Chrna3 cholinergic receptor, nicotinic, alpha polypeptide 3	34.3816	5.37E-06
Chrna4 cholinergic receptor, nicotinic, alpha polypeptide 4	2.04296	0.000169
Chrna5 cholinergic receptor, nicotinic, alpha polypeptide 5	8.45178	1.29E-05
Chrna7 cholinergic receptor, nicotinic, alpha polypeptide 7	2.91922	0.000299
Chrnb2 cholinergic receptor, nicotinic, beta polypeptide 2	3.16554	0.001204
Chrnb4 cholinergic receptor, nicotinic, beta polypeptide 4	18.4492	7.35E-06
Htr2b 5-hydroxytryptamine (serotonin) receptor 2B	3.10588	0.000223
Htr3a 5-hydroxytryptamine (serotonin) receptor 3A	8.89596	0.000113
Htr3b 5-hydroxytryptamine (serotonin) receptor 3B	2.23312	0.000913
Cnr1 cannabinoid receptor 1	4.6952	0.000568
Tacr3 tachykinin receptor 3	2.0943	0.003219
Gabrb3 gamma-aminobutyric acid	4.13815	0.000249
Gabrg2 gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	2.26019	0.000242056
Gabrg3 gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3	2.48097	0.00190814
Grik2	16.0038	5.72E-06

glutamate receptor, ionotropic, kainate 2 (beta 2)		
Grik3 glutamate receptor, ionotropic, kainate 3 (beta 3)	6.74	8.16E-05
Gria2 glutamate receptor, ionotropic, AMPA2	9.07007	5.50E-06

Table 4.2. Enrichment of enteric neuron subtype-specific transcripts. Fold differences and p-values for genes expressed only in some enteric neuron subtypes. Data validates sensitivity of microarray to lower abundance transcripts.

Table 4.3

Gene	Fold Difference (ENS/Non-ENS)	P-value	Known Ligands/Receptors
Ncam2* neural cell adhesion molecule 2	12.2577	1.94E-05	Ncam2 (homophillic)
L1cam* L1 cell adhesion molecule	11.6346	6.82E-05	L1cam (homophillic), Sema3A , Nrp1, Cntn2
Mdga2 MAM domain containing glycosylphosphatidylinositol anchor 2	10.444	9.15E-05	Mdga2 (homophillic)
Nrcam neuron-glia-CAM-related cell adhesion molecule	7.30816	3.67E-05	Sema3B, Sema3F, Sema6D, Nrp1
Plxna4 plexin A4	4.69611	4.23E-05	Sema3A, Sema3F
Slit1 slit homolog 1	3.44642	1.22E-05	Robo1, Robo2
Plxnb1 plexin B1	3.05096	3.85E-05	Sema3A, Sema3C
Plxna2 plexin A2	2.69268	0.000238	Sema3A, Sema3C, Sema3F
Sema3c semaphorin 3C	2.57924	0.000674	Nrp1, Nrp2, Plxna1, Plxna2, Plxnb1, Plxnd1,
Ncam1* neural cell adhesion molecule 1	2.4047	9.88E-05	Ncam1 (homophillic), GDNF

Table 4.3. Enrichment of genes with known roles in axon pathfinding. ENCDC enriched transcripts encoding cell surface receptors, adhesion molecules, or ligands with known roles in axon pathfinding. Asterisks indicate genes previously described in the ENS.

C) as well as in a subset of Iba1-positive muscularis macrophages in adult tissue (Fig. 4.2 D-F). Using this antibody we also found that Plexin A4 was localized to a subset of calretinin-positive axons present both in the myenteric plexus (Fig 4.3 A-C) as well as in the circular muscle (Fig 4.3 D-F). Unfortunately, IHC of the bowel of *Plexin A4* knockout mice that do not produce any Plexin A4 protein (Yaron et al. 2005) revealed identical staining patterns in the WT and KO using this antibody (Fig. 4.4). We attempted to clarify which neuronal subtypes express *Plexin A4* using two other published, non-commercial Plexin A4 antibodies (Yamashita et al. 2014; Sun et al. 2013), but were unable to obtain reliable signal in the ENS using either antibody.

4.3.3 Plexin A4 KO mice have no gross defects in ENS fiber patterning or neuron number.

Since both microarray and ISH results suggested *Plexin A4* enrichment in ENCDC, we decided to analyze neurite patterning in the ENS of *Plexin A4 KO* mice. No difference in TuJ1-positive fiber density was detected in the myenteric or submucosal plexus of *Plexin A4* KO mice (Fig.4. 5A-H). Additionally, we performed automated branch-point analysis using AngioTool software (Zudaire et al. 2011) but did not detect any differences in branching patterns of TuJ1-positive circular muscle fibers (Fig. 4.5I-K). Similarly, no differences in HuC/D-positive neuron numbers were seen in the myenteric or submucosal plexus of *Plexin A4* KO mice (Fig. 4.5.L-Q). To perform a more detailed analysis of ENS patterning in *Plexin A4* KO mice, we also analyzed the fibers of calretinin and nNOS expressing neurons. These neurotransmitters are found in largely non-overlapping enteric neuron subsets, with nNOS labeling primarily inhibitory motor neurons and calretinin labeling primarily excitatory motor neurons. Both neurotransmitters are also present in various interneurons, and calretinin is found in some IPANs (Furness et al. 2004). Using IHC to visualize calretinin and enzymatic NADPH-diaphorase staining to visualize nNOS

Figure 4.2

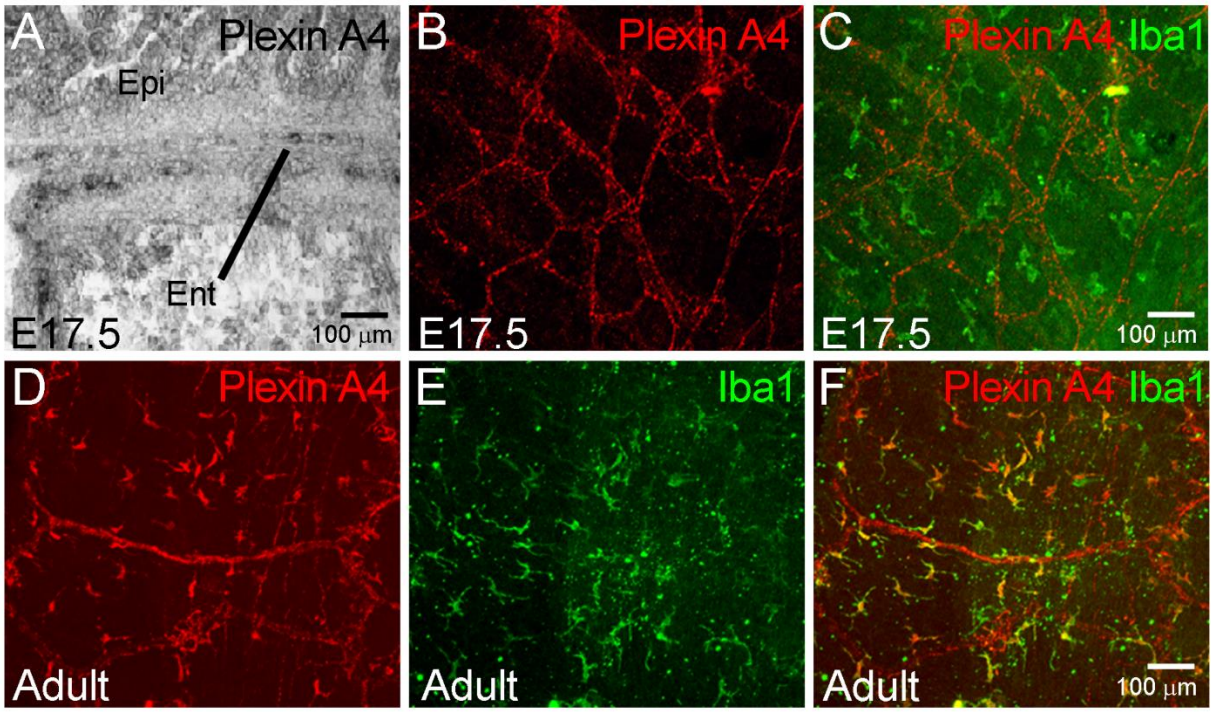

Figure 4.2. Confirmation of Plxna4 expression in the enteric nervous system.

(A) ISH demonstrating Plxna4 expression in E17.5 bowel sections in the region of the developing enteric nervous system (Ent) but not in epithelium (Epi). (B,C) Plexin A4-immunoreactivity was detected exclusively in a subset of enteric neuron fibers at E17.5. (D-E) Plexin A4-immunoreactivity in a subset of neuronal fibers persists into adulthood, but was also detected in Iba1-positive cells (D-F).

Figure 4.3

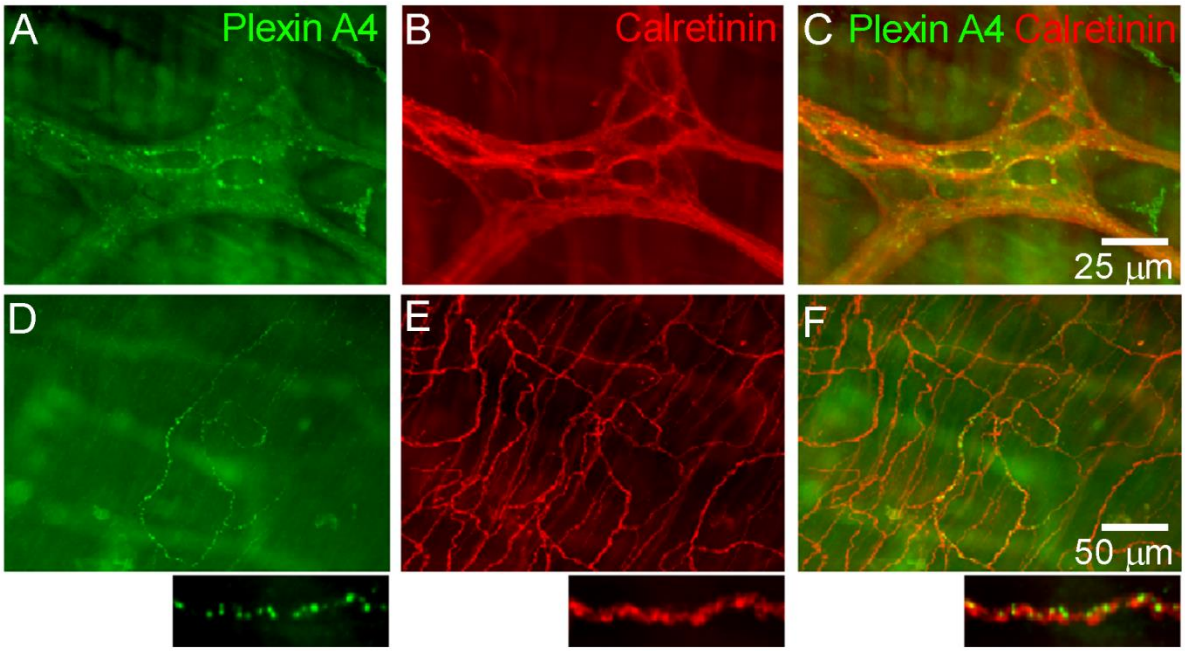

Figure 4.3. Plexin A4-immunoreactivity is found in a subset of calretinin-positive fibers in the ENS.

(A-C) Plexin A4-immunoreactive puncta co-localize with some but not all calretinin-positive fibers in the myenteric plexus as well as in the circular muscle (D-F). Insets represent 4X magnifications of fibers in D-F.

Figure 4.4

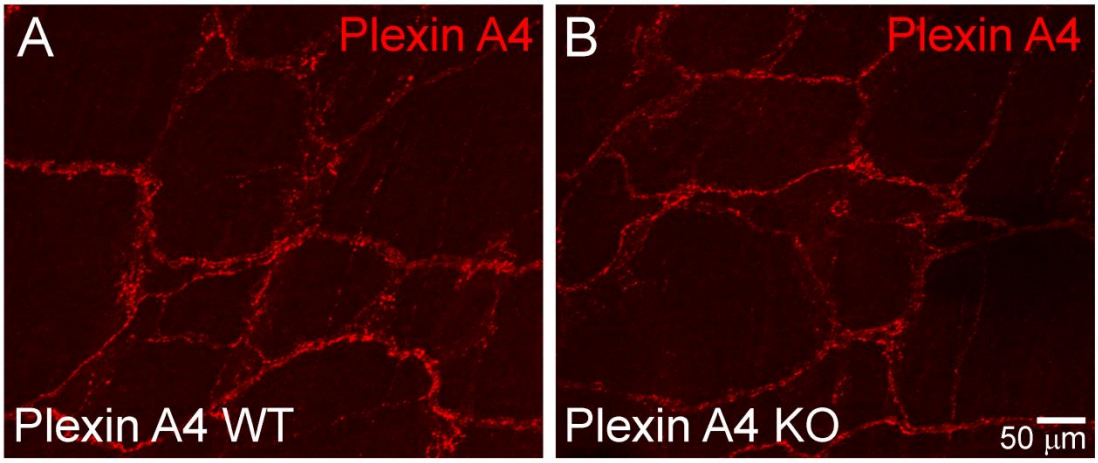

Figure 4.4. The Plexin A4 antibody, Novus NBP1-85128, is not specific to Plexin A4 protein.

(A-B) Immunoreactivity to the Novus NBP1-85128 anti-Plexin A4 antibody is unchanged in Plexin A4 knockout mice that are entirely missing Plexin A4 protein.

Figure 4.5

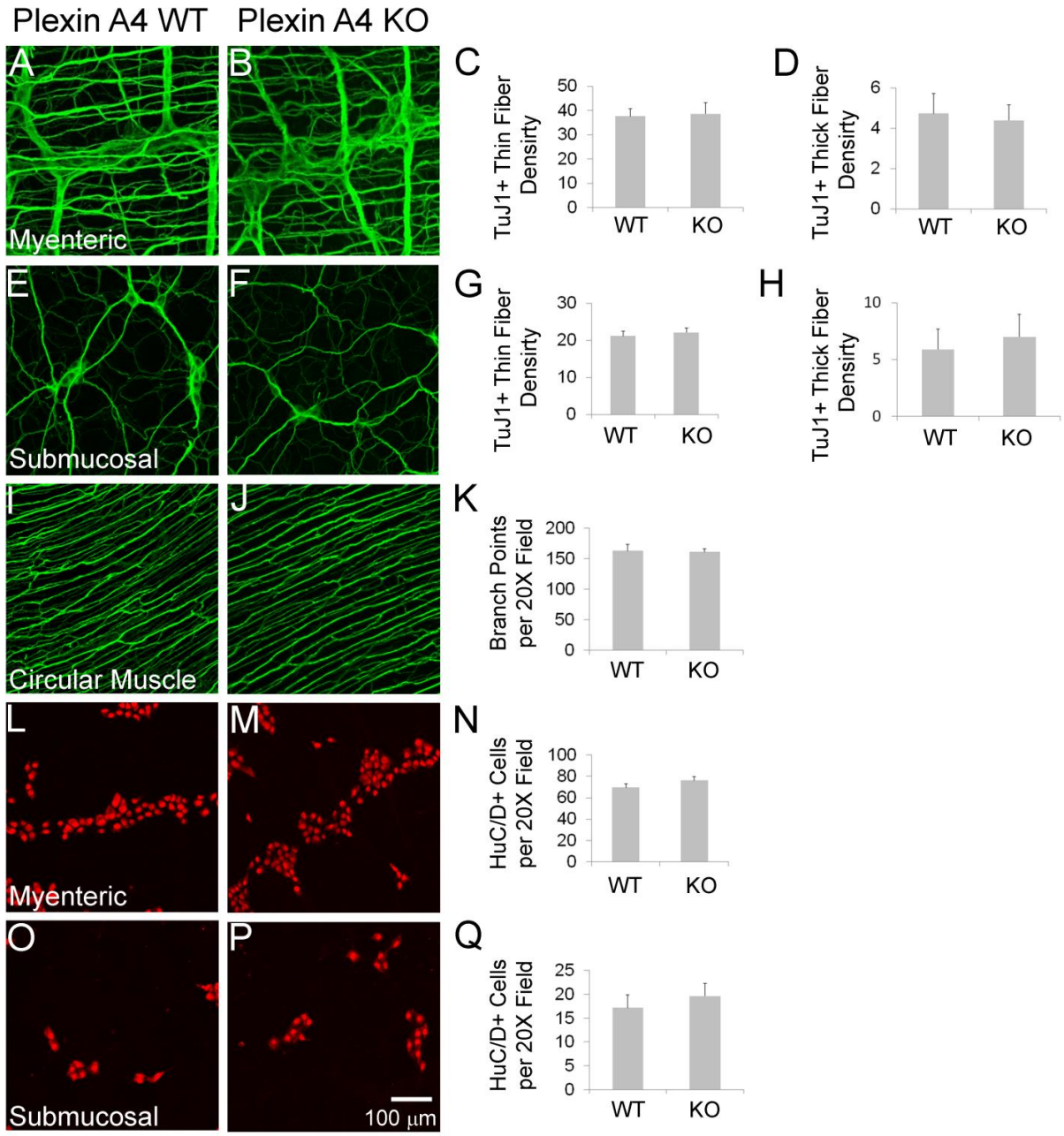

Figure 4.5. The enteric nervous system of Plexin A4 KO mice has no gross defects in neurite patterning or neuron number.

The myenteric (A-D) and submucosal (E-H) plexuses of Plexin A4 KO mice have normal density of TuJ1-positive thick fiber bundles and thin fibers. (I-K) Plexin A4 KO mice have no alterations in TuJ1-positive fiber branching in the circular muscle. Plexin A4 KO mice have normal numbers of HuC/D-positive neurons in the myenteric (L-M) and submucosal (O-P) plexuses. N = 3 KO, 3 WT.

neurons, we find no difference in the fiber density or neuron number of either neuronal population in *Plexin A4* KO mice (Fig. 4.6).

4.3.4 Plexin A4 KO mice have an intact peristalsis reflex.

Despite a lack of gross anatomic defects in the myenteric or submucosal plexus of *Plexin A4* KO ENS, it is possible that analysis with the broad markers used would not detect subtle patterning defects affecting only a small subset of neurons. Such subtle defects can still translate to severe motility problems, as is the case in *Celsr3* mutants (Sasselli et al. 2013). We therefore assessed motility in *Plexin A4* KO mice using an established *ex-vivo* peristalsis assay that tests ascending contraction and descending relaxation in response to stretch or to mechanical stimulation of the mucosal lining. We find that all sensory and motor components of the peristaltic reflex were intact in *Plexin A4* KO mice (Fig. 4.7 A-D).

Figure 4.6

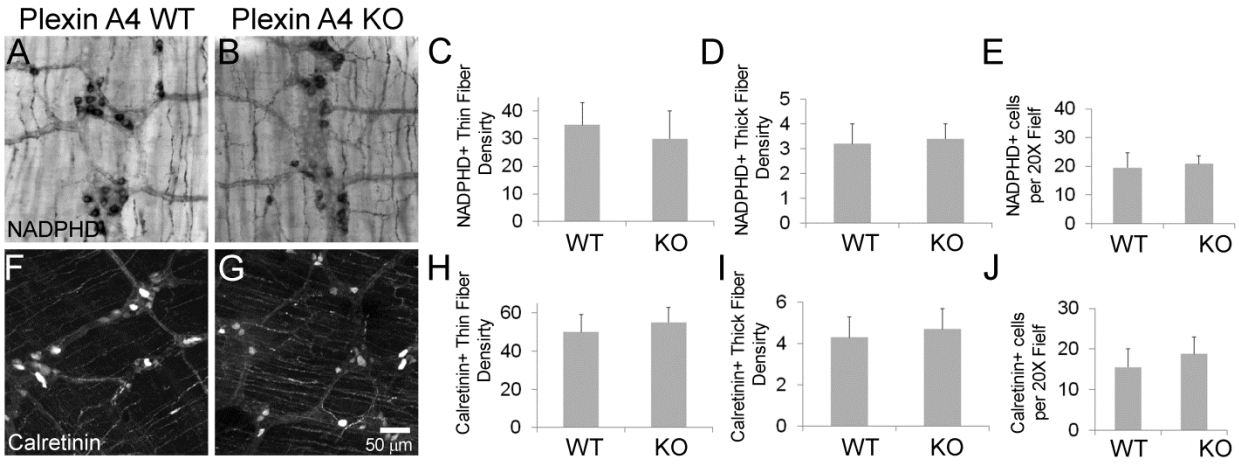

Figure 4.6. Plexin A4 KO mice have no defects in calretinin or nitrergic neurite patterning or neuron number.

(A-B) NADPHD labeling of nitrergic neurons shows no difference in fiber density (C-D) or nitrergic neuron number (E) in *Plexin A4* KO mice. (F-G) IHC for calretinin shows no difference in fiber density (H-I) or calretinin neuron number (J) in *Plexin A4* KO mice. N=3 KO, 3 WT.

Figure 4.7

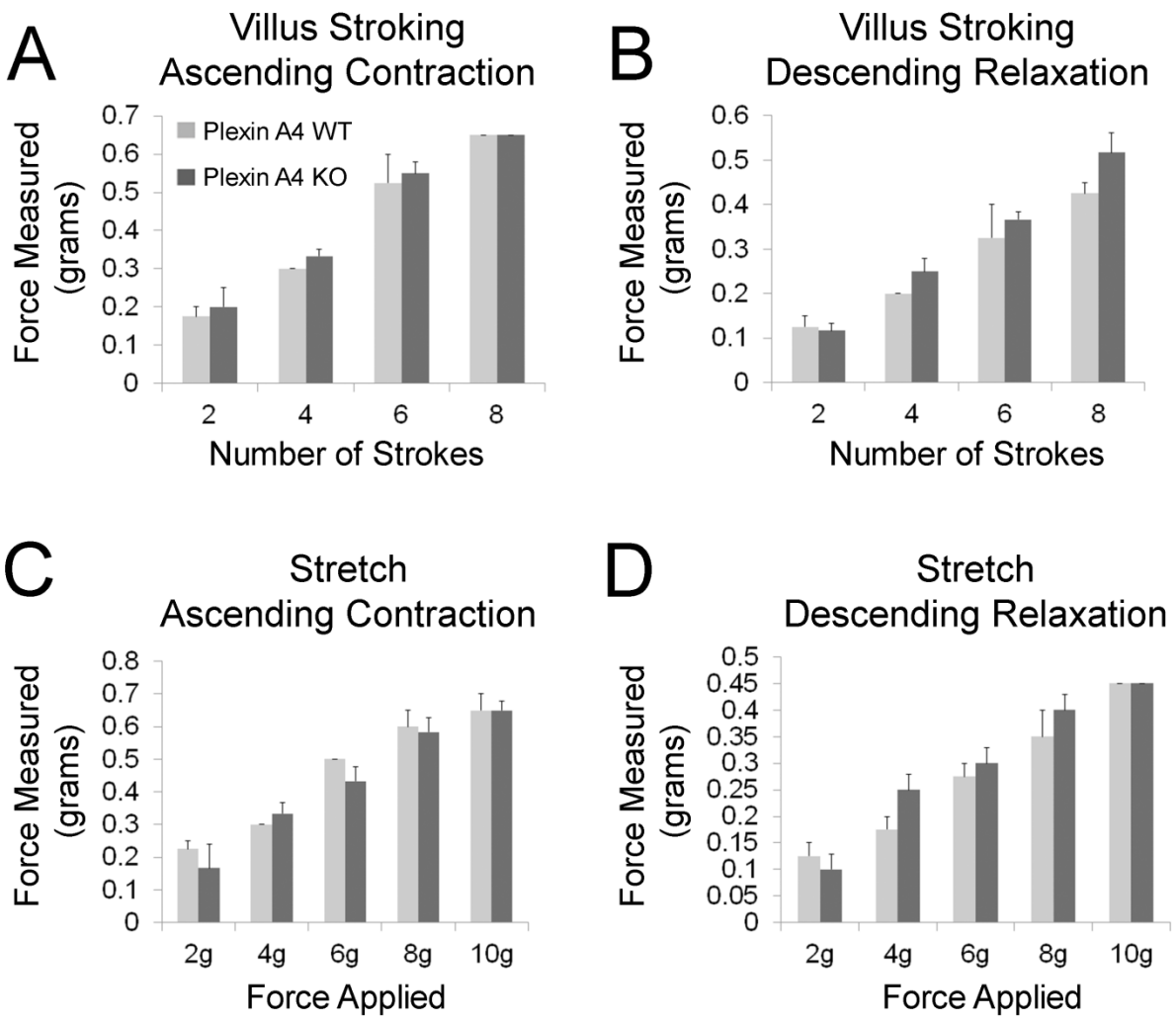

Figure 4.7. *Plexin A4* KO mice have an intact peristaltic reflex.

Mice lacking Plexin A4 exhibit normal ascending contraction and descending relaxation in response to mechanical stimulation of the villi (A-B) or bowel stretch (C-D). N=3 KO, 2 WT.

4.4 Discussion

Axons belonging to different neuronal subtypes in the ENS differ in their rostro-caudal and longitudinal vs. radial orientations, as well as in their ultimate targets (Sang et al. 1997; Hendriks et al. 1990) suggesting that neurons are equipped with mechanisms to sense directionality within the bowel and grow towards a particular target during development. The nature of these mechanisms remains largely unknown. We sought to identify which genes with known roles in axon guidance are enriched in the developing ENS, and assessed the role of one such gene, *Plexin A4*, in establishing ENS connectivity.

4.4.1 Novel axon guidance genes in the ENS.

Axon pathfinding relies on the coordinated interplay of guidance cues providing directional information and cell adhesion molecules that enable axonal progression. We therefore screened for genes encoding both guidance cue receptors and ligands, as well as adhesion molecules with known roles in axon navigation. We identified seven genes encoding cell surface receptors, adhesion molecules, or ligands with known roles in axon pathfinding that are enriched in the ENS at E17.5, a time when neural connectivity is being established in the bowel. Four of these genes encoded plexins or semaphorins. The remaining three included the cell adhesion molecules MDGA2 and NrCAM, and the ROBO receptor ligand, SLIT1.

MDGA2, a cell adhesion molecule of the IgCAM superfamily, is highly expressed in DRG as well as in dorsolateral commissural spinal interneurons (Joset et al. 2011). In response to chemoattractive cues produced by floorplate cells in the ventral midline of the developing spinal cord, dorsolateral interneurons project ventrally, cross the floorplate, and turn orthogonally to run alongside the floorplate into the longitudinal axis of the spinal cord (Kaprielian et al. 2000;

Joset et al. 2011). RNAi and binding studies suggest that homophilic MDGA2 interactions between ipsilaterally projecting axons and post-crossing dorsolateral interneuron axons are required for the orthogonal projection of axons following commissure crossing (Joset et al. 2011). Interestingly, many long-range guidance cues that instruct commissural crossing (Sonic hedgehog, netrins, Wnt) in concert with cell adhesion molecules, are also present during ENS development (Lake & Heuckeroth 2013; Avetisyan et al. 2015; Obermayr et al. 2013).

NrCAM, a cell adhesion molecule of the L1 family of immunoglobulin super family, has many diverse roles in neural development, including a role in axon guidance (Sakurai 2012). Mice missing NrCAM display wiring defects in several CNS circuits and exhibit mis-projection of cerebellar granule cells (Stoeckli 2010), defects in commissural axons crossing the ventral midline of the spinal cord (Charoy et al. 2012), defects in optic chiasm formation (Sakurai 2012), and mis-targeting of thalamocortical projections (Demyanenko et al. 2011). Interestingly, NrCAM can complex with the semaphorin co-receptor Neuropilin1 to mediate semaphorin signaling by Sema3B, Sema3F, and Sema6D (Pasterkamp 2012). NrCAM also represents a curious intersection between semaphorin and GDNF signaling. GDNF produced by floorplate cells in the ventral midline induces responsiveness of certain commissural spinal neurons to the midline repellent, Sema3B (Charoy et al. 2012). This is accomplished through suppression of calpain-mediated processing of the Sema3B co-receptor, Plexin A1. This effect on spinal neurons is mediated by GDNF signaling through Gfrα1/NrCAM complexes in a RET-independent manner (Charoy et al. 2012). GDNF-mediated regulation of enteric neuron competence to respond to various guidance cues is an interesting and unexplored area of research.

SLIT1 is a secreted glycoprotein guidance cue that signals through ROBO1/2 receptors to regulate axon guidance in a variety of systems (Long et al. 2004). SLIT/ROBO signaling controls formation of the lateral olfactory tract (Nguyen-Ba-Charvet et al. 2002), guidance of midbrain dopaminergic axons through the diencephalon (Dugan et al. 2011), axon pathfinding within the postoptic commissure in the forebrain (Miquelajáuregui et al. 2014), motor axon pathfinding in the hindbrain (Hammond et al. 2005), retinal ganglion cell axon guidance (Thompson et al. 2006), and midline commissural axon crossing in the spinal cord (Long et al. 2004). In all of these examples, SLIT1 is produced in the surrounding environment and acts on ROBO receptors in neurons. However, we see an enrichment of the SLIT1 ligand in developing ENS cells. It is possible that *Slit1* is enriched and secreted by enteric glia, which are also labeled in the *Wnt1Cre; R26R-EYFP* reporter mouse. Alternatively, *Slit1* expression by neurons has been shown to mediate migration of newly born neurons in the adult rostral migratory stream (Kaneko et al. 2010). SLIT1 production by these new neurons triggers ROBO-mediated repulsion of astrocyte processes, creating "astrocytic tunnels" that allow for neuronal migration in the adult brain (Kaneko et al. 2010). *Slit1* expression by enteric neurons could serve a similar clearing function, allowing for movement of cell bodies or axons within an already formed plexus.

4.4.2 Plexin A4 signaling in the ENS

Plexins and semaphorins represent the largest group of axon guidance molecules enriched in the developing ENS, with Plexin A4 enriched nearly 5-fold compared to non-ENS surrounding cells in the bowel. Binding of semaphorins to plexin and neuropilin receptors induces activates the plexin GTPase-activating protein (GAP) domain (Hota & Buck 2012; Pasterkamp 2012) and downstream signaling through protein kinases, GTPases and cytoskeleton-associated proteins that cause alterations in growth cone dynamics. Multiple

semaphorin ligands can activate a single plexin receptor, and multiple plexin receptors are responsive to a single semaphorin ligand (Pasterkamp 2012). *Plexin A4* knockout mice have mild but detectable defects in the trajectories of peripheral sensory axons, sympathetic axons, and cranial axons. These defects are far more severe in *Plexin A4/A3* double knockouts (Yaron et al. 2005). *Plexin A4* KO mice also have severe defects in the anterior commissure that are not enhanced in *Plexin A4/A3* double knockouts (Yaron et al. 2005), suggesting that plexins function redundantly in some but not all neural circuits.

Since ISH of E17.5 bowel sections confirmed high levels of Plexin A4 expression in the ENS, we decided to assess the ENS of *Plexin A4* KO mice. However, we were unable to detect any morphological defects in axon patterning or functional motility defects in peristalsis. It is possible that Plexin A4 functions redundantly with Plexin A2, which is also enriched in the ENS. Plexin A2 and A4 are both capable of transmitting signals from Sema3A, Sema3C, Sema3F, as well as Sema6A (Hota & Buck 2012), so it is conceivable that unmasking an ENS phenotype would require *Plexin A2/A4* double knockouts. Alternatively, *Plexin* A4 single knockouts may have an ENS phenotype that is too subtle to detect with the broad staining methods used in our studies. Detection of subtle patterning defects, such as those that may be present if only a small subset of enteric neurons is affected, may require tracing of individual axons from *Plexin-A4* expressing neurons.

4.4.3 ENS connectivity and disease.

Defects in ENS connectivity may be present in several enteropathies characterized by subtle abnormalities in plexus organization or smooth muscle innervation (Di Nardo et al. 2008) . An example of such an enteropathy is the rare congenital disorder called chronic idiopathic

intestinal pseudoobstruction (CIIP). CIIP is a poorly understood but highly morbid syndrome characterized by impaired GI propulsion and symptoms of intestinal obstruction in the absence of any lesion or physical blockage in the gut lumen (Amiot et al. 2009; Faure et al. 1999). Altered connectivity may also contribute to certain cases of irritable bowel syndrome (IBS), a disorder that features abdominal pain and altered intestinal motility and affects 10-15% of the USA population (Ohman & Simrén 2010; Agarwal & Spiegel 2011). Additionally, enteric neuron death can occur as a consequence of infectious agents, neurodegenerative disorders such as Parkinson's disease, and metabolic diseases such as diabetes (Obermayr et al. 2013). Successful regenerative therapies for these conditions must include strategies to reestablish proper connectivity in addition to replenishing neuron number. Such strategies would be greatly informed by knowledge of how ENS connectivity is established during development.

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Chapter 5: Muscularis macrophage development and the ENS.

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5.1 Introduction

Muscularis macrophages (MMs) are tissue resident macrophages found within and between the circular and longitudinal muscle layers of the bowel wall. These cells interact closely with enteric neurons in the myenteric plexus (Gabanyi et al. 2016; Phillips & Powley 2012) and represent a unique population of bowel macrophages that differs morphologically (Mikkelsen et al. 2011), transcriptionally (Gabanyi et al. 2016), and likely functionally from neighboring macrophages in the bowel mucosa and lamina propria.

Tissue resident macrophages were initially thought to be derived from adult blood circulating monocyctes. Elegant lineage tracing studies have subsequently shown that beginning at E8.5, all fetal tissues are initially seeded by macrophage progenitors from the yolk-sac (Hoeffel & Ginhoux 2015). At E12.5, a second wave of progenitors originating in the fetal-liver seeds all fetal tissues, with the exception of the brain which is isolated by the blood-brain barrier by E13.5 (Hoeffel et al. 2015; Daneman et al. 2010). These fetal-liver progenitors differentiate into macrophages and eventually outnumber yolk-sac derived macrophages in all tissues except the brain (Hoeffel & Ginhoux 2015; Ginhoux & Jung 2014; Ginhoux et al. 2010). Embryonically-derived macrophages retain the capacity to self-renew *in situ*, and at steady state will not be replaced by circulating bone-marrow derived monocytes (Hoeffel & Ginhoux 2015). The bowel is a notable exception to this developmental scheme, and it appears that constant

monocyte recruitment is required to maintain the mucosal and lamina propria macrophage population over time (Bain et al. 2014). Lineage tracing studies indicate that numbers of embryonically-derived macrophages in the bowel decrease with age in mice (Bain et al. 2014). Additionally, aged mice lacking the CCL2 chemokine receptor, CCR2, which is required for monocyte infiltration into the bowel (Kurihara et al. 1997) have dramatically reduced numbers of intestinal macrophages (Bain et al. 2014). Interestingly, we find that numbers of MMs in aged CCR2 knockout mice remain stable, suggesting a different maintenance mechanism for this macrophage population in the bowel. These findings further highlight the emerging idea that MMs represent a unique population of bowel macrophages.

Initial characterizations of muscularis macrophages in the muscle layers of the bowel reported frequent tight contact points between macrophage processes and the surface of myenteric neurons in a "lock-and-key" configuration (Mikkelsen et al. 2004; Phillips & Powley 2012) This suggested close communication between these two cell types, however the exact signals being exchanged are just beginning to be characterized. A recent study reported that muscularis macrophages produce BMP2 which alters enteric neuron activity and subsequently bowel motility (Muller et al. 2014). The same study reports that enteric neurons appear to be the main source of the MM survival factor CSF1 in the adult bowel. Several groups have now reported that neuronal signaling within the bowel skews MM phenotype towards an anti-inflammatory, tissue protective one (Matteoli et al. 2014; Gabanyi et al. 2016). A cholinergic anti-inflammatory pathway has been described in which vagal parasympathetic efferents signal onto cholinergic enteric neurons to reduce Ca^{2+} influx and cytokine production within MMs in a α7nAChR-dependent manner (Matteoli et al. 2014). Additionally, sympathetic efferents from the celiac and superior mesenteric ganglia release norepinephrine onto β2-adrenergic receptors on

MMs to initiate an "anti-inflammatory" transcriptional profile (Gabanyi et al. 2016). However, there is also evidence that neuronal signals, such as CGRP, serve as pro-inflammatory agents for muscularis macrophages (Glowka et al. 2015), and it is likely that MMs integrate both pro- and anti-inflammatory cues originating in specific neuronal subtypes.

Investigation of neuronal signaling onto MMs has thus far been restricted to analysis of adult animals. However, MMs and enteric neurons exist in close contact for days within the developing bowel. *Op/Op* mice with mutations in *Csf1* and consequent macrophage depletion have defects in adult ENS patterning (Muller et al. 2014; Thuneberg 1999), suggesting that developmental signaling between these two cell types can have lasting consequences. Clarifying the developmental interplay between enteric neurons and muscularis macrophages may be of particular relevance to understanding what role, if any, MMs have in the pathogenesis of Hirschsprung's associated enterocolitis (HAEC). Hirschsprung disease (HSCR) is a birth defect where the enteric nervous system is absent from distal bowel. HAEC is severe and persistent bowel inflammation in the context of Hirschsprung's disease that is the leading cause of death in babies with this disease (Gosain 2016).

To determine if enteric neurons are important for aspects of MM development, we analyzed the bowel of *Ret* KO mice that are missing enteric neurons throughout the digestive tract (Schuchardt et al. 1994). These mice provide a good model for studying muscularis macrophage development in the absence of enteric neural crest derived cells (ENCDC), including enteric neurons. Sympathetic innervation in the bowel of these animals is also defective in these animals (Enomoto et al. 2001). We find that, surprisingly, Ret KO muscularis externa devoid of enteric neurons contains normal numbers of well-patterned MMs that are phenotypically similar to control neonatal MMs. Consistent with this, we show that the main

source of CSF1 in the developing bowel is non-neuronal. LPS stimulation of muscularis externa also failed to produce a differential response in *Ret* KO vs control tissue. Together these findings argue that the neuronal circuitry responsible for modulating MM activation status in adults is not present or fully mature in the newborn bowel. This in turn suggests that there is a critical post-natal time period during which this neuro-immune circuitry develops and may be perturbed by environmental factors.

5.2 Materials and Methods

5.2.1 Animals

The use and care of mice were accredited and approved by the Children's Hospital of Philadelphia Research Institute Institutional Animal Care and Use Committee. The morning of vaginal plug was considered embryonic day 0.5 (E0.5). Ret-TGM mice (RRID:MGI_3623107, called Ret KO), were previously described (Enomoto et al. 2001). Ccr2 KO mice have been previously described (Boring et al. 1997) and were from Jackson Labs (stock #017586). Cx3cr1-EGFP mice were from Jackson Labs (stock #005582).

5.2.2 Generation of Ednrb-GFP-L10a mice

Ednrb-GFP-L10a mice were made by cloning a GFP-L10a fusion construct (Heiman et al. 2008) into the multiple cloning region of a vector containing the promoter and enhancer sequences of endothelin receptor B fused to splicing donor and acceptor sequences of the rabbit β-globin gene containing an intron and polyA sequences (Nishiyama et al. 2012) using In-Fusion (Clontech) cloning reagents (Sup. Fig 5.2). This vector was 87 injected into the pronucleus of hybrid CBA;C57BL/6 fertilized oocytes. Founders were screened by PCR using the primers 5'-GCACGACTTCTTCAAGTCCGCCATGCC-3' (forward) and 5'-

CGGATCTTGAAGTTCACCTTGATGCC-3' (reverse). Sixteen founder mice were obtained and screened for adult and embryonic ENS expression. A single founder line (line 16) was bred to C57BL/6 mice to produce embryonic mice for these studies. *Ednrb-GFP-L10a* transgenic mice were subsequently identified by GFP fluorescence.

5.2.3 Antibodies and reagents

Primary antibodies: Iba1 (rabbit, Wako Cat# 019-19741, RRID:AB_2313566, 1:500), TuJ1 (rabbit, Covance Research Products Inc Cat# PRB-435P-100, RRID:AB_10063850, 1:10,000), TuJ1 (chicken, Aves Labs Cat# TUJ, RRID:AB_2313564, 1:500), HuC/D (human ANNA–1 antiserum kindly provided by Vanda Lennon, Mayo Clinic, RRID:AB_2313944, 1:2000), Secondary antibodies: donkey anti-rabbit, goat anti-chick, goat anti-human Alexa fluor-488, -594, or -647, Invitrogen, 1:400).

5.2.4 Whole mount muscularis externa preparation and immunohistochemistry

Whole mount immunohistochemistry (IHC) was performed using P0 or 8-week old mice as previously described (Wang et al. 2010). Briefly, gut was opened along the mesenteric border, pinned to Sylgard®, fixed (4% paraformaldehyde (PFA), 30 minutes, 25^{o}C), and then dissected to separate muscle layers from submucosa. Peeled muscle layers were blocked in 5% donkey serum in PBS with 0.5% Triton (PBST) for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C. Samples were washed in PBS before incubating with secondary antibodies. After rinsing with PBS, samples were mounted in VECTASHIELD® mounting medium with DAPI (Vector Laboratories H-1200). For P0 bowel, only distal small intestine muscle layers could be dissected to reliably generate samples for immunohistochemistry (IHC) and imaging.

5.2.5 Image acquisition and analysis for *in situ* quantification of muscularis macrophages

Stained specimens were imaged using confocal microscopy (Zeiss LSM 710) to capture the full thickness of the muscle layers of the bowel. Macrophages were counted in three separate planes within the muscularis (circular muscle plane, myenteric plexus plane, and longitudinal muscle plane) by counting cells within twenty randomly selected 20x-fields per mouse. For P0 bowel only ten randomly selected 20x fields were counted because of the small amount of tissue available. At least 4 mice of each genotype were analyzed. Image processing was performed using ImageJ software and was limited to rotation, cropping, uniform brightness and contrast adjustments, and maximum intensity projection of volumes.

5.2.6 Flow cytometric analysis of muscularis macrophages from P0 bowel

P0 bowel muscle layers were dissected and digested in collagenase (0.5 mg/mL) and dispase (0.5 mg/mL) in HBSS at 37 ^{o}C for 20 minutes. Digested samples were triturated using a P1000 pipette and filtered through a 40 µm cell strainer before proceeding to staining. Cells were stained with LIVE/DEAD Aqua fixable viability dye (ThermoFisher) in PBS for 30 minutes at 4 ^{o}C, Fc blocked with purified CD16/CD32 antibody (clone 2.4G2) for 15 minutes at 4 ^{o}C, washed, and stained with fluorophore-conjugated antibodies in PBS/2%FBS for 30 minutes at 4 ^{o}C. The following antibodies were used for staining: CD11b APC Cy7 (clone M1/70), CD40 FITC (clone 3/23), CD86 APC (GL1), and MHC Class II (I-A/I-E) PE (clone M5/114.15.2) from BD Pharmingen; F4/80 PerCP Cy5.5 (clone BM8) and CD115 (CSF1R) PE Cy7 (clone AFS98) from eBioscience. All samples were acquired on a MACSQuant flow cytometer (Miltenyi Biotec) and analyzed using FlowJo software version 9.9 (Tree Star). Gating strategy was adapted from (Koscsó et al. 2015).

5.2.7 *Ex vivo* LPS stimulation

P0 distal small bowel muscularis whole mount preparations were performed in designated Sylgard plates to avoid LPS contamination. Unfixed peeled muscularis samples were briefly dipped in 3% hydrogen peroxide in 1X PBS, then rinsed with PBS and placed into 24-well plates with 200 μL Iscove's DMEM with or without 100 ng/mL LPS (Sigma #L4391). After incubation at 37°C for 6 hours gut specimens were processed for qRT-PCR. Supernatants were analyzed for IL-6 and TNFα by OptEIA ELISA (BD Biosciences #555268, #555240, and #555256, respectively) according to the manufacturer's instructions.

5.2.8 qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen #74104) and reverse-transcribed using SuperScript II Reverse Transcriptase for cDNA synthesis (Invitrogen #18064014). qRT-PCR was performed on at least three biological replicates with three technical replicates per run using SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad #1725211) and Bio-Rad Cycler CFX96. Primers are listed in Table 5.1.

Table 5.1

Primers for qRT-PCR				
Accession Number	Gene Symbol	Unigene Title		Sequences [a]
NM_023279.2	*Tubb3*	Tubulin, beta 3 class III	f r	TAGACCCCAGCGGCAACTAT GTTCCAGGTTCCAAGTCCACC
NM_011437.1	*Sox10*	SRY-box containing gene 10	f r	GACCAGTACCCTCACCTCCA CCTCCAGAGCTTGCCTAGTG
NM_001136061.2	*Ednrb*	Endothelin receptor type B	f r	ACCGTGGTTTAACGCCATAG GTATGCCTCCGAGAGAGCTG
NM_007392	*Acta2*	Alpha smooth muscle actin	f r	GTCCCAGACATCAGGGAGTAA TCGGATACTTCAGCGTCAGGA
NM_001113529.1	*Csf1*	Colony stimulating factor 1	f r	AGTATTGCCAAGGAGGTGTCAG ATCTGGCATGAAGTCTCCATTT
NM_001314054.1	*Il6*	Interleukin 6	f r	TGGGGCTCTTCAAAAGCTCC AGGAACTATCACCGGATCTTCAA
NM_001278601.1	*Tnfα*	Tumor necrosis factor	f r	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
NM_008084	*Gapdh*	glyceraldehyde-3-phosphate dehydrogenase	f r	AAC TTT GGC ATT GTG GAA GG GTC TTC TGG GTG GCA GTG AT
[a] 5’ to 3’sequences; f = forward primer; r = reverse-strand primer				

Table 5.1. Primers for qRT-PCR

5.3 Results

5.3.1 Muscularis externa colonization by muscularis macrophages during development does not require enteric neural crest derived cells (ENCDC).

ENCDC that become neurons and glia of the ENS first invade the bowel foregut at E9.5 and then migrate through the bowel in a rostrocaudal direction towards the end of the colon (Burns & Thapar 2006). By E12.5 ENCDC reach mid-colon and by E14.5 the bowel is fully colonized by ENS precursors. MMs have been reported in the bowel as early as E15.5 (Mikkelsen et al. 2004), but whether MMs are present in bowel prior to ENCDC colonization was not known. Since adult enteric neurons produce CSF1 that supports MMs, we hypothesized that ENCDC should colonize fetal bowel before MM to create a hospitable environment. To test this hypothesis, we used the neuronal marker TuJ1 to label enteric neurons. TuJ1 identifies all regions of the bowel that contain ENCDC (Bergner et al. 2013; Lake et al. 2013; Schill et al. 2015) since enteric neurons differentiate alongside migrating ENCDC. MMs were identified with the Iba1 antibody that is commonly employed for microglia staining in the central nervous system. We confirmed that Iba1 antibody labels all MMs in developing bowel by staining *Cx3cr1-EGFP* reporter mice that express EGFP in all bowel macrophages (Sup. Fig. 5.1). Iba1+ cells were present in the outer bowel wall in the region where muscle layers develop as early as E9.5 (Fig 5.1 A). At E12.5, muscularis macrophages are present throughout the small bowel alongside enteric neurons (Fig. 5.1B). MMs are also present in the distal colon, which is devoid of ENCDC at this age (Fig. 5.1C) suggesting that colonization of fetal bowel by MM does not require the ENS. To more definitively determine if the ENS is needed for MMs to colonize the bowel, we analyzed the muscularis externa of neonatal (P0) *Ret* knockout (*Ret* KO) mice that are completely missing

Figure 5.1

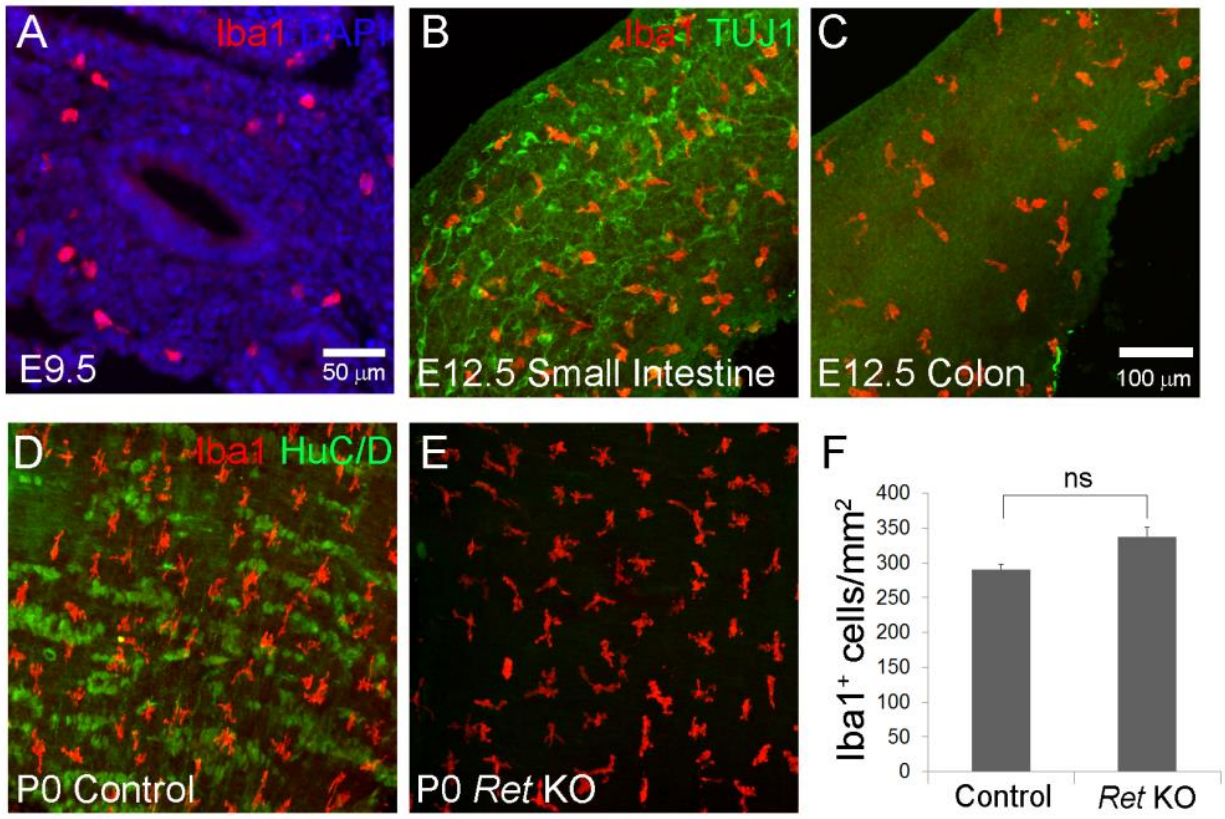


Figure 5.1. Muscularis macrophages colonize the bowel with or without enteric neurons.

(A) Iba1+ macrophages are present in the foregut as early as E9.5. **(B)** At E12.5, macrophages exist alongside the developing ENS in the small intestine and are also present in the colon, which is not yet colonized by ENCDC **(C). (D-E)** Distal small intestine of *Ret* knockout bowel is devoid of enteric neurons or other ENS lineage cells, but has well patterned muscularis macrophages present in normal abundance **(F)** (Student's T-test $p > 0.05$. N=4 mice per genotype). Scale bar in C applies to B-E.

enteric neurons and glia in the small bowel and colon. Surprisingly, *Ret* KO mice had normal numbers of MMs and the MM distribution in P0 bowel closely matched that in littermate controls where enteric neurons were visualized with HuC/D antibody (Fig. 5.1 D-F.)

5.3.2 The developing bowel contains a non-neuronal source of the muscularis macrophage survival factor, CSF1.

CSF1 is a key survival factor for many macrophage populations, including those in the muscularis externa. Mice lacking CSF1 (*Op/Op* mice) are completely devoid of MMs (Thuneberg 1999). It has been suggested in adults that enteric neurons are the primary source of CSF1 for MMs (Muller et al. 2014). However, our finding of well patterned MMs in mice lacking enteric neurons strongly suggests that alternative or additional sources of CSF1 exist, at least in neonatal and fetal mouse bowel. To determine whether enteric neurons are the main source of CSF1 in the developing bowel we used fluorescence activated cell sorting (FACS) to separate enteric nervous system cells from other components of the E12.5 mouse bowel wall. For this analysis we used a new mouse line (*Ednrb-EGFP-L10a*) in which all enteric neurons and precursors express EGFP fused to ribosomal protein L10A (Fig. 5.2 A-E). qRT-PCR on RNA isolated from EGFP-positive and EGFP-negative cells of the E12.5 bowel demonstrated successful enrichment for ENS expressed transcripts and depletion of non-ENS transcripts in the EGFP+ bowel cells (Fig. 5.2 F). We find that *Csf1* is highly enriched in the non-ENS EGFP-negative fraction (Fig. 5.2 G), consistent with a primarily non-ENCDC source of CSF1 during fetal development.

5.3.3 Absence of enteric neurons does not affect the surface phenotype or baseline activation state of neonatal muscularis macrophages.

Enteric neurons appeared to be dispensable for the survival and patterning of muscularis macrophages, but it was unclear whether they were required for normal macrophage phenotype

Figure 5.2

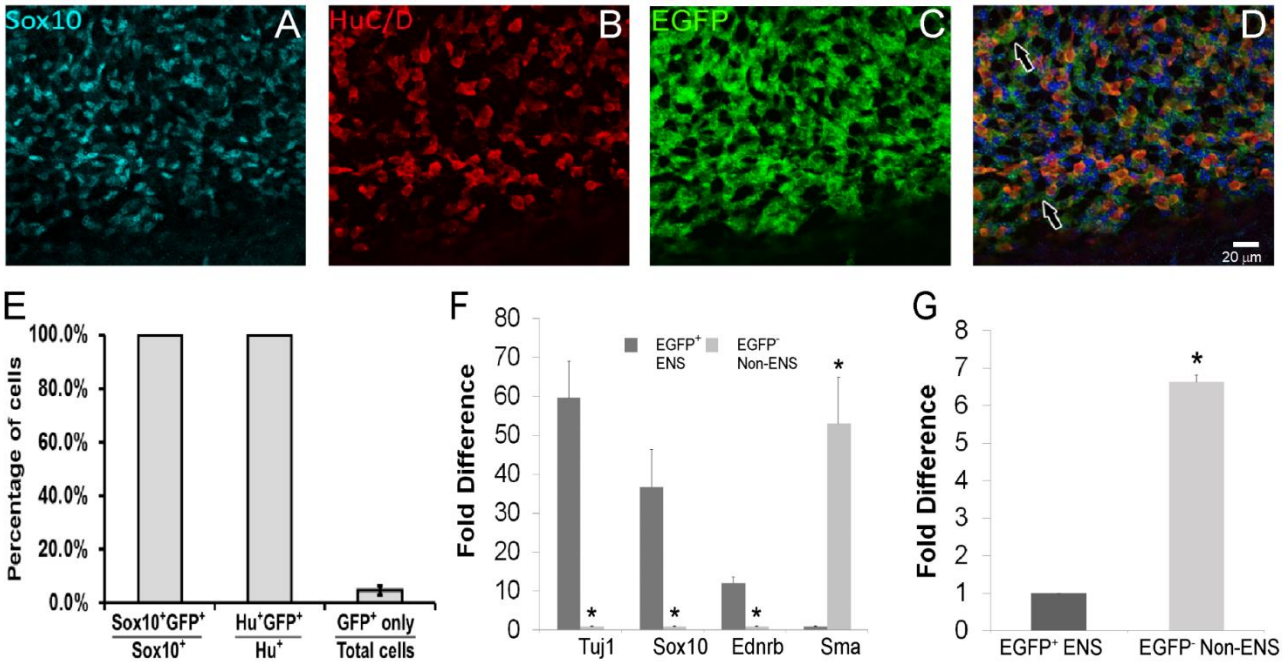


Figure 5.2. *Csf1* is predominantly expressed in non-ENS cells of fetal bowel as shown by FACS sorting ENCDC from *Ednrb-EGFP-L10a* mice that express *EGFP* in all ENS precursors.

(A-D) Immunohistochemistry for SOX10 and HuC/D, almost completely overlaps EGFP-L10A fluorescence in E12.5 small bowel of *Ednrb-EGFP-L10a* mice. 100% of SOX10 or HuC/D-positive cells express EGFP-L10A. **(D)** Rare EGFP-L10A-positive cells are SOX10- and Hu C/D-negative (arrows). **(E)** Quantification of immunohistochemistry data (N=3 mice). **(F)** Analysis of mRNA levels for *TuJ1*, *Sox10* and *Ednrb* (ENS lineage markers) and for Sma (expressed in smooth muscle) by qRT-PCR demonstrated that FACS sorting bowel cells from *Ednrb-EGFP-L10a* mice using EGFP-L10A fluorescence efficiently separated ENS precursors from other cells in the bowel wall. **(G)** *Csf1* is expressed at higher levels in non-ENS cells as compared to ENS cells in the E12.5 bowel wall based on qRT-PCR analysis (N=4 biological replicates for each gene). * $p<0.01$, Student's T-test.

or function. To assess the baseline activation state, and thus the immunostimulatory potential of muscularis macrophages developing in the absence of enteric neurons, we analyzed MMs from control and *Ret* KO mice by flow cytometry. $CD11b^{+}F4/80^{+}$ muscularis macrophages were readily detectable (Fig. 5.3A) and were present at similar frequencies in both control and *Ret* KO mice (Fig. 5.3B), consistent with our immunohistochemistry data. As further confirmation of their macrophage identity, *Ret* KO $CD11b^{+}F4/80^{+}$ cells expressed equivalently high levels of CSF1R as control macrophages (Fig. 5.3C), suggesting these cells are equally poised to respond to the key survival factor CSF1. Interestingly, expression of the co-stimulatory molecules CD40 and CD86, as well as major histocompatibility complex (MHC) class II, was unaltered in mice lacking enteric neurons (Fig 5.3D). Similar to control macrophages, *Ret* KO macrophages expressed these activation markers at relatively low levels, consistent with a non-activated phenotype. Of note, while MMs have previously been characterized as MHC II^{high} in adult mice (Muller et al. 2014), the low levels of MHC II seen in our study are likely a result of age-related differences, as MHC II is generally not expressed by MMs in neonatal mice (Mikkelsen et al. 2004). Thus, our data demonstrate that muscularis macrophages developing in the absence of enteric neurons are phenotypically intact. Furthermore, enteric neurons are dispensable for establishing the resting phenotype of muscularis macrophages in neonatal mice.

5.3.4 Muscularis macrophages from neonatal mice lacking enteric neurons did not differ from control mice in their activation by LPS stimulation.

To determine if muscularis macrophages from mice without an ENS would manifest a different response from WT MMs when challenged with LPS, we dissected out P0 *Ret* KO or littermate control muscularis externa and cultured for 6 hours in the presence or absence of LPS. Following treatment, the tissue was assayed for expression of IL-6 and TNFα cytokines using

Figure 5.3

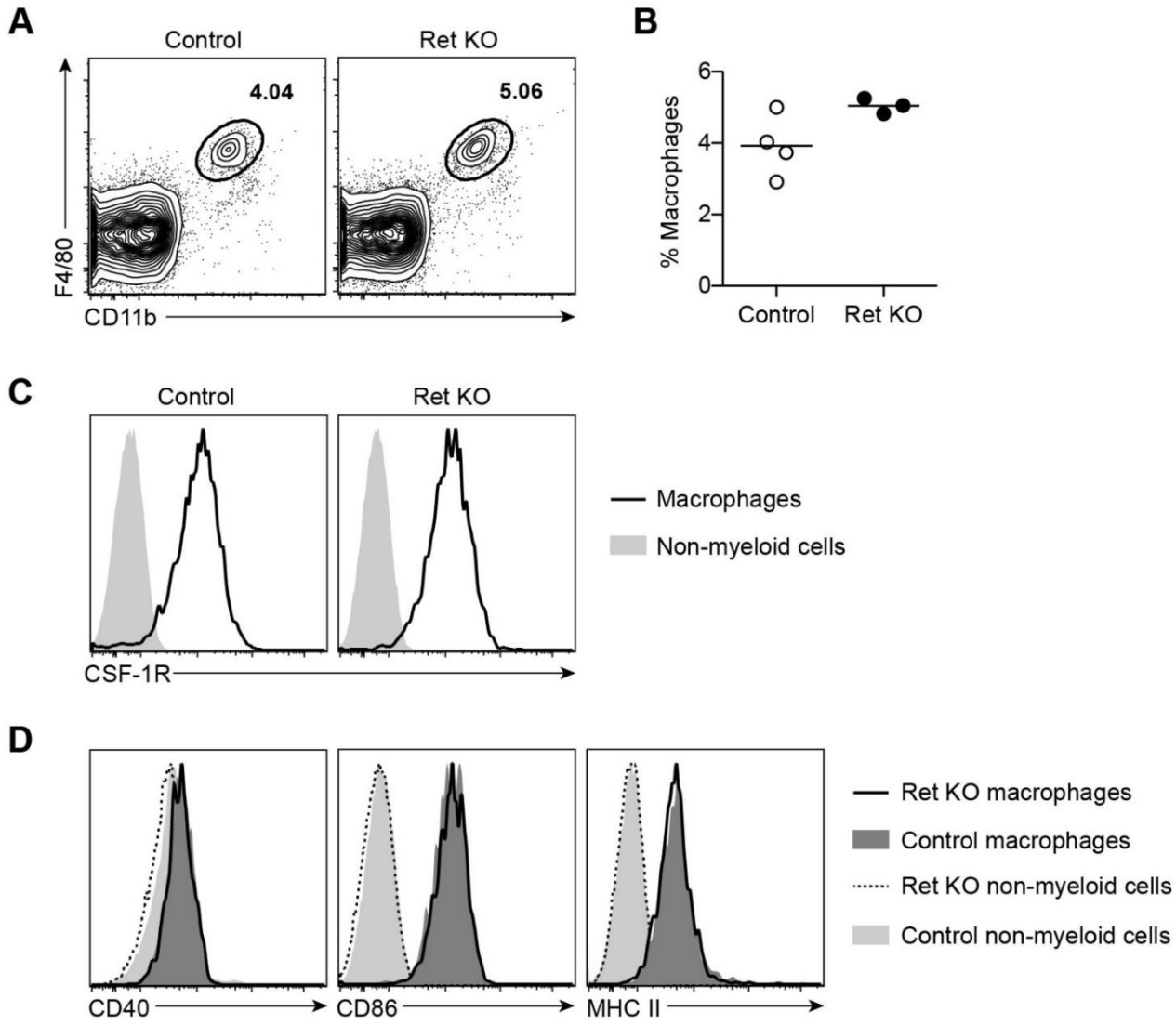


Figure 5.3. Absence of enteric neurons does not affect the surface phenotype or baseline activation state of muscularis macrophages.
P0 bowel muscle layers from control and *Ret* KO mice were digested into single cell suspensions and analyzed for expression of surface markers by flow cytometry. **(A)** Representative flow cytometry plots pre-gated on live cells by forward and side scatter and exclusion of LIVE/DEAD dye. Numbers indicate the frequency of $CD11b^{+}F4/80^{+}$ macrophages among live cells. **(B)** Summary data showing macrophage frequencies among live cells. No statistically significant differences between control and *Ret* KO by Student's *t*-test. **(C)** Representative histograms showing CSF1R expression on $CD11b^{+}F4/80^{+}$ macrophages compared to non-myeloid ($CD11b^{-}$) cells. **(D)** Representative histograms comparing expression of CD40, CD86, and MHC Class II in control and *Ret* KO macrophages. No statistically significant differences between control and *Ret* KO by Student's *t*-test. Control N=4, *Ret* KO N=3 mice.

qRT-PCR and for secretion of these cytokines by ELISA. Similar to our observations at baseline, we find that the LPS response, as measured by increased expression of IL-6 and TNFα (Fig. 5.4A-B) and increased secretion of IL-6 (Fig. 5.4 C), is unaltered in muscularis devoid of enteric neurons. TNFα protein levels were below the limit of detection in both *Ret* KO and control stimulated and un-stimulated samples.

5.3.5 Maintenance of the muscularis macrophage population in the steady state adult bowel does not require CCR2-mediated monocyte recruitment from bone marrow.

The presence of Iba1+ cells in the outer bowel wall mesenchyme as early as E9.5 suggests that the macrophages interacting with the developing ENS are yolk-sac derived. Prior studies have shown that although bowel macrophages in newborn mice are of embryonic origin (Bain et al. 2014) the majority of colonic macrophages in adult and aged animals are replaced by bone-marrow derived cells in a CCR2-dependent process (Bain et al. 2014). Interestingly, we find this not to be the case for muscularis macrophages, which are present in normal numbers in aged 8-week old *Ccr2* KO small intestine and colon (Fig. 5.5 A-F). In contrast, macrophages in the submucosa of *Ccr2* KO mice are modestly, but statistically significantly, reduced (Fig. 5.5 G-I). This suggests that mechanisms governing the maintenance of muscularis macrophages differ from those applicable to macrophages elsewhere in the bowel.

Figure 5.4

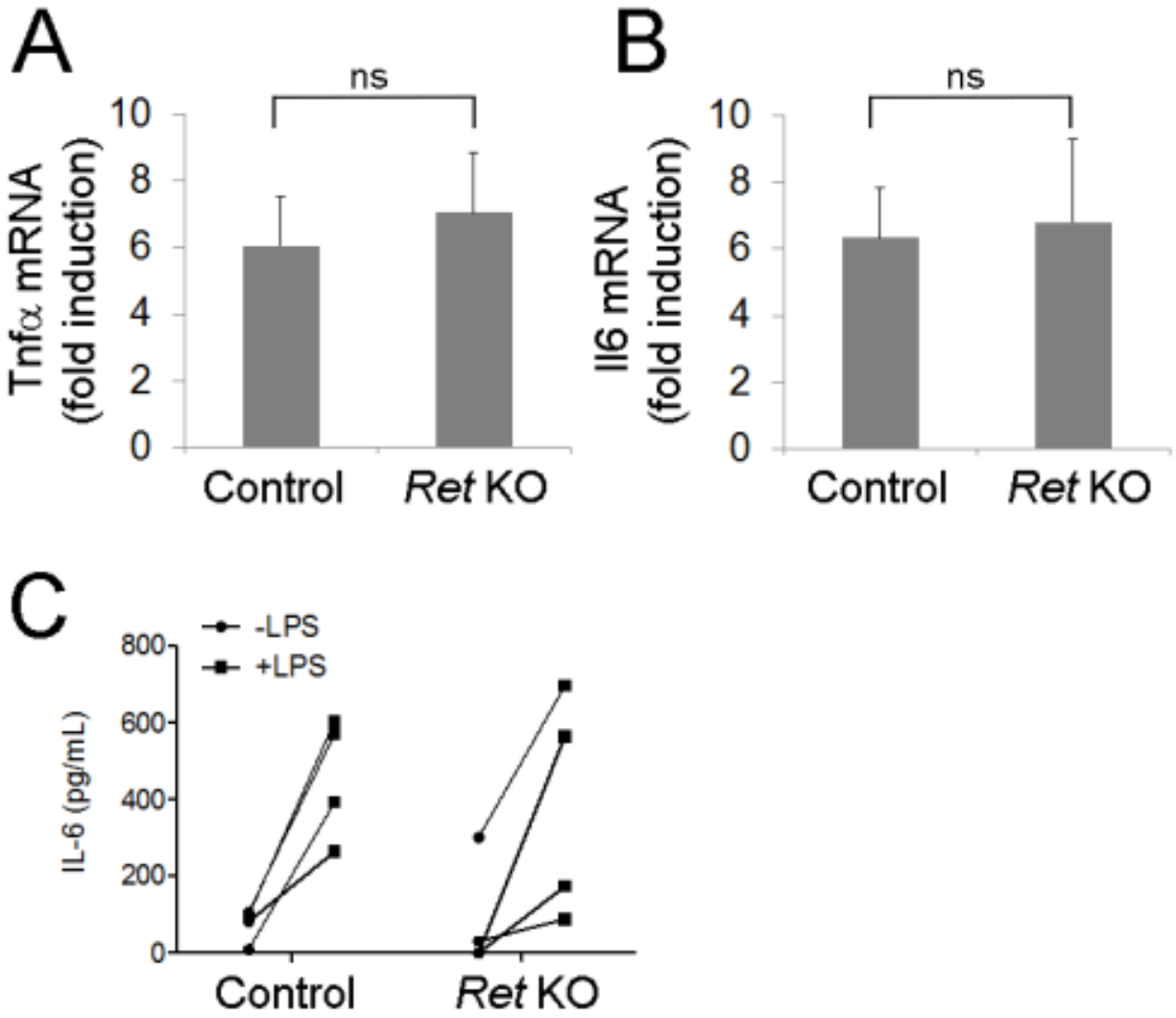


Figure 5.4. Muscularis macrophages from neonatal mice lacking enteric neurons do not differ from control mice in their activation by LPS stimulation.

(A-C) LPS stimulation of muscularis externa from *Ret* knockout bowel induces similar increases in cellular IL-6 and TNFα cytokine mRNA levels (A-B) and in IL-6 protein secretion (C) as control animals. (qRT-PCR control N=6, *Ret* KO N = 4 mice; ELISA N=4 mice from each group).

Figure 5.5

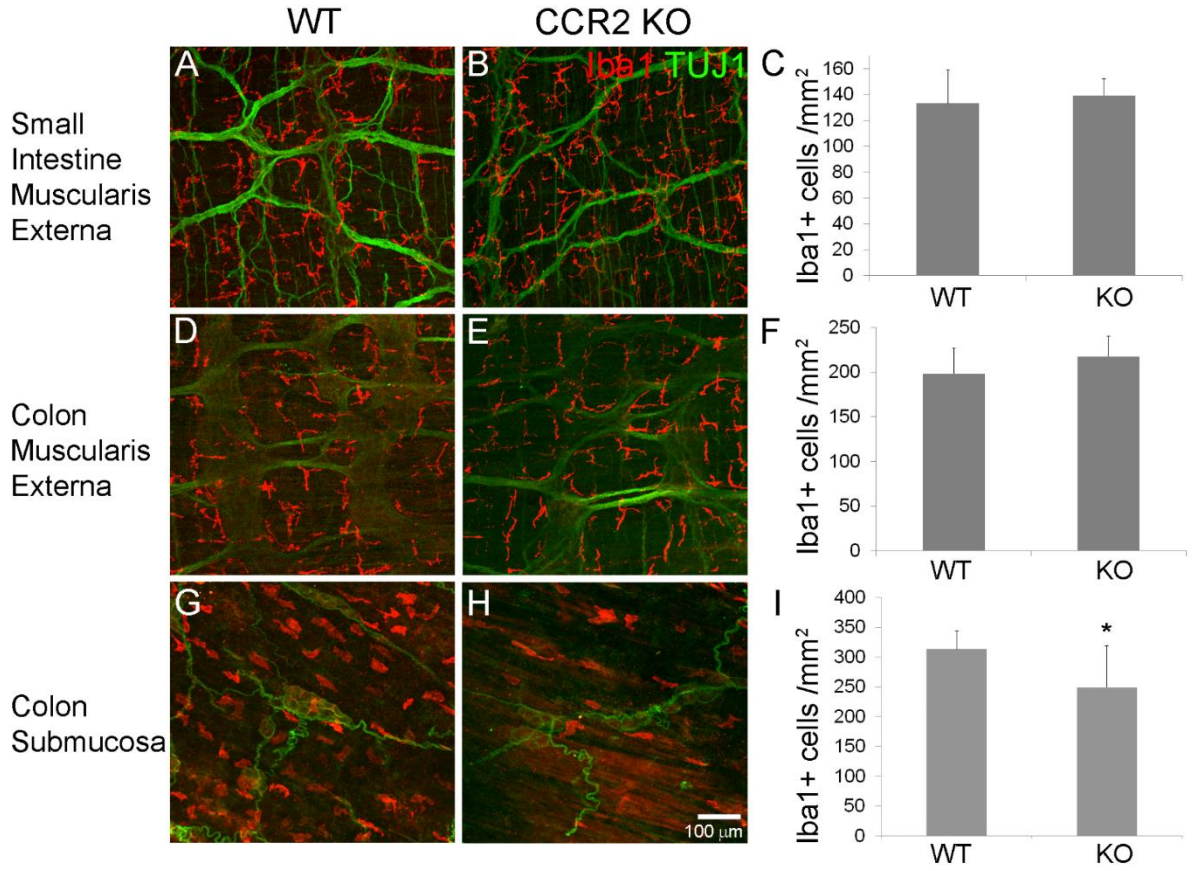


Figure 5.5. CCR2-mediated recruitment of bone marrow monocytes is not required for maintenance of muscularis macrophages.

(A-F) Iba1+ muscularis macrophages in 8-week old *Ccr2* KO mice are present in normal numbers in small intestine and colon compared to WT ($p > 0.05$, Student's T-test) **(G-I)** In contrast, Iba1+ macrophages in the submucosa are modestly, but statistically significantly, reduced in 8-week old *Ccr2* KO mice as compared to controls ($p > 0.05$, Student's T-test) (E-F). N=4 mice for each genotype.

5.4 Discussion

5.4.1 ENCDC are dispensable for muscularis externa colonization and patterning by muscularis macrophages during development.

Muscularis macrophages have been reported in the bowel as early as E15.5 (Mikkelsen et al. 2004), confirming their identity as true tissue resident macrophages. These cells are found along the entire length of the gastrointestinal tract both within and between the circular and longitudinal muscle layers. MMs residing in between the bowel layers, alongside enteric neurons, exhibit clear patterning, with cell bodies spaced at regular intervals and generally non-overlapping processes (Phillips & Powley 2012; Muller et al. 2014; Gabanyi et al. 2016). MMs in the myenteric plexus plane are also frequently seen contacting myenteric neurons, as well as extrinsic neuronal projections (Muller et al. 2014; Phillips & Powley 2012; Cailotto et al. 2014; Matteoli et al. 2014). In adults it has been suggested that enteric neurons are a major source of the MM survival factor, CSF1. Unexpectedly we found that muscularis macrophage colonization of the bowel precedes colonization by enteric neurons, and Iba1-positive MMs are observed in the area of the developing muscle layers as early as E9.5. At E12.5 Iba1-positive MMs are observed throughout the colon, which does not yet contain ENCDC or enteric neurons. This time course suggests that developing MMs do not require enteric-neuron derived survival factors. Indeed, we find that *Csf1* expression in the E12.5 bowel is highest in non-neuronal tissue. Consistent with this, *Ret* KO mice in which ENCDC fail to colonize the bowel have normal numbers of well-patterned MMs at birth. This suggests that enteric neurons are dispensable for the initial patterning of the muscularis externa by MMs. Several non-ENCDC cell types may serve as a local source of CSF1 in the developing bowel, including smooth muscle cells, interstitial cells of Cajal (ICC), epithelial cells, endothelial cells lining blood vessels, as well as

extrinsic sympathetic and parasympathetic nerve fibers terminating in the bowel. At E9.5 and E12.5 in the distal colon, many of these cells, including smooth muscle, ICC, and epithelium have yet to differentiate (de Santa Barbara et al. 2003; Young 2008; Garcia-Lopez et al. 2009), and extrinsic innervation is not yet present (Obermayr et al. 2013), suggesting that endothelial cells or undifferentiated mesenchyme is the most likely source of local CSF1 at this age. Alternatively, it is possible that a local source of CSF1 is not required, and circulating CSF1 in the embryo and fetus are sufficient to keep MMs alive. The aforementioned potential sources of CSF1 may also provide cues for establishing the regular patterning of MMs. Alternatively, MM patterning may be achieved in a cell-autonomous fashion, via homophilic repulsion mechanisms such as those regulating self-avoidance and tiling of neuronal cell bodies and processes in brain and retina (Hughes et al. 2013; Hattori et al. 2008).

5.4.2 Development of neuro-immune interactions in the bowel muscularis externa.

Multiple examples of neuronal modulation of muscularis macrophages have now been recorded. Notably, vagus nerve stimulation of cholinergic enteric neurons that are in close contact with MMs decreases the ATP-induced Ca^{2+} response in MMs and reduce muscularis externa inflammation in the context of post-operative ileus (POI) (Matteoli et al. 2014). This effect relies on α7nAChR cholinergic nicotinic receptors on immune cells and occurs independently of the spleen or T cells (Matteoli et al. 2014). An anti-inflammatory MM phenotype is also induced by sympathetic nor-epinephrine signaling in response to bacterial infection (Gabanyi et al. 2016). Signaling via β2AR beta-adrenergic receptors has been shown to alter gene expression in MMs, resulting in upregulation of tissue-protective genes such as *Chi3l3* (chitinase 3-like 3) and *Arg1* (arginase 1). Whether intrinsic enteric neurons play a role in mediating this extrinsic sympathetic response remains unclear.

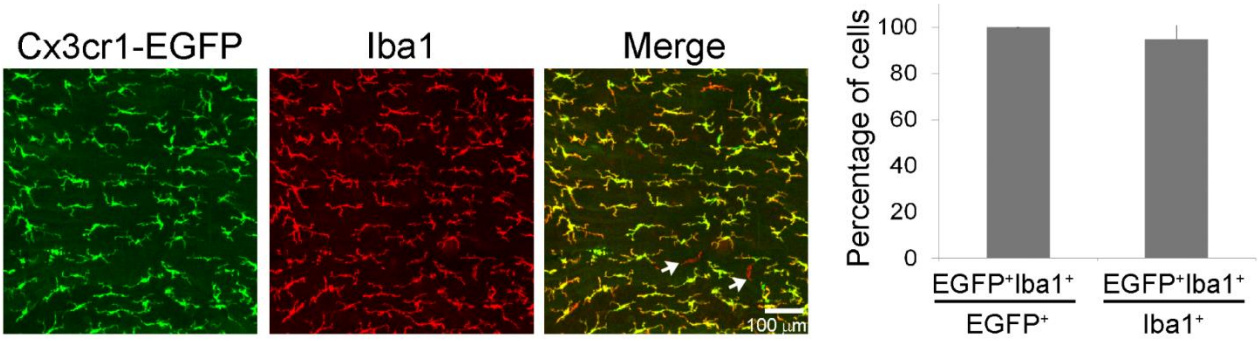
In light of these studies we hypothesized that MMs in *Ret* KO mice would be hyper-activated either at baseline or in response to LPS since *Ret* KO mice are missing enteric neurons and have defects in extrinsic sympathetic innervation of the bowel. However, we were unable to detect phenotypic differences in MMs sorted from neonatal *Ret* KO muscularis externa, or differences in cytokine production in response to LPS-stimulation. The lack of a differential response to LPS in *ex-vivo* muscularis externa may highlight the requirement of intact extrinsic sympathetic and parasympathetic connections for neuronal modulation of MMs, as these connections are severed in both *Ret* KO and control *ex-vivo* preparations. Alternatively, the unaltered *in vivo* baseline phenotype of MMs from *Ret* KOs may indicate that the mature neuro-immune circuitry responsible for modulating MM activation is not yet in place at P0. Importantly, the ENS retains a significant amount of plasticity following birth. In rodents the proportion of cholinergic neurons increases until P36 (de Vries et al. 2010) and some enteric neurons do not exhibit mature dendritic and electrophysiological properties until adulthood (Foong et al. 2012). Various environmental signals, such as intraluminal lipid exposure have already been shown to impact post-natal enteric neuron plasticity (de Quelen et al. 2011) and may have important roles in modulating neuro-immune circuit development in the bowel. Bacterial exposure is another well-established environmental signal that governs some features of MM phenotype. Specifically, bacterial exposure is required for expression of MHCII by MMs, and MMs from neonatal and germ-free mice lack MHC II expression (Mikkelsen et al. 2004). Clarifying which aspects of MM maturation are neuronally mediated is an important task for future studies and will likely require ablation of the ENS at different post-natal time points.

5.4.3 Developmental origin and maintenance of muscularis macrophages.

In most organs, tissue resident macrophages initially arise from embryonic yolk sac progenitors but over time are partially or completely replaced by progenitors from the fetal liver (Hoeffel & Ginhoux 2015). Two notable exceptions to this paradigm are microglia, which are the tissue resident macrophages of the brain, and bowel macrophages. Whereas, at steady state, microglia remain entirely yolk-sac derived throughout adulthood (Ginhoux et al. 2010), bowel macrophages are continuously replaced by circulating blood monocytes in a CCR2-dependent process (Bain et al. 2014), with very few embryonically-derived macrophages remaining in the bowel of aged mice (Bain et al. 2014). Muscularis macrophages, which make up a tiny percentage of total bowel macrophages (Mikkelsen et al. 2011) closely resemble microglia in their morphology and intimate interactions with neurons. Interestingly, we find that muscularis macrophage abundance in aged 8-week old *Ccr2* KO mice remains comparable to WT, in contrast to the reduced number of submucosal macrophages seen in *Ccr2* KOs. This suggests that mechanism of MM homeostasis differ profoundly from those regulating the majority of bowel macrophages. At the very least, steady state maintenance of MMs does not require CCR2. An intriguing possibility is that adult MMs represent an embryonically-derived (perhaps even a yolk-sac-derived) self-renewing macrophage population, similar to microglia. This question could be directly addressed using lineage tracing of yolk sac and fetal liver macrophage progenitors in adult muscularis externa.

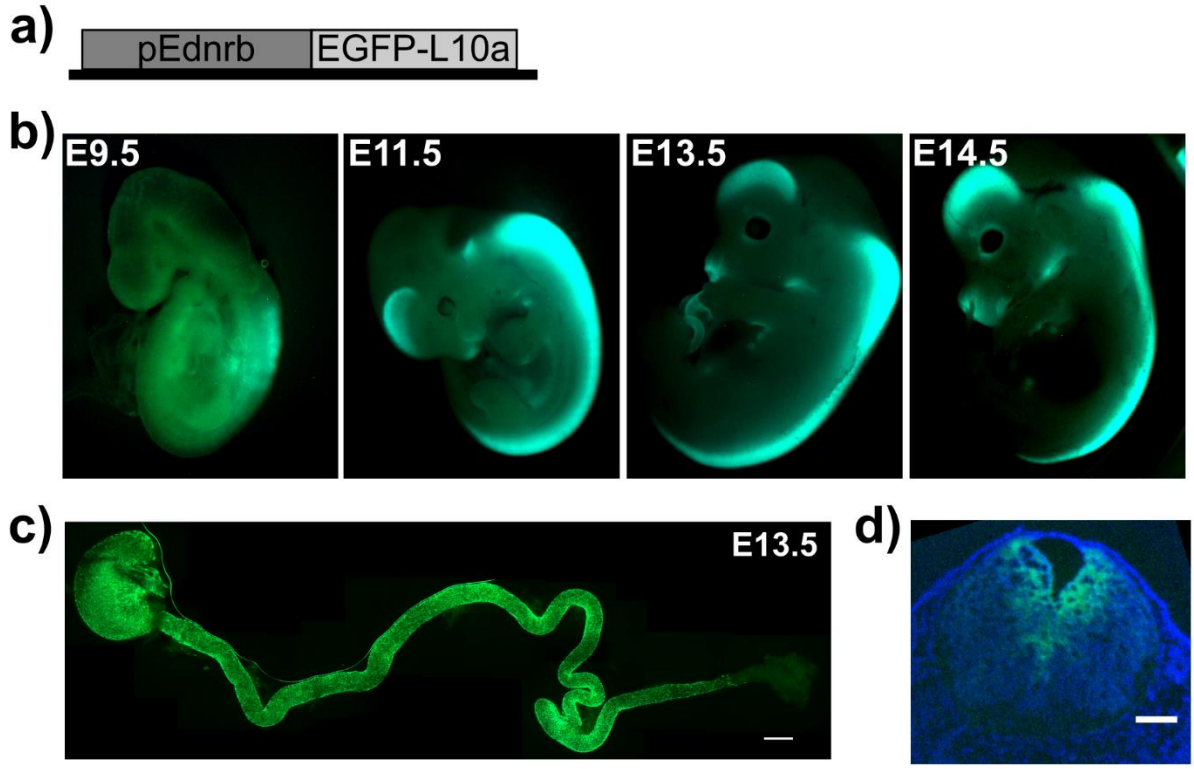
5.5 Supplemental Data

Supplemental Figure 5.1



Supplemental Figure 5.1. Iba1 effectively labels muscularis macrophages.
(A-C) Immunohistochemistry for Iba1 and *Cx3cr1-EGFP* fluorescence in muscularis externa of *Cx3cr1-EGFP* reporter mice shows that almost all EGFP+ cells are Iba1+. A small percentage of Iba+ cells are EGFP negative (arrows). **(D)** Quantitative analysis of Iba1+ and EGFP+ cell populations. N=3 mice.

Supplemental Figure 5.2



Supplemental Figure 5.2. ENCDC-specific Ednrb-EGFP-L10a mouse.
(A) Transgenic construct used to make ENCDC-specific Ednrb-EGFP-L10a mouse. pEdnrb, Endothelin receptor B promoter; EGFP-L10a, enhanced green fluorescent protein- ribosomal protein L10a fusion. **(B)** The EGFP-RPL10A fusion protein is expressed in the neural tube of developing transgenic mice at E9.5, E11.5, E13.5, and E14.5. (C) EGFP fluorescence in the developing bowel at E13.5. Scale bar, 500 μm. (D) Transverse section of an Ednrb-EGFP-L10a mouse at E12.5 demonstrating EGFP expression in the neural tube. Scale bar, 20 μm.

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Chapter 6: Conclusions and Future Directions

6.1 HGF/MET Signaling in the ENS

The enteric nervous system (ENS) has many neuronal subtypes that coordinate and control intestinal activity. Trophic factors that support these neuron types and enhance neurite growth after fetal development are not well understood. We show that Met is expressed broadly in the ENS during early development but becomes restricted to a subset of CGRP –positive intrinsic primary afferent neurons (IPANs) in adult animals. Met cKO mice missing functional MET protein in their ENS have shorter neurites in Met-expressing neurons and display an abnormal peristaltic response triggered by mucosal deformation. Met cKO mice also have increased susceptibility to DSS induced bowel injury and reduced levels of bowel epithelial proliferation. These observations raise a number of additional questions.

We need to define the interplay between MET and RET signaling in the developing ENS. Low levels of GDNF/RET signaling *in vitro* induces Met transcription, but unlike in the kidney, this effect did not rely on Etv4/Etv5 signaling. Despite this initial induction, the MET and RET receptor tyrosine kinases become restricted to non-overlapping neuronal populations by adulthood. Which neuronal populations retain Ret expression in the adult ENS is currently unknown. The mechanisms and significance of this restriction are also unknown and warrant further investigation.

Perhaps the largest number of questions arising from this work pertain to how HGF/MET signaling in a set of IPANs regulates epithelial response to mucosal injury. Although several

mechanisms could underlie this observation, one intriguing hypothesis involves impaired CGRP signaling from Met-positive IPANs onto macrophages in the bowel. CGRP has previously been shown to regulate gene expression in bowel macrophages (Baliu-Piqu et al. 2014; Glowka et al. 2015), and bowel macrophages are known to promote survival and proliferation of epithelial progenitor cells during colonic wound healing (Lundgren et al. 2011). It would be interesting to directly test whether CGRP treatment of specific bowel macrophage populations (i.e. muscularis macrophages vs. lamina propria macrophages) or activation of Met-positive IPANs can trigger epithelial proliferation.

Overall, our studies on HGF/MET signaling in the ENS may be relevant for understanding and treating intestinal motility disorders and also suggest that enhancing the activity of MET expressing CGRP neurons might be a useful strategy to reduce inflammatory bowel injury.

6.2 Axon pathfinding in the ENS

Although much progress has been made in understanding early events in ENS development, almost nothing is known about how axons of enteric neurons navigate to their targets to generate functional neural circuits. Defects in these processes may contribute to the pathophysiology of various enteric neuropathies such as chronic idiopathic intestinal pseudo-obstruction (CIIP) and certain cases of irritable bowel syndrome (IBS) (Di Nardo et al. 2008; Faure et al. 1999). Furthermore, understanding mechanisms regulating enteric neuron connectivity will be crucial for the generation of effective regenerative stem cell therapies that

might be used to treat not only rare congenital defects, but also common acquired enteric neuropathies such as those associated with Parkinson's disease and diabetes (Rivera et al. 2011).

We identified several genes encoding classical axon guidance receptors that are enriched in the developing ENS, with Plexin A4 exhibiting the greatest enrichment. Although the ENS of *Plexin A4* knockout mice appears grossly normal, it is possible that these mice harbor subtle defects in neurite patterning that cannot be detected with broad staining methods. Characterization of the ENS of these mice would be greatly aided by a reliable antibody to Plexin A4 that would allow us to determine which enteric neuron subtypes (if any) produce Plexin A4. Additionally, sparse genetic labeling of *Plexin-A4* expressing neurons would allow us to definitively identify any defects in axonal trajectories of these neurons in *Plexin A4* KO mice.

While *Plexin A4* KOs have no defects in peristalsis, there are a number of other motility patterns, such as those involved in mixing zand segmentation of luminal content (Furness 2008) that could be affected in these animals. Alternatively, Plexin A4 may be expressed in neurons that are part of the secretory or vasodilatory reflexes in the bowel. More thorough functional testing may reveal gastrointestinal disturbances in *Plexin A4* KO mice.

Several cell adhesion molecules with known roles in regulating axon trajectories were also identified as enriched in the developing ENS. These include MDGA2, NrCAM, NCAM1, NCAM2, and L1CAM. Enteric neurons are clustered into ganglia which serve as basic units of connectivity that are repeated around and along the length of the bowel. Given this arrangement, long range axon guidance cues may not be the best means of establishing connectivity within a plexus. Homophilic or heterophilic interactions via cell adhesion molecules on adjacent axons may represent another mechanism of axon targeting within the ENS.

6.3 Muscularis Macrophage Development and the ENS

Understanding neuro-immune interactions with bowel macrophages may provide insight both into developmental bowel disorders, such as Hirschsprung's associated enterocolitis (HAEC), as well as into adult inflammatory bowel disease. Our work on muscularis macrophages shows that this population of macrophages colonizes the bowel before the arrival of ENS precursors, and that enteric neurons are dispensable for muscularis macrophage survival and patterning of the bowel. Additionally, the absence of enteric neurons in the neonatal bowel does not alter the surface phenotype of muscularis macrophages or their response to LPS. Finally, we also demonstrate that maintenance of the muscularis macrophage population does not require CCR2-mediated recruitment of monocytes, as is the case for mucosal and lamina propria macrophages.

Further investigation is required to determine the time course during which enteric neurons begin to influence muscularis macrophage phenotype and activation status. This can be accomplished using conditional *Ret* KO mice that are only missing RET in neural crest derivatives, to avoid neonatal lethality caused by kidney agenesis in germline *Ret* KO mice. Additionally, the effect of individual neurotransmitters on muscularis macrophage activation can be assessed systematically.

Our findings that muscularis macrophage abundance is not altered in aged *Ccr2* KO mice does not rule out the possibility that adult muscularis macrophages are still bone marrow derived but are recruited to the muscularis externa in a CCR2-independent manner. To definitively assess whether adult muscularis macrophages are primarily derived from bone marrow, fetal liver, or

yolk sac, we could perform lineage tracing experiments using Cx3cr1Cre^{ER} mice bred to R26tdTomato reporter mice. A single tamoxifen treatment at E8.5 would irreversibly label macrophages arising from CX3CR1+ yolk-sac progenitors, but not from fetal liver or adult bone marrow. This strategy has been used to successfully label microglia but not peripheral monocytes and macrophages (Parkhurst et al. 2013; Ginhoux et al. 2010). If muscularis macrophages are constantly replaced by circulating bone marrow derived monocytes, the percentage of fluorescently labeled muscularis macrophages should decrease with age, as is the case for lamina propria macrophages (Bain et al. 2014). However, if muscularis macrophages resemble microglia, in that they are yolk sac derived cells capable of self-renewal *in situ*, the percentage of fluorescently labeled muscularis macrophages would remain comparable at different ages.

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