Altered Gastrointestinal Motility in Multiple Sclerosis

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ALTERED GASTROINTESTINAL MOTILITY IN MULTIPLE SCLEROSIS

A Dissertation Presented
by
Estelle Trego Spear
to
The Faculty of the Graduate College
of
The University of Vermont

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For the Degree of Doctor of Philosophy
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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system that causes motor, visual, and sensory symptoms. Patients also experience constipation, which is not yet understood, but could involve dysfunction of the enteric nervous system (ENS). Autoimmune targeting of the ENS occurs in other autoimmune diseases that exhibit gastrointestinal (GI) symptoms, and similar mechanisms could lead to GI dysfunction in MS. Here, we characterize GI dysmotility in the experimental autoimmune encephalomyelitis (EAE) model of MS and test whether autoantibodies targeting the ENS are present in the serum of MS patients.

Male SJL or B6 mice were induced with EAE by immunization against PLP$_{139-151}$, MOG$_{35-55}$, or mouse spinal cord homogenate, and monitored daily for somatic motor symptoms. EAE mice developed GI symptoms consistent with those observed in MS. In vivo motility analysis demonstrated slower whole GI transit, and decreased colonic propulsive motility. EAE mice had faster rates of gastric emptying, with no changes in small intestinal motility. Consistent with these results, ex vivo evaluation of isolated colons demonstrated that EAE mice have slower colonic migrating myoelectric complexes and slow wave contractions. Immunohistochemistry of EAE colons exhibited a significant reduction in GFAP area of ENS ganglia, with no changes in HuD, S100β, or neuron numbers.

To test whether antibodies in MS bind to ENS structures, we collected serum samples from MS patients with constipation and without constipation, and healthy control patients without constipation. Immunoreactivity was tested using indirect immunofluorescence by applying serum samples to guinea pig ENS tissue. MS serum exhibited significantly higher immunoreactivity against guinea pig ENS than control patients, which was particularly evident in MS patients who did not experience constipation. There was no significant difference in immunoreactivity between MS patients with and without constipation. Targets of human MS and mouse EAE serum include enteric glia and neurons.

Taken together, these data validate EAE as a model for constipation in MS, and support the concept that this symptom involves changes within the neuromuscular system of the colon. EAE mice develop symptoms consistent with constipation that affects functional ENS networks and may result in structural or phenotypic changes at the cellular level. Serum immunoreactivity suggests that autoantibodies could play a role in the development of constipation in MS by targeting the ENS itself.
DEDICATIONS

This dissertation is dedicated to two family members who have inspired my research in the field of multiple sclerosis.

Gregory Alan Spear

Mary Spear Thornton
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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Organization of the Gastrointestinal Tract

The GI tract is composed of a series of tube-like organs that span from the mouth to the anus, and it also includes several accessory organs that form as outpouchings from the embryological gut tube. Most of these organs are organized as a central, hollow lumen surrounded by the epithelial mucosal layer, with alternating connective tissue, nervous, and muscular layers building on top of one another, away from the lumen. The GI tract can be described in terms of both the longitudinal axis, from mouth to anus (Figure 1.1), and the radial axis, from the inner mucosal layer directly opposed to the lumen, to the outermost epithelial serosa (Figure 1.2). The primary function of the gut is to break down food in order to absorb nutrients and water, and to excrete the digestible waste. Therefore, each organ of the gut tube has anatomical and physiological adaptations that are optimized to carry out different aspects of digestion.

Longitudinal Organization of the GI Tract

The first cavity of the GI tract is the mouth, through which food and liquids are consumed (Figure 1.1). The mouth provides the early stages of digestion by mechanically breaking down food through chewing, and breakdown of sugars begins to occur through the release of amylase in saliva. After chewing, the tongue moves food to the back of the throat, and the muscles of the pharynx help to push food into the esophagus. The esophagus is the conduit to the stomach, and a bolus of food is moved into the stomach by a wave of muscle contractions above the bolus, and relaxation below it. The
esophagus and stomach are separated by the muscular lower esophageal sphincter, which must relax to allow the passage of food.

In the stomach, food is churned and further broken down by hydrochloric acid, and proteins are digested by the enzyme, pepsin. After one to two hours of mixing in the stomach, the thick, liquid contents, now called chyme, are emptied through the pyloric sphincter into the first segment of the small intestine, named the duodenum. Bicarbonate secretion in the duodenum has to quickly shift the pH of the chyme to prevent injury to the mucosa of the small intestine. Additional digestive enzymes that break down sugar, protein, and fat, are secreted into the duodenum through the sphincter of Oddi; the outlet of both the common bile duct that originates in the gallbladder and liver, and the pancreatic duct. Chyme continues to mix with these digestive juices as it propagates along the latter two segments of the 20-foot-long small intestine, named the jejunum and ileum, respectively. The mucosa of the small intestine is characterized by villi; small, finger-like projections into the lumen, that are decorated with even smaller projections called microvilli. Together these specializations increase surface area for maximal contact of the absorptive mucosa with nutrient-rich chyme. The small intestine contracts back and forth to constantly mix the luminal contents and further promote absorption. At the end of the ileum, the ileocecal valve allows for the release of chyme into the first part of the large intestine, called the cecum.

The cecum stores liquid waste until it is released into the ascending colon, and subsequently into the transverse, descending, and sigmoid colon, and finally the rectum. The purpose of the colon is also absorption, although unlike the small intestine, it is almost entirely specialized for absorption of water, not nutrients. Therefore, the colonic
mucosa does not possess villi or microvilli, but instead is covered in crypts that are lined with mucus-secreting goblet cells to help lubricate the passage of solid waste. The patterns of movement in the colon are also different from those of the small intestine; instead of mixing contents back and forth, propulsive motility or peristalsis in the colon propels waste in the anal direction. At the end of the colon, waste is stored in the rectum, where the sensation of rectal distension is relayed to the brain. Relaxation of the smooth-muscle internal anal sphincter is involuntary, but defecation itself is normally under voluntary control. Defecation occurs following voluntary relaxation of the external anal sphincter and puborectalis muscles. If defecation does not occur, reverse peristalsis pushes feces back into the colon.
Figure 1.1

The GI tract is organized longitudinally from the mouth to the anus. Luminal contents move through continuous hollow organs, each serving a unique purpose in the stages of digestion. Used with permission from (Mescher, 2016).
Radial Organization of the GI Tract

In addition to the longitudinal organization of the GI tract from mouth to anus, it is also organized in layers that are stacked radially outward from the lumen (Figure 1.2). The major components of radial organization include the innermost mucosa, followed by the submucosa and submucosal nerve plexus, circular muscle, myenteric plexus, and longitudinal muscle.

The mucosa is the innermost layer of the GI tract, and throughout the majority of the gut, it is comprised of simple columnar epithelium, except for the mouth, esophagus, and anus which are comprised of stratified squamous epithelium. Deep to the epithelial layer resides the lamina propria and muscularis mucosa. The most prevalent cell of the GI epithelium is the enterocyte, responsible for absorption of both nutrients and water, as well as water and electrolyte secretion. There are four classes of secretory cells in the gut; mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, Paneth cells involved in defense against pathogens, and bicarbonate-secreting tuft cells (Circu et al., 2012). The prevalence of these cell types varies in each region of the gut; there is approximately three times the density of goblet cells in the colon compared to the small intestine, but few if any Paneth cells. The ileum has an additional epithelial cell type called microfold ‘M’ cells that present antigen to immune cells located in the lamina propria, including those in specialized lymphoid tissue called Peyer’s patches (Jung et al., 2010).

Deep to the epithelium is the lamina propria; a connective tissue layer that contains both fenestrated capillaries and lymph vessels and, in the small intestine, extends into the villi. The lamina propria contains many immune cells, including lymphocytes, mast cells,
and macrophages that are recruited from the blood following upregulation of chemoattractants and adhesion molecules in the vasculature (Habtezion et al., 2016). The outermost component of the mucosa is the muscularis mucosa, a thin smooth muscle layer that aids in glandular secretion and fine manipulations at the level of the epithelium.

Outside of the mucosa, the next major layer of the GI tract is another connective tissue layer named the submucosa. The submucosa contains rich networks of blood and lymph vessels, and like the lamina propria, also serves an immune function. In the ileum, the submucosa contains lymphoid follicles termed Peyer’s patches that extend into the mucosa. These are comparable to lymph nodes found throughout the body, in that they contain T cell follicles and germinal centers in which B lymphocytes proliferate and undergo somatic hyper-mutation and class-switching.

Embedded within the submucosa is one of two major divisions of the enteric nervous system, the submucosal (Meissner’s) plexus. The major function of this plexus is neural control of secretion and vasodilation at the level of the mucosa.

Outside of the submucosa is the muscularis propria that contains an inner circular and outer longitudinal muscle layer. The patterns of coordinated contractions and relaxations generated by these muscular networks, termed GI motility, potentiate mixing and movement of luminal contents of the gut. Sandwiched between the circular and longitudinal muscle layers is the second division of the enteric nervous system, named the myenteric (Auerbach’s) plexus. The major function of this ganglionic plexus is the control of GI motility, and this process will be described in detail in the section titled Regulation of GI Motility. The outermost layer of the gut, adjacent to the longitudinal muscle, is an epithelial layer termed the serosa. Serosa from any given segment of the GI
tract is often in direct contact with serosa from another segment, and this tissue is surrounded by lubricating serous fluid that allows the organs to glide across one another during movement.

**Figure 1.2**

The GI tract is organized in radial layers that include an inner mucosa involved in absorption and secretion. The submucosa is rich in vasculature and immune cells, and contains the submucosal nerve plexus. The outer *muscularis propria* contains an inner circular and outer longitudinal muscle layer. Between these muscle layers is the myenteric nerve plexus that is involved in gastrointestinal motility. Reproduced with permission from (Mescher, 2016).
Regulation of Gastrointestinal Motility

Extrinsic Motor Innervation of the GI Tract

The GI tract receives relatively little extrinsic innervation in comparison to the several hundred million intrinsic enteric neurons that are contained within the wall of the gut (Furness et al., 2004). The extrinsic efferent innervation is composed of input from the sympathetic and parasympathetic divisions of the autonomic nervous system (ANS), and vagal and spinal nerves comprise the extrinsic afferent population (Figure 1.3).

Sympathetic pre-ganglionic fibers arise from cells located in the lateral horn of the spinal cord at levels T1-L2, the thoracolumbar division of the ANS. The sympathetic pre-ganglionic nerves that innervate the GI tract exit the sympathetic chain ganglia as splanchnic nerves and synapse on ganglia located anterior to the vertebral bodies called the pre-vertebral ganglia. These ganglia are named the celiac, superior mesenteric, and inferior mesenteric ganglia, respectively, and innervate defined anatomical distributions of the GI tract. All sympathetic pathways have in common a two-neuron system, at which acetylcholine (ACh) is released by the pre-ganglionic neuron in a sympathetic ganglion, stimulating nicotinic ACh receptors (nAChRs) on the body or dendrites of the post-ganglionic neuron. The post-ganglionic neuron of a sympathetic pathway typically secretes norepinephrine (NE) onto α or β adrenergic receptors of the target muscle or gland; in the gut, these synapse on neurons or glia of the intrinsic enteric nervous system. The sympathetic pathway is characterized by the typically short pre-ganglionic neurons and long post-ganglionic neurons, the former of which are lightly myelinated (B fibers) and the latter of which have no myelin (C fibers). Sympathetic post-ganglionic neurons release NE in the myenteric ganglia to decrease activity of the enteric nervous system by
relaxing smooth muscle, reducing GI motility, and constricting sphincters (Furness et al., 2004; Miolan et al., 1996). They also cause vasoconstriction of arteries to shunt blood to skeletal muscle, and inhibit actions of secretomotor neurons in the submucosal plexus (Furness et al., 2004).

Figure 1.3
The GI tract receives extrinsic innervation by the sympathetic and parasympathetic branches of the autonomic nervous system. The sympathetic component (left) arises from the lateral horn of the spinal cord between T1-L2 and decreases digestive processes by constricting sphincters, and decreasing blood flow, luminal secretions, and motility. The parasympathetic innervation (right) to the GI tract arises from vagus nerve or S2-S4, and activates digestive processes. Reprinted, with permission, from R.S. Behnke, 2012, Kinetic Anatomy, 3rd ed. (Champaign, IL: Human Kinetics), 190.
Parasympathetic pre-ganglionic nerves that innervate the GI tract arise from vagus nerve and spinal levels S2-S4; for this reason, they are also referred to as the craniosacral division of the ANS. Cell bodies of vagal efferents are located in the dorsal nucleus of vagus located in the medulla; in the sacral spinal cord, parasympathetic cell bodies are located in the intermediate region of the grey matter. Vagus nerve supplies parasympathetic innervation from the lower esophagus through the GI tract until the splenic flexure of the colon, and S2-S4 supplies parasympathetic innervation from the splenic flexure through the rectum and internal anal sphincter. Similar to the sympathetic pre-ganglionic nerves, the axons of the parasympathetics are also lightly myelinated. The parasympathetic division of the ANS is different from the sympathetics in that the pre-ganglionic fibers are long, and the ganglia are located in the wall of the organ itself, opposed to the pre-vertebral ganglia of the sympathetic division, which are located outside of the target organ. In the wall of the GI tract, parasympathetic pre-ganglionic neurons synapse directly on intrinsic neurons of the ENS. Parasympathetic innervation of the GI tract acts to increase gastric and intestinal activity and glandular secretion. It is important to note that, while most, if not all gastric neurons receive direct input from parasympathetic preganglionic neurons, most neurons of the ENS do not receive extrinsic inputs, and their synaptic inputs instead originate from other enteric neurons.

The GI tract is also innervated by approximately 50,000 extrinsic primary afferent neurons (Furness et al., 2004). The extrinsic visceral primary afferent fibers are pseudounipolar neurons of the vagus nerve, with the cell bodies located in the nodose ganglion (inferior ganglion of the vagus nerve), and the spinal primary afferent fibers are pseudounipolar nerves whose cell bodies are located in the dorsal root ganglia (Furness et
Both sets of primary afferent fibers have peripheral sensory endings in the gut mucosa along with other layers of the gut, and terminals in either the brainstem or dorsal horn of the spinal cord. Additionally, collateral branches of the primary afferents may synapse in the submucosal or myenteric plexus, or directly on the sympathetic post-ganglionic cell body in the pre-vertebral ganglion. Intestinofugal nerves are intrinsic to the myenteric plexus, but also contain afferent projections to the pre-vertebral sympathetic ganglia, serving as an inhibitory relay circuit (Furness et al., 2004).

While the number of extrinsic motor fibers that innervate GI ganglia is in the thousands, the number and extent of collateral synapses is uncertain. Historically, Langley suggested that, because so few sub-diaphragmatic vagal fibers project to the GI tract, that there must exist intrinsic command and/or pacemaker neurons that relay and distribute the extrinsic signals (Langley, 1922). Using anterograde labeling techniques, a more recent study found that vagal collaterals may synapse with 0-100% of myenteric ganglia, depending on the region of the GI tract (Powley, 2000). Enteric innervation via vagus nerve is highest in the stomach and cecum, intermediate in the small intestine, and lowest in the colon, at which point most parasympathetic innervation would derive from the sacral spinal cord.

It has long been known that the ENS can function independently of the CNS (Bayliss et al., 1899), therefore many studies have tried to elucidate the role of extrinsic autonomies on GI motility. Hashmonai and colleagues investigated the effect of sympathetic and parasympathetic innervation on the migrating motor complex (MMC) in a canine model by performing a sympathectomy or decentralization surgery (Hashmonai et al., 1987). In control animals, almost all MMCs were initiated in the stomach, with
little to no initiation of MMCs in the duodenum or jejunum. The percentage of MMCs initiated in the stomach was reduced by three-fold in sympathectomized animals, with the initiation of electrical activity shifted towards the small intestines. In decentralized dogs, this shift was even more prevalent, with nearly no MMCs initiating in the stomach, and most occurring in the duodenum. This indicates that while the CNS is not required for MMC initiation, it does influence their pattern and initiation, especially in the stomach. In addition to the MMC, reflex signaling through the sympathetic pre-vertebral ganglia is sufficient to signal contraction proximal to small intestinal or colonic distension, independent of intact ENS connections, paravertebral ganglia, or spinal cord (as reviewed by (Miolan et al., 1996)).

*Intrinsic Innervation of the GI Tract: The Enteric Nervous System*

The enteric nervous system (ENS) is composed of two ganglionated neuronal plexuses, although aggregates of cell bodies can be found outside of these ganglia as well. There also exist non-ganglionated plexuses that will not be discussed here. The myenteric (Auerbach’s) plexus is located between the outer longitudinal muscle layer and the inner circular muscle layer of the gut. The submucosal plexus is located between the inner circular muscle layer and the GI mucosa. The enteric plexuses also contain supporting enteric glia with important functional and homeostatic roles (Iantorno et al., 2007).

The myenteric plexus contains large, cigar shaped ganglia, and is mostly involved in regulation of the longitudinal and circular muscles. In the myenteric plexus, large bundles of axons speckled with nerve cell bodies form longitudinal connections (parallel to the
longitudinal muscle) between ganglia; these are called primary (interganglionic) connectives. Smaller bundles of axons called secondary connectives run in a circumferential direction (parallel to the circular muscle layer), often connecting the primary connectives but not necessarily entering a myenteric ganglion. Even smaller tertiary connectives lack geometric orientation, and connect ganglia to primary and secondary connectives.

The submucosal plexus in humans is composed of multiple layers of ganglia that are functionally distinct. The outer submucosal plexus mostly innervates the circular muscles, whereas the inner plexus (Meissener’s plexus) is generally involved in mucosal innervation, including secretomotor and vasodilator functions. The connectives of the submucosal plexus are smaller and less organized than those of the myenteric plexus.

**Stages of GI Motility**

In humans, there exist two primary states of motility: digestive and interdigestive, also referred to as postprandial and interprandial. The interdigestive state predominates between meals, and rapidly shifts to the digestive state upon food consumption. The interdigestive state is characterized by four phases (I-IV) of myoelectrical activity that results in smooth muscle contraction. Little activity occurs during phase I, and sporadic contractions begin in phase II. Phase III consists of regular, spontaneous, oral to anal contractions termed the migrating myoelectrical complex (MMC). During phase IV of digestion, the myoelectrical activity decreases and returns to quiescent phase I.

Although considered to occur during the interdigestive phase, spontaneous MMC generation is an important component of motility. The MMC occurs every 1.5 hours in
humans, and takes no more than ten minutes to propagate the length of the GI tract (Kunze et al., 1999). In mice, the MMC occurs approximately once per minute (Dickson et al., 2010). The MMC is under neural control, as it is greatly reduced by mAChRs (Bush et al., 2001). Denervation studies have indicated that the MMC occurs even in the absence of extrinsic sympathetic and parasympathetic innervation (Hashmonai et al., 1987; Kunze et al., 1999). Further, disruption of MMC propagation is associated with constipation (Dickson et al., 2010).

The digestive state of motility terminates the spontaneous MMC, and is characterized by the peristaltic reflex. A common starting point of the peristaltic reflex is a stimulus in the lumen, either chemical or mechanical, that is sensed by intrinsic primary afferent neurons (IPANs). The IPANs then stimulate excitatory and inhibitory motor neurons to cause an upstream contraction of circular muscle and downstream contraction of longitudinal muscle, in addition to relaxation of the circular muscle distal to the stimulus. This peristaltic reflex generates a pattern referred to as propulsive motility that is characterized by the net movement of luminal contents in the oral to anal direction. Each peristaltic contraction only propagates a short distance so that there is adequate time for nutrient absorption. Another pattern of motility, termed segmentation, occurs when contents are mixed via successive cycles of upstream contraction and downstream relaxation followed by upstream relaxation and downstream contraction.

A final pattern of smooth muscle activity in the GI tract is that of slow waves generated by the intestinal pacemaker cells; interstitial cells of Cajal (ICC). These small, slow wave oscillations paradoxically occur at high frequency, with approximately 30-35 cycles per minute, or one event every 1.7-2 seconds in mice. They do not generate proper
contractions of the gut tube, but occur as inconspicuous ripples along the gut. Still, ICC are involved in modulating enteric neurotransmission, and are necessary for GI motility (Klein et al., 2013).

**Morphology and Physiology of Enteric Nervous System Cells**

The neurons of the ENS are morphologically multipolar, unmyelinated cells, classified as Dogiel type neurons, based on the descriptions by Jan von Dogiel in 1899. There are two main structural classifications, although others have been added throughout the years. The Dogiel types are not functionally distinct, and one subtype may constitute motor, afferent, or interneurons. ENS neurons are characterized as one of two main physiological subtypes, ‘S’ or ‘AH’ neurons (Hirst et al., 1974). S-type neurons exhibit rapid depolarization and repolarization, without the long after-hyperpolarization that is characteristic of the AH neurons.

Dogiel Type I neurons are located in the myenteric plexus and are formed by an elongated cell body with many short, spiny dendrites and one axon. Most of the dendrites project circumferentially and longitudinally from the cell body within the plexus, although some may extend radially to innervate other layers of the gut tube. Physiologically, Dogiel Type I neurons are of the S type. The functional class of these cells includes excitatory or inhibitory motor to longitudinal and circular muscle and enteric interneurons. (Brehmer et al., 1999).

Dogiel Type II neurons have long filamentous projections, several of which are axonal (Brehmer et al., 1999; Furness et al., 2004). These neurons may be located in both the myenteric and submucosal plexuses, and project circumferentially or
longitudinally to synapse on other ganglia, or radially inward to innervate the mucosa. They often contain long projections in the anal direction, which are thought to aid in downstream relaxation during peristalsis. Physiologically, most Dogiel Type II neurons are of the AH type. Functionally, they are mostly classified as intrinsic primary afferent neurons (Furness et al., 2004).

Figure 1.4

Depiction of the ENS networks involved in GI motility. IPANs sense chemical or mechanical stimuli and communicate to upstream or downstream interneurons. The IPANs then stimulate excitatory and inhibitory motor neurons to cause an upstream contraction of circular muscle and downstream contraction of longitudinal muscle, in addition to relaxation of the circular muscle distal to the stimulus. Reproduced with permission. (Furness, 2012)
Afferent Cells of the ENS

Multiple cells relay afferent signals from the GI tract, some of which are intrinsic to the ENS, and others that are extrinsic. Afferents of the GI tract sense muscle stretch and tension, whereas the chemical contents of the lumen are sensed by non-neuronal enteroendocrine cells. The most common afferent cell of the ENS is the intrinsic primary afferent neuron (IPAN, Figure 1.4). IPAN cell bodies are located either in the myenteric or submucosal plexus, and they are of Dogiel Type II morphology with AH physiology (Bornstein et al., 2004). IPANs form synapses with other IPANs, allowing for a localized stimulus to excite the surrounding cells. Additionally, IPANs send signals to motor neurons and interneurons. The primary neurotransmitters utilized by these cells include acetylcholine and tachykinins (Furness, 2000).

Intestinofugal cells are intrinsic to the ENS and located mainly in the myenteric plexus, but unlike other cells of the ENS, which communicate with each other, axons of intestinofugal cells project out of the GI tract to synapse in the pre-vertebral sympathetic ganglion. They secrete acetylcholine to modulate sympathetic innervation based on signals they receive from other ENS neurons (Furness, 2000). For example, in response to a stimulus in the lumen, an intestinofugal cell may communicate with sympathetic neurons to contribute to relaxation downstream from the stimulus to allow for movement of the luminal contents.

Other afferent cells are entirely extrinsic to the ENS, as described above. These include visceral afferents that travel on vagus nerve and have cell bodies in the nodose ganglion, as well as spinal afferents that have cell bodies in the dorsal root ganglion.
Efferent Cells of the ENS

Efferent innervation of the GI tract involves both intrinsic and extrinsic neurons. The intrinsic cells are classified as either excitatory or inhibitory motor neurons, secretomotor/vasodilator, or secretomotor neurons. Excitatory and inhibitory motor neurons are typically Dogiel Type I cells with a single axon, similar to multipolar spinal cord motor neurons (Figure 1.4). Those that innervate longitudinal muscle are located almost entirely in the myenteric plexus, whereas those that innervate circular muscle are located in the myenteric plexus, and in larger species such as humans, the outer layer of the submucosal plexus as well. Efferent cells that innervate muscularis mucosa are located in the inner submucosal plexus (Meissener’s).

Excitatory motor neurons express choline acetyltransferase (ChAT), an enzyme responsible for acetylcholine (ACh) synthesis, secreting both ACh and tachykinins (substance P and neurokinin A). Excitatory motor neurons of the ENS project orally where they act on muscarinic ACh receptors (mAChRs) or tachykinin receptors (Bornstein et al., 2004; Furness, 2000). Inhibitory motor neurons express nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP), and pituitary adenylyl cyclase activating peptide (PACAP) (Lecci et al., 2002). Inhibitory neurons project anally to allow for downstream relaxation of the gut tube (Bornstein et al., 2004). The projection of excitatory and inhibitory neurons supports their function so that a stimulus in the lumen can activate both types of neurons. Simultaneous upstream contraction and downstream relaxation of the gut tube results in a pressure gradient that propels luminal contents in an aboral direction, and the sequential activation of repeated circuitry along the gut tube is the basis of peristalsis.
Additionally, the submucosal ganglia contain secretomotor, vasodilator, and secretomotor/vasodilatory neurons that regulate gastric acid, mucous, hormonal secretion and local blood flow.

**Enteric Interneurons**

A major role of enteric interneurons is their involvement with reflexes of gastrointestinal motility (Bornstein et al., 2004). These cells are further divided into ascending and descending interneurons (Figure 1.4).

There is one class of ascending interneurons in the guinea pig ileum that secretes ACh, tachykinins (substance P), and calretinin. ACh release onto other interneurons or excitatory motor neurons stimulates nAChRs; the latter also involves NK_3_ receptors (Bornstein et al., 2004). The distal colon contains additional cell projections that have not been well characterized. Most synaptic input onto ascending interneurons arises from IPANs (Kunze et al., 1999).

There are multiple classes of descending interneurons, with the most common neurotransmitters being NO, ATP and the neuropeptides, VIP and gastrin-releasing peptide (GRP) (Bornstein et al., 2004). Descending NO interneurons project to other interneurons as well as inhibitory motor neurons that project anally. Another descending interneuron is somatostatin reactive (SOM) interneurons. They primarily relay between descending NOS interneurons and inhibitory motor neurons. It is believed that interneurons expressing both ChAT and SOM are primarily involved in propagation of the MMC because they mostly receive input from other ChAT/SOM interneurons (Pompolo et al., 1998). Neurons that express both ChAT and NOS are believed to be
those that modulate descending inhibition by exciting inhibitory motor neurons, leading to downstream relaxation during peristalsis (Yuan et al., 1995).

**Interstitial Cells of Cajal**

The interstitial cells of Cajal (ICC) represent the pacemaker population of the GI tract. Located within almost every layer of the gut, including the longitudinal and circular muscle, and the myenteric and submucosal plexuses, ICC form an interface between the ENS and smooth muscle. ICC can vary greatly in morphology (ie. number of processes) and ultrastructure (protein expression), and certain ICC share characteristics with smooth muscle cells. They all have in common abundant neuronal contacts, mitochondria, and gap junctions through which depolarizations spread to other ICC and smooth muscle cells. ICC are immunoreactive for the receptor tyrosine kinase, c-kit, and are identified by its expression histologically.

Gut smooth muscle cells have few gap junctions between one another; instead they rely mostly on ICC for signal propagation (Seki et al., 1998). The role of ICC slow wave contractions in GI motility was demonstrated by Klein and colleagues, who found that mice deficient in c-kit developed delayed gastric emptying, and slow small intestine and colonic transit (2013). The mechanism underlying this altered motility was described as a loss of slow wave contractions due to disruption of excitatory neurotransmission on smooth muscle cells. This finding has implications in the understanding of both excitatory and inhibitory smooth muscle innervation of the gut, because it suggests that ICC’s are a necessary modulator for this type of signaling.
Similarly, altered ICC morphology and function has been associated with GI
dysmotility and diseases of the GI tract, including slow transit constipation (Cohen et al.,
2017; He et al., 2000), Crohn’s disease (Wang et al., 2007), diabetic gastroparesis (Ordog
et al., 2000), and paraneoplastic syndrome (Pardi et al., 2002), among others.

*Enteric Glia*

Similar to CNS astrocytes, enteric glia outnumber ENS neurons and were, until
recently, thought to play a minimal role in ENS signaling. In fact, much that has been
presumed about the structure and function of these cells has been overturned in recent
years. Enteric glia were originally thought to be most similar to peripheral nervous
system (PNS) Schwann cells, largely based on their location outside of the CNS and their
neural crest embryological origins, but unlike Schwann cells, enteric glia do not produce
myelin. It was then recognized that these cells morphologically resembled astrocytes, and
also express glial fibrillary acidic protein (GFAP) (Jessen et al., 1983). More surprising
was the recent demonstration that enteric glial cells exhibit a unique transcriptional
profile, with characteristics of neurons, Schwann cells, astrocytes, and even
oligodendrocytes (Rao et al., 2015). For example, enteric glia cells express proteolipid
protein 1 (PLP-1), a major component of CNS myelin. It has since been shown that
enteric glia can be located within myenteric and submucosal ganglia, along the
connectives between ganglia, within the longitudinal and smooth muscle, and within the
mucosa (Gulbransen et al., 2012). The anatomical localizations of enteric glia reflect their
molecular subtypes, with each group differentially co-expressing common proteins and
transcription factors used for their identification; PLP-1, GFAP, S100β and Sox-10 (Rao et al., 2015).

Functionally, enteric glia form an incomplete capsule around enteric ganglia but do not ensheathe neuronal axons. Here, they are important regulators of neural integrity, extracellular $K^+$, intracellular $Ca^{2+}$ and are involved in neurotransmitter signaling in the gut (as reviewed by (Iantorno et al., 2007)). These cells also play an important role in maintaining integrity of the gut epithelial barrier (Savidge et al., 2007). It has been shown that similar to reactive astrocytosis, enteric glia demonstrate phenotypic plasticity by increasing GFAP expression in response to inflammation, altering their functional role in gut physiology (von Boyen et al., 2004). Reduced GFAP levels are also described in inflammatory GI diseases likely representing a loss of glial cells, further potentiating inflammation and dysfunctional GI homeostasis and physiology (Kleinschmidt et al., 2011). A role of enteric glia in forming a blood-ENS barrier is an attractive idea, given that CNS astrocytes are involved in the blood-brain barrier (Allen et al., 1994; Bagyanszki et al., 2012; Thi et al., 2008). However, little evidence currently supports this hypothesis, because the ENS is thought to be relatively permeable (Allen et al., 1994).

The role of enteric glia in the regulation of GI motility is gaining increased attention. For example, it was found that the number of enteric glia, but not neurons or ICC, was decreased in the ileum of patients suffering severe constipation (Bassotti et al., 2006a). One study selectively targeted enteric glia by the adoptive transfer of hemagglutinin (HA) specific CD8$^+$ T cells in to a GFAP-HA transgenic mouse (Aube et al., 2006). Following adoptive transfer, the transgenic mice had delayed small intestinal motility and increased intestinal permeability. In another study, abnormal glial $Ca^{2+}$ signaling in mice deficient
in connexin-43 hemichannels resulted in altered GI motility, including delayed colonic transit, altered fecal water content, and lower amplitude muscle contraction and relaxation events in response to electric field stimulation (McClain et al., 2014). Disrupted enteric glia cells could alter enteric physiology through promoting phenotypic plasticity of enteric neurons, altering their neurotransmitter release and regulation of smooth muscle contraction and relaxation (Aube et al., 2006).

**Autoimmune Diseases of Gastrointestinal Motility**

Perhaps due to its rich immune cell environment, vascular permeability, and homology of proteins between the CNS and ENS (Rao et al., 2015), many autoimmune diseases are associated with GI dysmotility. Some of these are primary autoimmune diseases of the gut, including idiopathic achalasia (Latiano et al., 2006; McMillan et al., 2010; Moses et al., 2003), Sjögren’s syndrome (Gordon et al., 2001; Hammar et al., 2010; Jin et al., 2012b; Kondo et al., 2009; Park et al., 2011), and Celiac disease (Volta et al., 2002), among others. Secondary autoimmune diseases of altered GI motility occur in parallel to autoimmune diseases that primarily target other tissues such as scleroderma, type I diabetes, and systemic lupus erythematosus. Autoimmune gastrointestinal dysmotility can also occur in paraneoplastic syndrome, during which an immune response is generated against a neoplasia, or Chagas’ disease, in which the immune response is generated against the parasite *T. cruzi*. Finally, there are lines of evidence suggesting that constipation in Multiple Sclerosis could occur through similar mechanisms.
**Idiopathic Achalasia**

Idiopathic achalasia is a disorder of the lower esophagus that results in loss of peristalsis and failure of lower esophageal sphincter relaxation during swallowing, ultimately causing dysphagia. Previously, our lab has shown that achalasia patients have serum antibodies that target enteric neurons, and in some patients the specificity extends to subpopulations of nitrergic (NO producing) inhibitory neurons (Moses et al., 2003). The presence of antibodies that target nitrergic neurons is consistent with poor esophageal sphincter relaxation and the previous finding that achalasia patients have a loss of esophageal and gastric inhibitory neurons (De Giorgio et al., 1999). The causative role of these antibodies in achalasia is unclear given that serum from patients with gastroesophageal reflux disease (GERD) has comparable specificity and antibodies were not found to correlate with achalasia risk alleles or symptomology. This prompts the question of whether the autoantibodies are pathological, or exist as an epiphenomenon of underlying disease (Latiano et al., 2006; Moses et al., 2003). Consistent with an autoantibody mechanism in achalasia, intravenous immunoglobulin (IVIg) has provided symptom relief in a case study of one patient with achalasia and autoantibodies specific for N-type calcium channels (McMillan et al., 2010). Further, application of achalasia antibodies to cultured human gastric fundus reduced the relative expression of NOS and VIP in myenteric neurons and functionally decreased the summative relaxation of gastric smooth muscle compared to healthy control serum (Bruley des Varannes et al., 2006). The mechanism responsible for altered NOS in this ex vivo study was probably not cell death, but could have been related to transcriptional down-regulation (Boeckxstaens,
Further, because the group used serum and not purified antibodies, these changes could also result from circulating inflammatory cytokines in the serum.

Interestingly, other groups suggest that autoimmune achalasia actually originates following infection with *Helicobacter pylori* (*H. pylori*), a bacterium with strong ties to both gastric autoimmunity and apoptosis (Kountouras et al., 2004). For example, ferrets that are naturally colonized with *Helicobacter mustelae* (*H. mustelae*), and humans colonized with *H. pylori*, develop autoantibodies against parietal cells located in the gastric mucosa (Croinin et al., 2001; Vorobjova et al., 2000). The autoantibodies generated in this animal model are not believed to develop through molecular mimicry, as they do not cross-react with *H. mustelae*, but infection alone could lead to release of sequestered host antigen that propagates the development of autoimmune disease. However, the pathogenicity of these antibodies is unclear, and so far, *H. pylori* infection has not been shown to generate anti-neuronal antibodies that could contribute to loss of nitrergic neurons. Further research is warranted to investigate the relationship between this bacterium and the development of achalasia.

*Sjögren’s Syndrome*

*Sjögren’s* syndrome is an autoimmune disease that primarily targets lacrimal and salivary glands, leading to uncomfortable dryness of the eyes and mouth, a condition termed *sicca*. Autoantibodies identified in *Sjögren’s* syndrome include anti-muscarinic antibodies that target the M3 muscarinic acetylcholine receptors (M3R), Ro/La nuclear proteins, or anti-nuclear antibodies (ANA) (Gordon et al., 2001). In addition to salivary and lacrimal effects of *Sjögren’s* syndrome, some patients also experience symptoms
related to GI dysfunction, with 23% of patients suffering from constipation (Krogh et al., 2007), 43% showing evidence of impaired gastric emptying, and 29% of patients experiencing overt gastroparesis (Hammar et al., 2010). Pathogenicity of Sjögren’s syndrome antibodies on GI tissues has been tested through ex vivo passive transfer experiments during which serum application reduces the amplitude of contraction in response to electric field stimulation and inhibits frequency of colonic MMCs in both a neuronal and M3R-dependent manner (Park et al., 2011; Park et al., 2013). The antibodies inhibit surface expression of M3R through receptor internalization (Jin et al., 2012a). IVIg has been used as a therapy against pathogenic functional autoantibodies specific to M3R in Sjögren’s syndrome, celiac disease, and dermatomyositis and was found to improve bladder and bowel symptoms of the diseases, without altering disease-specific antibodies such as anti-La (SS-B) (Smith et al., 2005). It is thought that IVIg contains neutralizing anti-idioptypic antibodies, that bind to the variable region of M3R autoantibodies and block their activity.

**Celiac Disease**

Celiac disease is an immunological disease in which patients generate antibodies against the gluten protein, gliadin, as well as autoantibodies against the enzyme, tissue transglutaminase (TTG). Endoscopy and biopsy findings include villus atrophy, crypt hyperplasia and lymphocyte infiltration of the small intestine, leading to diarrhea and malnutrition (Boscolo et al., 2010). Due to the expression of TTG throughout the body, extra-intestinal organs including the liver, kidneys and heart are affected, and approximately 10% of patients exhibit neurological dysfunction (Caio et al., 2015). This
is observed at the level of the CNS manifesting as ataxia, epilepsy and other symptoms; and at the level of the ENS, manifesting as constipation and other GI dysmotility. Studies estimate that between 50-75% of patients with celiac disease produce anti-neuronal antibodies that are immunoreactive against CNS targets, and 25% produce autoantibodies immunoreactive against ENS targets (Boscolo et al., 2010; Caio et al., 2015). Caio and colleagues found that most patients who produced high titers of antibodies that targeted the ENS also experienced Rome III criteria for functional constipation (2015). Another group investigated pathogenicity of human autoantibodies in celiac disease by passively transferring human serum or IgG into naïve, T-cell deficient mice, and found that the autoantibodies bound to heart, kidneys, smooth muscle, and small intestinal mucosa, and induced intestinal pathology similar to that characteristic of celiac disease (Kalliokoski et al., 2015). Interestingly, anti-TTG antibodies themselves bind to cytoplasm and nuclei of CNS neurons, and transfer of these antibodies are sufficient to cause ataxia in naïve mice (Boscolo et al., 2010). Further, anti-gliadin antibodies cross-react with CNS targets (Hadjivassiliou et al., 2002). TTG is expressed in enteric neurons of both the human and rat suggesting that the pathogenicity of these autoantibodies could extend to the ENS and promote GI dysmotility (D'Argenio et al., 1988; Thomazy et al., 1989). Further studies should investigate the effects of passive transfer of celiac disease serum on the ENS to determine its role in constipation.

**Paraneoplastic Syndrome**

Paraneoplastic syndrome is a condition that occurs in some patients with small-cell lung cancer, particularly those with brain metastases, and develops when an aberrant
immune response against the malignancy attacks other organs, often targeting the brain, spinal cord, peripheral nerves, or even the ENS (Lewerenz et al., 2015). ENS symptoms of paraneoplastic syndrome present as patients develop GI dysmotility related to underlying changes in the ENS structure and signaling, referred to as intestinal pseudo-obstruction. This phenomenon was described multiple times in the literature as patients with intractable constipation were found to have late stage metastatic cancer associated with bowel and autonomic symptoms. The autoimmune component of this symptom development was not discovered until years later (Ogilvie, 1948; Sodhi et al., 1989). Approximately ten autoantibodies have been associated with small cell lung cancer and paraneoplastic syndrome, but nearly all patients with paraneoplastic syndrome generate antibodies against the Hu family of RNA-binding proteins. Hu protein is found within the tumor itself, but is also highly expressed in healthy neurons (Dalmau et al., 1990; King et al., 1999). Dalmou and colleagues found that only 16% of patients with small-cell lung cancer without paraneoplastic syndrome generate these antibodies, suggesting their involvement in the symptom etiology (Dalmau et al., 1990). The autoantibodies themselves are referred to as anti-Hu or type I anti-neuronal nuclear antibody (ANNA-1). It has been shown that patients with paraneoplastic-associated GI dysmotility had the highest serum titers against all four Hu proteins, in comparison to patients with other neurological involvement including sensory neuropathy, limbic or brainstem encephalitis, cranial neuropathies, or sensorineural deafness (King et al., 1999). Hu proteins are so widely expressed in the ENS that the use of anti-Hu antibodies is standard for immunohistochemical labeling of neuronal cytoplasm.
Histological evaluation of the small intestine of a single patient with paraneoplastic syndrome showed normal lymphocyte and neuron populations, but a general loss and disorganization of interstitial cells of Cajal and some reduced neurites compared to a healthy control sample (Pardi et al., 2002). Evidence for the functional role of anti-Hu antibodies in paraneoplastic syndrome gut dysfunction was demonstrated by Li and colleagues in 2016. This group found that purified IgG from patients with paraneoplastic syndrome activated visceral afferent neurons in mouse, and elicited an excitatory effect when applied to guinea pig and human enteric neurons (Li et al., 2016).

**Chagas’ Disease**

Chagas’ disease occurs 10-30 years following acute infection with *Trypanosoma cruzi*, a protozoan parasite endemic to central and south America, and causes gastrointestinal and cardiac symptoms, including megaesophagus, megacolon, and Chagasic cardiomyopathy. In one study of twelve Brazilian Chagas’ patients, GI pathology associated with the disease included fibrosis, loss of enteric glia, decreased ICC in the myenteric plexus, but increased ICC in the muscular layers, and increased lymphocytes throughout the layers of the colon (Iantorno et al., 2007). Disease pathogenesis in chronic Chagas’ is thought to occur as a result of molecular mimicry to protozoan antigens, promoting autoimmunity; in addition to T cell involvement, autoantibodies have been associated with cardiac and GI involvement of the disease (Girones et al., 2005). Autoantibody targets in Chagas’ disease that could contribute to both cardiac and GI pathology include the muscarinic acetylcholine receptor (mAChR) (Goin et al., 1994; Sterin-Borda et al., 2001), acetylcholinesterase, β-adrenergic
receptors, and voltage-gated potassium channels (VGKCs) (Hubball et al., 2012). Purified serum antibodies from Chagasic patients were found to increase muscle tone when incubated with rat and human GI smooth muscle strips or rat cardiac tissue \textit{ex vivo} in a pattern consistent with muscarinic blockade, suggesting the functional role of these autoantibodies in the clinical condition (Goin et al., 1994; Sterin-Borda et al., 2001). The serum of patients with Chagas’ disease and megacolon is more immunoreactive against colonic mAChRs and smooth muscle compared to those without megacolon, and the antibodies were found to predominantly target the M2 mAChR extracellular loop (Sterin-Borda et al., 2001). A subset of patients representing approximately 14% of the Chagas’ population, also produce VGKC autoantibodies specific for the Kv1 subtype that is distributed throughout the GI tract, including the neuronal cell bodies of myenteric and submucosal ganglia (Hubball et al., 2012).

\textit{Systemic Sclerosis}

Systemic sclerosis (scleroderma) is a multi-system autoimmune disease of connective tissue, that causes fibrosis of skin, heart, lung, and vasculature (Mitchell et al., 1997). Patients also develop altered esophageal, gastric, and small intestinal motility (Di Ciaula et al., 2008), in addition to colonic and anorectal dysmotility (Heyt et al., 2004; Plastiras et al., 2007; Thoua et al., 2011). One mechanism of GI involvement could be related to fibrosis of digestive organs. In a murine model of scleroderma in which transgenic mice express a constitutively active, fibroblast-specific TGF-\(\beta\) receptor, mice were found to have increased levels of non-cross-linked collagen in the colon, and lower levels in the small intestine, compared to wild type mice (Thoua et al., 2012). Further, this fibrosis was
found to cause reduced smooth muscle contractility of the colon \textit{ex vivo}; transgenic mice generated lower amplitude cholinergic-dependent contractions compared to wild type mice. However, there was no evidence of altered GI motility \textit{in vivo}, but the group speculated whether aspects of the disease process could target the neuromuscular circuitry of the gut itself. Further, the time course of the model could have been too short to observe these \textit{in vivo} alterations of motility.

Approximately half of scleroderma patients produce circulating antibodies that positively stain myenteric ganglia from rat GI tissue at high titers, but these antibodies do not predict clinical GI dysmotility (Howe et al., 1994; Kumar et al., 2016). Serum IgG bind to GI smooth muscle at half the intensity of IgG that binds myenteric ganglia (Kumar et al., 2016). However, passive transfer of scleroderma IgG that binds myenteric ganglia is sufficient to prolong the duration and interval of rat MMCs, suggesting that these antibodies could elicit a functional role in the clinical population (Eaker et al., 1999). Purified IgGs isolated from the serum of scleroderma patients have been shown to reduce GI smooth muscle contractility in response to acetylcholine by reducing the activity of M\textsubscript{3} muscarinic receptors, and additionally reduces acetylcholine release itself; both effects were neutralized in the presence of pooled human IgG (Kumar et al., 2016; Singh et al., 2012; Singh et al., 2009). Together, these data suggest that scleroderma IgG target M\textsubscript{3} muscarinic receptors in both enteric neurons and smooth muscle cells, although it is believed that neurons are targeted earlier in the disease process and smooth muscle is involved in later stages (Kumar et al., 2016). Similar to the use of pooled human IgG that reverses the effect of scleroderma IgG in \textit{ex vivo} assays, IVIg has been used as a clinical therapy. While some studies report no change, others describe improvement of GI
symptoms following IVIg administration, supporting the role of antibodies as a mechanism of GI involvement in scleroderma (Allen et al., 1994; Clark et al., 2015; Raja et al., 2016; Sanges et al., 2017).

Diabetes Mellitus

Diabetes Mellitus, both type I and type II, have long been associated with disturbances in GI motility that includes gastroparesis, diarrhea, and constipation. The etiology of these symptoms is thought to occur, in part, from degeneration of small peripheral nerves due to increased glucose levels and oxidative stress. Studies of the ENS in animal models of diabetes show that there is a loss of enteric neurons and axonal degeneration throughout the GI tract, particularly affecting nitrergic neurons (Chandrasekharan et al., 2011; Chandrasekharan et al., 2007; Zandecki et al., 2008). Type I diabetes is associated with the production of autoantibodies, including those that target glutamic acid decarboxylase (GAD), an enzyme that is expressed in both pancreatic β islet and also in neurons. GAD antibodies are thought to be associated with peripheral neuropathies in diabetes (Kaufman et al., 1992). It is also suspected that functional autoantibodies that alter channel conductance could play a role. Evidence for such antibodies was demonstrated by Jackson and colleagues, who found that both ex vivo application and in vivo passive transfer of serum or IgG from patients with Type I Diabetes, significantly altered the generation of MMCs in the colons of naïve mice compared to healthy control serum or serum from other autoimmune diseases (Jackson et al., 2004; Wan et al., 2008). These antibodies elicited effects similar to, and share a binding site with, a smooth muscle L-type calcium channel
dihydropyridine agonist. The effect of IgG on MMCs was normalized following administration of neutralizing, anti-idiotypic antibodies present in IVIg (Wan et al., 2008).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multi-systemic autoimmune disease that can cause symptoms including nephritis, pregnancy loss, thrombosis, neuropsychiatric symptoms, and cutaneous symptoms (Yaniv et al., 2015). Gastrointestinal dysfunction occurs commonly in lupus, presenting as intestinal pseudo-obstruction (Nguyen et al., 2004), mesenteric vasculitis and thrombosis, small intestinal swelling, colonic and rectal lesions, and protein losing enteropathy, together leading to symptoms including constipation, diarrhea, abdominal pain, and bloody stool. While many of these symptoms, including gastro-duodenal mucosal lesions, appear to be related to pharmaceutical intervention, the remainder are believed to occur as part of the disease process.

Over 180 different autoantibodies have been identified in SLE patients, many of which have been associated with specific organ pathologies (Yaniv et al., 2015). A common pathology in SLE patients is vasculitis and thrombosis that could indirectly cause altered GI motility as a consequence of smooth muscle inflammation or hypoxia. For example, autoantibodies specific for $\beta_2$-glycoprotein I, exert their mechanisms through binding of endothelial cells and promoting coagulation (Del Papa et al., 1997). This pathology is broadly defined as anti-phospholipid syndrome, and is not necessarily specific to SLE, but can occur in any patient that develops lupus anti-coagulant (LAC) or anti-cardiolipin (aCL) antibodies. Interestingly, among the autoantibodies that have been identified in SLE, several of them overlap with antibodies of GI pathology in other autoimmune diseases,
including anti-nuclear antibodies (ANA) that are well-documented to promote GI
dysmotility in paraneoplastic syndrome (King et al., 1999). While no studies have
investigated the relationship between SLE GI dysmotility and autoimmune targeting of the
ENS itself, and it is possible that antibodies in SLE could contribute to symptoms such as
intestinal pseudo-obstruction.

Multiple Sclerosis

Few studies have investigated autoimmune mechanisms of MS that could target
the ENS and lead to the gastrointestinal dysmotility that is observed across the patient
population. However, over 20 specific autoantibodies have been identified in the serum
of MS patients, and many of these target proteins that are expressed both in the CNS and
the ENS (Rao et al., 2015; Schirmer et al., 2014). As described above, Rao and
colleagues recently published a transcription analysis of enteric glial cells, and they
reported that proteolipid protein 1 (PLP-1), a major constituent of CNS myelin, is also
one of the most ubiquitously expressed surface markers for enteric glia (2015).

Recently, Wunsch and colleagues published their findings that support the role for
autoantibodies in constipation in MS (Wunsch et al., 2017). The group used a modified
protocol of the murine experimental autoimmune encephalomyelitis (EAE )model, in
which mice were immunized against a fusion protein, MP4, that contains components of
myelin basic protein (MBP) and proteolipid protein (PLP), and induces a B-cell mediated
disease (Kuerten et al., 2006). The small intestine ENS of EAE mice was infiltrated with
macrophages, B cells, and T cells in pre-clinical, acute, and chronic EAE, compared to
non-immunized or hen-egg lysozyme-immunized controls. Pathology within the GI tracts
was similar between EAE mice and clinical samples, and included myenteric plexus axonal degeneration and gliosis detected through increased GFAP immunoreactivity; smooth muscle atrophy replaced by fibrotic tissue was also found within the GI tract of the mouse model. Further, the mice exhibited altered GI motility, as evidenced through reduced transit time, and reduced cholinergic and nitric oxide signaling recorded through smooth muscle myography. ELISA using EAE and clinical serum samples against enteric proteins demonstrated evidence that these samples may contain autoantibodies that target structures of the ENS and promote constipation.

In summary, the wide panel of autoimmune diseases associated with gastrointestinal dysmotility, including the recent evidence in EAE, suggests that constipation in MS could be considered an additional cause of autoimmune gastrointestinal dysmotility.

Multiple Sclerosis

Epidemiology of Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the central nervous system (CNS). Common symptoms of MS include visual, motor, sensory, cognitive, and autonomic disturbances in cardiac, sexual, bladder, and bowel function. MS is diagnosed at a relatively young age, with onset occurring between the third and fifth decade of life (Kister et al., 2013). The sex ratio varies with location and over time, but the most recent worldwide estimate is that females are diagnosed with MS approximately three times more frequently than males (Koch-Henriksen et al., 2010; Trojano et al., 2012). Identical twin concordance rate is between 25-30%, demonstrating that there is a genetic link to MS (Fagnani et al., 2015; Hawkes et al., 2009), with certain
human leukocyte antigen (HLA) genes strongly associated with MS susceptibility and others with resistance (Moutsianas et al., 2015). Environmental and lifestyle factors, including the latitude of residence, vitamin D levels, socioeconomic status, and exposure to certain pathogens are also associated with development of the disease (as reviewed by (Koch-Henriksen et al., 2010; Milo et al., 2010)). One meta-analysis found that of the environmental risk factors associated with MS, the most threatening include smoking, exposure to Epstein-Barr virus, and development of infectious mononucleosis (Belbasis et al., 2015). It is thought that MS is positively correlated with latitude, but a better indicator may be ultraviolet radiation as measured by lifetime sunlight exposure; immunomodulatory Vitamin D3 synthesis in the skin is mediated by sunlight (Koch-Henriksen et al., 2010). Consistent with the relationship between MS and latitude is the finding that MS prevalence is highest in countries farthest from the equator, and latency between symptom relapse rate is shorter (Koch-Henriksen et al., 2010; Spelman et al., 2014). Interestingly, MS risk is also higher in individuals born in April opposed to those born in October or November, suggesting a role for maternal sun exposure during pregnancy (Dobson et al., 2013). The relationship between race or ethnicity and MS is also unclear; although 90% of MS patients are Caucasian, Black individuals have a higher incidence than Caucasians, and those of Asian or Hispanic descent have a lower incidence (Kister et al., 2013; Langer-Gould et al., 2013). The degree of sexual dimorphism of the disease is also different between the racial and ethnic groups. Potential genetic and hormonal contributions to sexual dimorphism in MS have been studied extensively in the experimental autoimmune encephalomyelitis (EAE) mouse model (Butterfield et al., 1999; Fillmore et al., 2004; Spach et al., 2009).
Multiple Sclerosis Disease Course

MS is believed to exist in two stages, the first of which involves demyelinating lesions in the CNS that could theoretically be reversed; the second phase of axonal degeneration results in irreversible damage because CNS neurons are largely incapable of regeneration. No therapies exist to repair CNS degeneration due to MS, as most pharmaceuticals aim to prevent disease progression by inhibiting inflammatory responses or migration of lymphocytes past the blood-brain barrier (BBB). The progression of MS can follow a number of disease courses, distinguished by their chronicity (Figure 1.5). Relapse-remitting MS (RRMS) is the most common form, affecting 85% of the MS population. It is characterized by periods of symptom exacerbation (relapses) followed by a partial improvement of the symptoms (remissions), which never entirely revert to baseline. Secondary progressive MS (SPMS) occurs after a patient with RRMS ceases to experience remissions, but symptoms become increasingly debilitating. About half of RRMS patients will transition to the SPMS form, and SPMS is regarded as treatment-resistant. Primary progressive MS (PPMS) occurs when a patient has symptoms with no remissions; the disease severity increases constantly from onset. Progressive relapsing MS (PRMS) is characterized by chronically progressive disease, similar to PPMS, but with bouts of severe relapses. Whether these different progressions represent a spectrum of the same disease, or different diseases all together, is debated in the field.
Diagnosis of Multiple Sclerosis

Early symptoms of MS, such as optic neuritis – an inflammation of the optic nerve that results in partial loss of the visual field, often cause patients to seek medical attention. Diagnosis of MS typically involves magnetic resonance imaging (MRI) studies, and is made based on the McDonald criteria that require CNS lesions separated, or disseminated, by space and time. Dissemination in space is identified as lesions occurring in different regions of the CNS, such as juxtacortical, periventricular, cerebellar, or located in the brainstem or spinal cord (Polman et al., 2011). The lesions must not be related to other pathologies such as spinal cord or brainstem syndrome caused by infarct or injury. Relapses are defined as an exacerbation of symptoms lasting at least 24 hours,
unrelated to infection, and which correlate to a demyelinating lesion of a CNS region. Dissemination in time is determined one of two ways; 1) the patient initially presents with multiple lesions, including both gadolinium-enhancing and non-enhancing lesions or 2) the patient initially presents with only gadolinium-enhancing or non-enhancing lesions, in which case he/she must develop additional lesions no sooner than 30 days from onset of symptoms.

Oligoclonal bands (OB) of immunoglobulin-G proteins (IgG) in cerebrospinal fluid (CSF) are also associated with MS and may aid in diagnosis, as 95% of MS patients are positive for CSF OB (Link et al., 2006). Demonstration of OB requires collection of CSF via lumbar puncture, followed by separation of proteins by gel electrophoresis (Link et al., 2006). The name ‘oligoclonal bands’ describes the presence of more than two bands of IgG following protein separation. While other diseases are associated with CSF OB, MS is different in that the CSF OB have distinct antigen specificity from those of the blood serum. This suggests that clonal expansion of antibody-secreting B lymphocytes, known as plasma cells, occurs locally within the CNS (Link et al., 2006).

*Immunopathology of Multiple Sclerosis*

The etiology of MS is a topic of contention within the research community, and it is quite likely that there are multiple causes that, in combination with genetic susceptibility, activate the disease. MS could initiate when the peripheral immune system outside of the CNS becomes autoreactive against a CNS antigen, with the impetus for this autoreactivity caused by molecular mimicry to a foreign pathogen, exposure of the immune system to CNS proteins, or failure to remove lymphocytes of high avidity to
CNS antigens during T and B cell maturation (as reviewed by (Dendrou et al., 2015)).
The EAE model of MS represents features of this mechanism of MS pathogenesis, during which an experimental animal, typically a mouse, mounts an immune response to a myelin protein against which the animal is immunized. MS could be initiated by a viral infection of the CNS, and evidence for this possibility is supported through the use of Theiler’s murine encephalomyelitis virus (TMEV) that causes a release of CNS protein into the periphery (as reviewed by (Dendrou et al., 2015)). In humans, it is speculated that a CNS infection related to Epstein-Barr virus (EBV) could initiate MS and trigger an adaptive immune response (as reviewed by (Dendrou et al., 2015)). It has also been proposed that MS could begin inside the CNS itself as a neurodegenerative disease, and only develops autoimmune features as a consequence of the immune processes involved in neurodegeneration. For example, it is known that antibodies specific to myelin proteins are generated following stroke (Becker et al., 2016), and that Alzheimer’s and Parkinson’s disease are associated with innate immune activation that are proposed to develop into an adaptive immune response (Monson et al., 2014; Theodore et al., 2008).

Regardless of the underlying factors that trigger the generation of MS, it is known that autoreactive immune cells including helper CD4⁺ and cytotoxic CD8⁺ T lymphocytes, regulatory T cells (Tregs), and B lymphocytes, are involved in pathogenesis (Figure 1.6). Restimulation of lymphocytes and cytokine and chemokine secretion involves macrophages, microglia, and astrocytes.

The T cell role in the disease is supported by the known genetic link of HLA genes that code for surface proteins involved in antigen presentation to T cells, stimulating their proliferation and cytokine secretion. MS has historically been regarded as a CD4⁺ T cell
driven disease, with evidence that pro-inflammatory IFNγ-secreting Th1 and IL-17A-secreting Th17 cells that recognize myelin peptides are recruited to the CNS and promote inflammation in MS lesions (Bielekova et al., 2004; Hellings et al., 2001). The upregulation of co-stimulatory factors expressed on the surface of T cells during states of inflammation help to stabilize the interaction of T cell receptors with antigen-HLA complexes, effectively allowing T cells to be stimulated by lower affinity antigens than normal (as reviewed by (Ridgway et al., 1999). Studies of murine EAE induced by adoptive transfer of CD8+ T cells specific for myelin basic protein (MBP) that secrete pro-inflammatory IFNγ have shown that these cells are sufficient to cause CNS autoimmunity (Huseby et al., 2001). In humans, these cytotoxic CD8+ T cells are found at high levels in CNS lesions and positively correlate with axonal degeneration (Frischer et al., 2009). Additionally, regulatory T cells (Tregs), whose general role is to prevent autoimmune disease by promoting tolerance to self-antigens through immunosuppression of Th1 and Th17 cells, are involved in MS pathogenesis. Specific populations of Tregs that suppress Th17 cells occur at lower frequency in RRMS patients, and the pharmacokinetics of Treg immunosuppression is abnormal in MS (Baecher-Allan et al., 2011; Fletcher et al., 2009).
Figure 1.6

Central nervous system pathogenesis of MS involves T lymphocytes, B lymphocytes, microglia, macrophages and astrocytes. Reproduced with permission from (Frohman et al., 2006), Copyright Massachusetts Medical Society.
B lymphocytes are known to play a role in MS in the form of both regulatory B cells and antibody secreting plasma cells. The presence of oligoclonal bands in the CSF of MS patients that represent a unique antibody profile compared to those in the blood, is evidence for clonal expansion of plasma cells occurring within the CNS (Link et al., 2006). CNS antibodies are thought to contribute to lesion formation, and specific subpopulations of MS patients are characterized by Type II lesions containing both immunoglobulins and complement (Keegan et al., 2005; Storch et al., 1998). The function of these antibodies has been demonstrated in vitro and in vivo. In one study, anti-MOG antibodies reduced the cell number when incubated with human glioblastoma cells that were virally transduced to express human MOG (Zhou et al., 2006). Passive transfer of human anti-MOG antibodies promoted demyelination, perivascular inflammation, and axonal degeneration of the rat brain. Many studies have suggested the importance of antibodies in experimental EAE, describing less severe disease in mice or rats deficient in B cells, antibodies, or complement (as reviewed by (Cross et al., 2001)). The role of antibodies in the blood of MS patients is unclear, although it is possible that they are associated with pathology both within and outside of the CNS. Plasma exchange has proven efficacious in improving MS symptoms, particularly in patients with the Type II pattern of demyelination that is characterized by antibody and complement localization in CNS lesions (Keegan et al., 2005). Further, new MS therapies including rituximab and ocrelizumab are monoclonal anti-CD20 antibodies that target mature B cells, although these therapies do not directly affect antibody secretion because plasma cells are believed to stop expressing CD20 during development. However, the expression of CD20 is thought to persists in certain plasma cell populations, and the used of CD20 antibodies
could result in decreased autoantibody production, especially of those secreted by short-lived plasmablasts (Huang et al., 2010).


tsembles of Multiple Sclerosis

The North American Research Committee on Multiple Sclerosis (NARCOMS) has tracked the patient-perceived severity of the eleven most common MS symptoms in 25,728 patients spanning disease duration of 0-30 years, to better understand the course of the disease. The prevalence of these symptoms at the time of onset and 30 years post-diagnosis, respectively, are: gait disability (50%, 91%); hand dysfunction (60%, 86%); visual impairments (55%, 80%); fatigue (81%, 84%); cognitive deficits (63%, 81%); bladder/bowel dysfunction (41%, 92%); sensory abnormalities (85%, 94%); spasticity (54%, 89%); pain (59%, 85%); depression (50%, 79%); and tremor/discoordination (38%, 81%) (Kister et al., 2013). Motor dysfunction of MS is typical of an upper motor neuron (UMN) disease. Hyperreflexia occurs because there is a loss of descending inhibition to the lower motor neuron (LMN) reflex arcs. Spasticity is associated with hyperreflexia and is characterized by tight, rigid muscles. The severity of MS disability is categorized as a 1-10 ranking defined by the expanded disability status scale (EDSS) (Buzzard et al., 2012).
Gastrointestinal Dysmotility in Multiple Sclerosis

MS patients can experience many patterns of GI dysmotility, but patients commonly experience paradoxical constipation (as described by the Rome IV criteria, Figure 1.7) combined with fecal incontinence, although each symptom can also occur independently. MS likely causes fecal incontinence due to loss of voluntary (somatic) control of the external anal sphincter; rectal hyperreactivity; and reduced anal sensation (Krogh et al., 2009). Dysfunctional pelvic floor muscles, such as the puborectalis and external anal sphincter, can also contribute to constipation (Gill et al., 1994a). Pelvic muscle tone and pudendal nerve dysfunction have been tested in MS patients by magnetic resonance defecography (Gill et al., 1994b; Law et al., 2008), evoked potentials (Brostrom et al., 2003), and rectal squeeze pressure in response to balloon distension (Wiesel et al., 2000). A comparison of mild and severe MS patients to supra-conal spinal cord injury patients demonstrated a correlation between increased rectal compliance and high EDSS (Preziosi et al., 2011). Similarly, constipation and incontinence associated with the pelvic floor are more likely to occur in conjunction with urinary retention or incontinence, likely related to the proximity of their pathways in the spinal cord (Preziosi et al., 2013). Importantly, abnormal control of pelvic floor muscles would not adequately explain how both symptoms occur simultaneously. Instead, the co-occurrence of constipation and fecal incontinence suggests that other factors are involved in the development of constipation in MS.
In MS patients, there is evidence of slowed GI transit throughout the length of the gut, at sites largely independent of pelvic floor influence, such as the stomach and proximal colon. Gastric emptying abnormalities in MS have been observed as both a decrease and increase in emptying rates, varying between patients (el-Maghraby et al., 2005). In one study using magnetic resonance defecography, five of seven MS patients exhibited slow-transit constipation, versus two of five constipated controls (Chia et al., 1996). ‘Slow transit constipation’ described patients in whom over 20% of tracers were still present in the colon after 120 hours. Of the five MS patients with this type of motility, two exhibited the tracers throughout the length of their colons, including ascending and transverse colon; sites far removed from pelvic floor involvement. This strongly suggests that patterns of dysmotility in MS could be associated with involvement of the enteric nervous system. Glick and colleagues investigated colonic myoelectrical

Figure 1.7

Rome IV Criteria for Functional Constipation

1. Must include two or more of the following:
   a. Straining during at least 25% of defecations
   b. Lumpy or hard stools in at least 25% of defecations
   c. Sensation of incomplete evacuation for at least 25% of defecations
   d. Sensation of anorectal obstruction/blockage for at least 25% of defecations
   e. Manual maneuvers to facilitate at least 25% of defecations (e.g. digital evacuation, support of the pelvic floor)
   f. Fewer than three defecations per week
2. Loose stools are rarely present without the use of laxatives
3. Insufficient criteria for irritable bowel syndrome
4. Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis
activity in MS patients and healthy controls, and found that the amplitude of motor activity was decreased in MS patients before and after a meal (1982). They also showed that there was no difference in the spike frequency of myoelectrical activity between MS patients and controls at baseline, but following a meal, control subjects exhibited an increase spike frequency that was not present in MS patients (Glick et al., 1982).

Interestingly, the myoelectrical activity of the colon is generated by the enteric nervous system (ENS) that is intrinsic to the GI tract. The researchers even suggested that the cause of dysfunction was due to visceral neuropathy, but neither they nor any other group conducted further research exploiting this possibility.

An intimate relationship exists between the ENS and immune structures of the GI tract; inflammation itself is known to alter GI motility and influence ENS plasticity and neurotransmitter repertoire (Mawe, 2015; Mawe et al., 2016). For example, inflammation in the gut mucosa, typical of colitis, can result in hyperexcitability of the intrinsic primary afferent neurons that innervate the mucosa (Linden et al., 2003). Experimental colitis is associated with an increase in the available neurotransmitter pool of ENS ganglia (Krauter et al., 2007); disruption of downstream inhibitory neuromuscular transmission (Strong et al., 2010); and disruption of colonic propulsive motility (Hoffman et al., 2011; Strong et al., 2010). Similar mechanisms may contribute to GI dysmotility in MS. It has previously been shown that the permeability of the GI barrier is compromised in MS and may contribute to intestinal inflammation (Yacyshyn et al., 1996). Recently, this loss of barrier function was also demonstrated in mice with experimental autoimmune encephalomyelitis (EAE), but was not observed in adjuvant-treated controls (Nouri et al., 2014). Compromised barrier integrity could act as a conduit for commensal bacteria or
pathogens to invade the layers of the GI tract and promote inflammation, thus affecting gut motility.

**Animal Models of Multiple Sclerosis**

*Experimental Autoimmune Encephalomyelitis*

The most common model of MS is experimental autoimmune encephalomyelitis, abbreviated as EAE. There are variations of EAE induction, but typically, animals are immunized against foreign CNS proteins, and generate an adaptive immune response that translates into autoimmunity against self-antigens. Classically, animals were inoculated using homogenized spinal cord or brain; most researchers now use purified peptides that are similar to known targets in MS. Two examples are myelin oligodendrocyte glycoprotein (MOG\textsubscript{35-55}) used in C57Bl/6 mice and proteolipid protein (PLP\textsubscript{139-151}) used in the SJL strain. Most often, the peptides are administered in an emulsion with complete Freund’s adjuvant (CFA) that contains inactivated *Mycobacterium tuberculosis* (MTB) and mineral oil. CFA is thought to activate peripheral antigen presenting cells and increase their production of co-stimulatory molecules and cytokines. Some induction protocols also include a separate injection of inactivated *Bordetella pertussis* toxin (PTX), which is thought to further stimulate the innate immune response and weaken the blood brain barrier; PTX tends to increase the symptom severity.

EAE can also be induced by adoptive transfer of T lymphocytes, during which activated T cells are isolated from immunized animals and injected into naïve mice, which go on to develop EAE themselves. Spontaneous genetic models of EAE have been developed, most of which contain a myelin-specific T cell receptor transgene in
combination with other T or B cell receptor mutations (Croxford et al., 2011). Genetic models have variable success and the animals sometimes fail to develop EAE without the administration of PTX or CFA.

Like all animal models, EAE does not perfectly replicate every aspect of the clinical disease. Regardless, the model has been invaluable for the study of MS. One strength of EAE is that it is strain-dependent, allowing for investigation of genetic influences on disease course. C57Bl/6 mice are susceptible and develop a monophasic disease course most comparable to progressive MS, whereas SJL/J mice develop a relapse-remitting disease, and BALB/C mice are resistant to EAE. EAE has led to the development of several MS therapies, including Glatiramer Acetate, Mitoxantrone, Fingolimod and Natalizumab (Hart et al., 2011; Mix et al., 2010).

Viral Models of Multiple Sclerosis

Other animal models include Theiler’s murine encephalomyelitis virus (TMEV) that induces a viral-mediated disease course in mice, opposed to inflammatory. It is therefore a beneficial alternative model in which to test MS therapies; except for Natalizumab, all approved therapies for MS also improve TMEV (Denic et al., 2011). TMEV results in an acute phase of axonal degeneration, followed by progressive oligodendrocyte apoptosis. Similar to EAE, it mainly affects the spinal cord opposed to the brain (Denic et al., 2011).

Cytotoxic Models of Multiple Sclerosis

Cuprizone is a toxic copper chelator that is administered to mice through their diet and causes death of oligodendrocytes. This model is best for studying remyelination.
because the process readily occurs following cuprizone withdrawal, unimpaired by the inflammation that characterizes MS and EAE (Torkildsen et al., 2008). Demyelination occurs in brain regions consistent with those affected in MS, including the corpus callosum, cerebellar peduncles, and cortex. It is also accompanied by axonal loss, but does not cause BBB disruption or T cell infiltration (Torkildsen et al., 2008).

Lysolecithin is a toxin that is injected into the CNS to cause focal demyelination. Lysolecithin causes an infiltration of T and B-lymphocytes, but instead of regulating the demyelination, they may be involved in recovery by increasing the influx of macrophages to clear myelin debris (Ousman et al., 2000). Similar to cuprizone, this model is commonly used to study the remyelination process in MS (Procaccini et al., 2015).

**Specific Aims**

Multiple Sclerosis (MS) is an inflammatory disease with autoimmune components that primarily targets myelinated regions of the brain and spinal cord. Common symptoms of MS include visual disturbances, muscle weakness, fatigue, pain and numbness. Patients also experience debilitating autonomic complications, including bowel dysfunction, that results in gastroparesis, constipation, and fecal incontinence. Patterns of gastrointestinal dysmotility observed in MS patients suggests that there may be dysfunction in the enteric nervous system (ENS) itself. The ENS is a distinct division of the autonomic nervous system that regulates GI motor, secretory, and vasodilatory functions independently of the brain and spinal cord through intrinsic reflex circuits.

One aspect of MS immunopathology is the production of autoantibodies that target CNS proteins, many of which are also expressed in the ENS and could represent an
additional target of MS immune processes. We hypothesize that the experimental
autoimmune encephalomyelitis (EAE) model of MS develops GI dysmotility similar to
that experienced in clinical populations, and secondly, that antibody production plays a
role in the development of GI symptoms in EAE and MS.

**Aim 1.** We will test the hypothesis that mice induced with EAE develop GI symptoms
consistent with those observed clinically, by predominantly evaluating aspects of
constipation. We will assess GI motility through *in vivo* analysis of gastric and small
intestinal transit, colonic transit, whole GI transit, fecal water content, and *ex vivo*
spatiotemporal mapping. To determine whether EAE is associated with ultrastructural
changes at the level of the ENS, we will perform neuronal counts of EAE ENS tissue, and
evaluate distribution of neuronal and glial proteins within ganglia.

**Aim 2.** We will test the hypothesis that antibodies in the sera of MS and EAE mice target
neuronal and/or glial cell proteins in enteric ganglia. Clinically, we will collect serum
samples from healthy control subjects without GI symptoms and from MS patients with
and without constipation. We will quantify the immunoreactivity intensity of sera using
indirect immunofluorescence against guinea pig ENS tissue preparations. Experimentally,
we will collect serum from EAE and control mice at the time of euthanasia and evaluate
patterns of immunoreactivity.
References for Chapter 1


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CHAPTER 2: ALTERED GASTROINTESTINAL MOTILITY IN MULTIPLE SCLEROSIS

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Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system that, in addition to motor, sensory, and cognitive symptoms, also causes constipation, which is poorly understood. Here, we characterize GI dysmotility in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS and test the immunoreactivity of MS and EAE serum against the enteric nervous system (ENS).

EAE was induced in male B6 or SJL mice by immunization against MOG\textsubscript{35-55}, PLP\textsubscript{139-151}, or mouse spinal cord homogenate. Animals were monitored daily for somatic motor symptoms. EAE mice developed delayed whole GI transit, slower colonic motility, and drier fecal pellets compared to controls. They also demonstrated faster gastric emptying. Consistent with dysfunctional ENS neurocircuitry, EAE mice had less frequent colonic migrating myoelectrical complexes and slow wave contractions. EAE colons exhibited decreased GFAP-positive area in myenteric ganglia. Human serum from MS patients exhibited increased immunoreactivity against the myenteric plexus of guinea pig whole mount preparations compared to healthy controls. There was no significant difference in serum immunoreactivity between MS patients with and without constipation. Cellular targets of MS and EAE serum included myenteric neurons and glia.
These data represent a comprehensive analysis of altered GI motility in an animal model of MS. Consistent with symptoms experienced in MS, we demonstrate that EAE mice widely exhibit features of GI dysmotility. EAE mouse colons develop functional changes and altered glial populations that persist in the absence of extrinsic innervation, suggesting the direct involvement of ENS neurocircuitry. Serum immunoreactivity against the ENS suggests that MS could be classified among other diseases known to induce autoimmune GI dysmotility.

Introduction

Multiple Sclerosis (MS) is an inflammatory disease that causes demyelination of central nervous system (CNS) axons, leading to altered neurotransmission and ultimately causing axonal degeneration and neuron cell death. The hallmark symptoms of MS include loss of somatic motor and sensory function, fatigue, and visual deficits. Less studied are the autonomic symptoms of MS, including cardiac, sexual, bladder and bowel dysfunction, with constipation affecting up to 73% of MS patients (Chia et al., 1995; Hennessey et al., 1999; Hinds et al., 1990; Nordenbo et al., 1996; Sullivan et al., 1983). While there is known pelvic floor involvement in MS, many features of the constipation are more indicative of enteric nervous system (ENS) involvement. For example, there is evidence of delayed colonic transit even in the proximal colon, whereas pelvic floor dysfunction manifests as the collection of feces in the sigmoid colon or rectum (Chia et al., 1996). Evidence of decreased amplitude and frequency of myoelectrical events associated with contractions in the colon of MS patients following meal consumption further suggests dysfunction of the ENS, the intrinsic autonomous nervous system of the
gut (Glick et al., 1982). The role of brainstem or spinal cord contribution to MS-related gastrointestinal (GI) dysmotility is unclear; gastroparesis is not correlated with lesion location (el-Maghraby et al., 2005), whereas some symptoms are positively correlated with the expanded disability status score (EDSS) that is indicative of spinal cord involvement (Hinds et al., 1990; Preziosi et al., 2013). Alternately, it has been observed clinically that constipation can precede diagnosis of MS, when patients have little to no dysfunction as identified by the EDSS scale and whether these symptoms represent an early manifestation of MS, or an independent condition, is unclear (Wiesel et al., 2001).

CNS lesions caused by MS pathology should not necessarily alter GI motility because motor, secretory, and vasodilatory functions of the gut are regulated by the ENS, a branch of the autonomic nervous system that is intrinsic to the gut. Innervation of the gut is unique from other organs because all components of the ENS, including afferent, inter- and motor neurons, are contained entirely in the wall of the GI tract and generate complicated functions independent of CNS innervation (Furness, 1987; Kunze et al., 1999). Additionally, every neurotransmitter found in the CNS is present in the ENS, and similar to the CNS, neuronal regulation of the ENS involves neuro-glial signaling. The ENS is entirely unmyelinated, but enteric glial cells share transcriptional similarities with astrocytes and oligodendrocytes, including their expression of glial fibrillary acidic protein (GFAP) (Jessen et al., 1980) and more recently discovered expression of proteolipid protein 1 (PLP-1) and myelin basic protein (MBP) (Rao et al., 2015). Signal propagation in ENS smooth muscle involves pacemaker cells, named interstitial cells of Cajal (ICC), that propagate depolarizations leading to ripples of contractions, termed slow waves. Larger contractions, termed colonic migrating myoelectrical complexes are
the result of coordinated activity of excitatory and inhibitory motor neurons, and drive propulsive motility (Dickson et al., 2010). Importantly, disruption within any population of ENS cells, including neurons (Mawe, 2015), glia (McClain et al., 2014), and ICC (Klein et al., 2013), can result in altered GI motility.

An entity associated with constipation in autoimmune disease is described as autoimmune gastrointestinal dysmotility (AGID), that results when antibodies bind to neurons or glia of the ENS, and cause disordered GI motility. AGID can occur idiopathically, or in association with a disease such as paraneoplastic syndrome (Dhamija et al., 2008), Chagas’ disease (Girones et al., 2005), diabetes (Jackson et al., 2004), and achalasia (Moses et al., 2003). In some instances, antibodies target proteins that are important for neuromuscular transmission, such as muscarinic receptors in Chagas’ disease (Goin et al., 1994). In other conditions, such as achalasia, the autoimmune disruption in motor function involves a loss of neurons (Csendes et al., 1992; De Giorgio et al., 1999).

Multiple Sclerosis is a disease with strong autoimmune characteristics, and it is thought to begin when autoreactive T and B lymphocytes cross the blood brain barrier and target CNS myelin and neuronal proteins. Over twenty antigens have been identified in MS, and many of these CNS targets are also found in the ENS, including myelin proteins PLP-1 and MBP, in addition to neurofilaments (Bartosik-Psujek et al., 2011; Berger et al., 2003; Rao et al., 2015; Schirmer et al., 2014; Silber et al., 2002). One study found that 28% of MS patients had at least one antibody that targeted common GI mucosal antigens, supporting the possibility that the GI tract is a target of antibodies produced during MS pathogenesis (Banati et al., 2013). A recent study investigated the
role of antibodies in GI dysfunction in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS that favors B lymphocyte involvement over T cell disease (Wunsch et al., 2017). Their findings suggest that EAE causes loss of integrity within the ENS and slower GI transit. Further, this group reported that both MS and EAE serum contains antibodies that target structures of the ENS.

Here we present an in-depth characterization of GI dysmotility in EAE by testing in vivo metrics of motility, in addition to ex vivo analysis of slow wave contractions and colonic migrating myoelectric complexes (CMMCs). Secondly, we provide evidence that GI dysmotility in MS may be caused by autoantibodies that target the ENS by testing the immunoreactivity of MS and EAE serum against cells of the ENS.

Methods

Animals

Male B6 (C57Bl/6J, Jackson Laboratories, Bar Harbor, Maine) or SJL (SJL/J, Harlan Laboratories, Indianapolis, Indiana for PLP induction or Jackson Laboratories for MSCH induction) mouse strains, 8 weeks of age, were housed in a temperature controlled room with 12-hour light:dark cycle, with food and water ad libitum. The University of Vermont Institutional Animal Care and Use Committee approved all experimental protocols.

EAE Induction

At 8 weeks of age, mice were divided into EAE or control groups. Mice were immunized against MOG_{35-55} or PLP_{139-151} peptide, or whole mouse spinal cord homogenate (MSCH). In total, four different EAE immunization paradigms are used in
this study; B6 + MOG, SJL + PLP, B6 + MSCH and SJL + MSCH. B6 + MOG and SJL + PLP paradigms were used as previously reported (Krementsov et al., 2015), and we further optimized MSCH induction based on established protocols (Butterfield et al., 2000).

**B6 + MOG**

B6 mice immunized against MOG peptide were injected subcutaneously (S.Q.) with an emulsion containing 200µg MOG35-55 and 200µg *Mycobacterium tuberculosis* in complete Freund’s adjuvant (CFA) H37RA (Difco Laboratories). This treatment was administered on day 0 and repeated on day 7.

**SJL + PLP**

SJL mice immunized against PLP peptide were injected S.Q. with an emulsion containing a total of 100µg PLP139-151 and 200µg *Mycobacterium tuberculosis* in CFA H37RA (Difco Laboratories). This treatment was administered on day 0 and repeated on day 7.

**B6 + MSCH**

B6 mice immunized against MSCH received S.Q. injection of 3.75 mg MSCH and CFA with 300µg *M. tuberculosis* divided between three sites on day 0. These mice received intraperitoneal injection containing 200ng of Pertussis Toxin (PTX, List Biological Laboratories, Inc.) on day 0 and again on day 2.
SJL + MSCH

SJL mice immunized with MSCH were injected with 100µL of an emulsion containing 2.0 mg MSCH and CFA with 200µg *M. tuberculosis* on day 0, and again on day 7.

Control Mice

For each induction paradigm, control mice received S.Q. or I.P. vehicle injections containing CFA or PTX but no MOG, PLP, or MSCH.

Post-procedural care and EAE symptom scoring

Following EAE induction, animals were weighed and scored daily. EAE scoring was recorded as follows: 0) no symptoms, 1) tail paralysis, 2) tail paralysis and mild-moderate hind limb weakness, 3) tail paralysis and hind limb paralysis, 4) a score of 3 with urinary incontinence, 5) moribund or death (Krementsov et al., 2015). When animals reached a level 3, food pellets and *Napa Nectar* were placed on the bottom of the cage to facilitate nutrient and fluid intake. Mice that maintained a level 4 for two days, or those that reached a level 5, were euthanized were recorded as a level 5 for the remainder of the experiment duration.

Assays of GI Motility

For mice immunized with MOG or PLP peptides, motility studies were initiated at 28 days following induction, a time point at which symptoms had typically peaked and recovered slightly. In mice immunized with MSCH, motility analyses were initiated two
days after the first mice began to exhibit symptoms. On separate days, mice sequentially underwent assays to assess fecal water content, colonic transit (MSCH mice only) and whole GI transit time. The last metric analyzed in all animals was upper GI transit, which took place just after mice had reached the peak symptom score and recovered slightly. All EAE mice were included in motility analyses, regardless of symptom development.

**Whole GI Transit**

Animals were administered, by oral gavage, 300µL of a solution containing 6% carmine red (Sigma Aldrich) and 0.5% methylcellulose (Sigma Aldrich) in tap water. They were placed in individual cages with no bedding, but were allowed food and water *ad libitum*. Fecal pellets were monitored, and whole GI transit was recorded as the time in minutes from gavage until carmine red was observed in the fecal pellets.

**Fecal Output**

Mice were placed in individual cages with no bedding, food, or water for one hour. Fecal pellets were collected regularly throughout the hour, and were weighed immediately. The pellets were dried for 24 hours at 50°C and re-weighed in order to calculate the percentage of water content.
Colonic Transit

Mice were lightly anesthetized with 2% isoflurane. A 2.3 mm bead was inserted 2.0 cm into the distal colon using a blunt gavage needle. When mice awoke from anesthesia, colonic transit was measured as the time in minutes until the bead was expelled.

Upper GI Transit

Mice were fasted and housed without bedding for 12 hours, and without water for 3 hours before the start of the experiment. Each mouse was administered with 100µL of 2.5mg/mL Rhodamine B Dextran (ThermoFisher Scientific) in 2% methylcellulose by oral gavage. After 20 minutes for C57Bl/6J mice and 24 minutes for SJL/J mice, the animals were euthanized by isoflurane and exsanguination. The stomach and small intestines were removed, and the small intestines were cut into 10 even pieces. Each segment was placed in a separate conical tube containing 4mL 0.9% NaCl, homogenized, and then centrifuged for at 4°C and 1500 rpm for 15 minutes. Then, 250µL of supernatant was pipetted into a black-bottomed 96-well plate. The level of fluorescence was measured on a BioTek Synergy H4 Hybrid Microplate Reader using Gen5 software (BioTek, Winooski, Vermont). Data were analyzed for percentage of gastric emptying, geometric center and leading edge.

Spatiotemporal Mapping

Control mice (n=5) or SJL mice induced under the MSCH EAE protocol (n=4), were euthanized by isoflurane and exsanguination 2-4 days following the onset of somatic motor symptoms. Their colons were removed and pinned loosely in an organ bath
containing 37°C physiological Krebs solution. A camera (DMK 41AF02, The Imaging Source, Charlotte, North Carolina) was oriented above the tissue and used to record videos 10 minutes in length (3.75 fps, 1280x960, 8-bit) using Astro IIDC software (Aupperle Services and Contracting, Calgary, Alberta). Analysis was performed using custom-written software (VolumetryG8d, Grant Hennig, University of Vermont). In brief, videos of the colons were transformed into particle formats, and a spline was fit longitudinally to measure the diameter of the tissue over time. Diameter was exported into spatiotemporal maps and color coded for diameter. From these maps, thresholds were set to select for long (83 frames, approximately 8 to 30 seconds) and short (9 frames, 0.8 to 3.2 seconds) intervals between contractions to identify CMMCs and slow wave contractions, respectively. First, a histogram was compiled from these maps to measure how frequently each interval duration is represented. However, because the length of tissue and total number of events varies between animals, interpretation of these histograms is limited. In order to make group-wise comparisons, data were normalized to create graphs of the cumulative distribution of interval durations. In these graphs, the number of intervals that occur at each interval duration is represented as a percentage.

Mouse Immunohistochemistry

SJL mice induced under the MSCH EAE protocol (n=5), or control mice (n=5), were euthanized by isoflurane and exsanguination 2-4 days after the onset of symptoms. Their colons were removed, opened longitudinally along the mesenteric border, and pinned flat in a sylgard coated petri dish. After overnight fixation in 2% paraformaldehyde and 0.2% picric acid the colons were dissected to expose the LMMP. The LMMP tissue was cut
into 1cm² sections and stained for either HuD, GFAP, or S100β (1:100 rabbit anti-HuD IgG, Santa Cruz Biotechnology, 1:500 rabbit anti-GFAP, EnCor Biotechnology, or 1:1000 rabbit anti-S100β) with a goat anti-rabbit IgG Cy3 secondary antibody. Tissue sections were counterstained with DAPI (1:10,000, Sigma).

Using the tissue stained with anti-HuD antibodies, a researcher blinded to the identity of the samples acquired images of 20 ganglia per slide using an Olympus AX70 microscope on an Olympus America camera and MagnaFIRE SP software. The number of neurons per ganglion was quantified by manually counting the cells. Using ImageJ software (NIH), a region of interest was drawn around the perimeter of each ganglion to quantify the area of each ganglion in pixels, which was converted to µm². The density of neurons within a ganglion was quantified by dividing the number of neurons in a ganglion by the ganglion area.

In order to evaluate the area constituted by the specific cell markers of neurons and glia present within a ganglion, we used Nikon C2 confocal microscope to acquire high-resolution images. A total of five images were collected for each sample, with each image focusing on one particular ganglion. Each slide was examined with a 40x oil immersion lens under DAPI and Cy3 filters and a scan rate of 10.3 pixel dwell time and a z-stack was acquired (0. 5µm depth) to capture the full thickness of each ganglion.

Image deconvolution was completed using AutoQuant X³ software (Media Cybernetics). A separate TIF file was created for each channel and analyzed using VolumetryG8d by calibrating the images to 160 µm × 160µm in area. A researcher blinded to the identity of the samples applied a fluorescent threshold reduce background noise, and drew a mask around each ganglion using custom software (VolumetryG8,
Grant Hennig, University of Vermont). The area of each fluorophore was calculated by overlaying the mask and the original fluorescent TIF, converting both into binarily represented colors, and determining the percentage of image overlap.

**Human Serum Collection**

Blood samples, 5mL in volume, were collected from three groups of 20 subjects, including constipated MS patients; non-constipated MS patients; and age- and gender-matched non-constipated controls. The blood was centrifuged and the plasma stored at -20° C. The Rome IV criteria were used to identify constipated MS patients. Additional patient information was collected using an author-derived GI questionnaire to better characterize symptoms and identify patterns of constipation. All protocols involving human blood were approved by the UVM institutional review board.

**Immunohistochemistry with EAE mouse and human serum samples**

Fresh guinea pig ileum was pinned flat in a sylgard coated petri dish and fixed in 2% paraformaldehyde and 0.2% picric acid for 24h. Whole mount preparations were then dissected to expose longitudinal muscle and myenteric plexus (LMMP). Human serum was pre-absorbed with 2% liver powder (Sigma Aldrich) and diluted to 1:100 or 1:200 in 1X PBS with 4% goat serum and 0.5% Triton-x 100 (Sigma Aldrich) and incubated with LMMP preparations overnight, followed by incubation with the secondary antibody (1:150, goat anti-human Cy3, Jackson Immuno). A researcher was blinded to the samples, and collected images of three ganglia for each different human serum sample, adjusting the microscope exposure time so that pixels just began to show saturation. The
intensity of immunoreactivity was measured using Image J software (NIH) by drawing a region of interest around three independent ganglia, measuring their intensity on the greyscale (0-256), and subtracting the intensity of the background staining. The average intensity of the three ganglia was recorded from each tissue section, and values were normalized to account for differences in microscope exposure time.

**Statistics**

All statistics were calculated using GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com). Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Comparisons between two groups were analyzed as one- or two-tailed t-tests and the F test was used to determine equal variance between groups. If significant differences in variance were detected, Welch’s correction was used. Data that were not normally distributed were analyzed using the non-parametric Mann-Whitney test. Data were Q-tested and outliers were removed if values were greater than 2 standard deviations (S.D.) from the mean. In cases of three or more groups, data were analyzed by one- or two-way ANOVA, with Bonferroni’s multiple comparisons test. Cumulative distributions of spatiotemporal mapping assays were analyzed by the Kolmogorov-Smirnoff test and also by non-linear regression. Significance is considered as * p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001.
Results

EAE mice exhibit features of constipation in vivo

To determine whether EAE mice exhibit symptoms of constipation, we first measured whole gastrointestinal transit time following oral gavage of carmine red dye. The time until expulsion of the dye was significantly longer in EAE mice compared to controls, including B6 mice induced with MOG (p<0.05), and in SJL mice induced with either PLP (p<0.05) or MSCH (p<0.05) (Figure 2.1 A, C, D). The difference in transit time between EAE and control B6 + MSCH mice was not significant (p<0.1022, Figure 2.1 B).

Fecal water content was calculated as an indicator of constipation as slower transit allows more time for water absorption. Fecal pellets from EAE mice were dryer than those of control mice in every induction method, including B6 mice induced with MOG (p<0.05) or MSCH (p<0.01), and SJL mice induced with PLP (p<0.01) or MSCH (p<0.0001) (Figure 2.2 A-D).

To measure colonic propulsive motility, we calculated the time until expulsion of a small glass bead inserted 20mm into the distal colon. Expulsion time was significantly longer in EAE mice compared to controls when tested in B6 mice (p<0.05) and SJL mice (p<0.05) both induced with MSCH (Figure 2.3). This analysis was not performed in B6 _ MOG mice or in SJL + PLP mice.

Gastric emptying and small intestinal transit were measured following oral gavage of rhodamine-B dextran. Gastric emptying was significantly accelerated in B6 + MOG mice (p<0.05), B6 + MSCH mice (p<0.05), SJL + PLP mice (p<0.001), and SJL + MSCH mice (p<0.05) (Figure 2.4 A-D). There were no differences in small intestinal transit as
measured by leading edge or geometric center in EAE mice versus control mice (Figure 2.5).

The colons of EAE mice have altered contractile activity ex vivo

Spatiotemporal analysis was used to measure the ex vivo interval time between contractile events in the colon. Spontaneous motility in ex vivo colonic preparations involves intrinsic neuromuscular activity and therefore allows for an evaluation of possible changes in enteric neuromuscular function. SJL + MSCH EAE mice demonstrated significantly altered patterns of slow wave contractions (Figure 2.6 A, p<0.0001) and of CMMCs (Figure 2.6 B, p<0.0001) compared to control mice. Non-linear regression analysis was used to determine whether one curve fits both data sets. The results of the regression indicate that there is a significantly different curve that fits EAE (R^2=0.92, Sy.x=6.55) and control (R^2=0.95, Sy.x=5.75) slow waves (Figure 2.6 A, p<0.0001). There is also a significantly different curve that fits EAE (R^2=0.98, Sy.x=3.80) and control (R^2=0.97, Sy.x=3.70) CMMCs (Figure 2.6B, p<0.0001). In both instances, there is a shift in intervals towards longer periods of quiescence in EAE mice.

EAE mice do not exhibit neuronal loss, but have alterations in a subpopulation of ENS glía

To determine whether constipation in EAE is associated with structural changes at the histological level, we stained EAE SJL + MSCH colons with anti-HuD antibodies. Cell counts and analysis of ganglion sized suggested that there was no difference in neuron number, ganglion size, or neuronal packing density within ganglia between EAE and
control mice (Figure 2.7). We used confocal microscopy and image deconvolution to determine the relative area of ganglia containing pixels that positively expressed HuD, S100β and GFAP. There were no significant differences between the area within a ganglion that was positive for HuD or S100β expression (Figure 2.8 A, B). However, there was a significant decrease in the area of positive GFAP expression within EAE ganglia compared to control ganglia (Figure 2.8 C, p<0.05). Representative images show the relative density and distribution of HuD, S100β and GFAP expression within the ganglia of control (Figure 2.8 D-F) or EAE mice (Figure 2.8 G-I).

*Antibodies in MS serum exhibit positive immunoreactivity against the cells of the ENS*

We measured the intensity of immunofluorescence of enteric ganglia exposed to serum from MS patients or healthy controls. The tissue exposed to MS serum (n=40) exhibited higher levels of immunofluorescence than that exposed to control serum (n=20) when diluted at either 1:100 or 1:200 (Figure 2.10 A&B, p<0.01). In particular, 17.5% (7/40) of MS patient serum samples exhibited immunoreactivity intensity greater than 3 S.D. from the mean of the control group.

The MS patients were dichotomized into those who met Rome IV criteria for functional constipation (MS + GI, n=21) and those who did not (MS no GI, n=19). One subject self-identified as non-constipated, but met the Rome IV criteria for constipation and was instead included in the MS + GI group. Constipated patients reported significantly fewer weekly bowel movements compared to MS patients without constipation or healthy controls (p<0.0001, Figure 2.9). Subjects who did not experience constipation produced serum antibodies that bound more intensely to guinea pig LMMP.
preparations when diluted at 1:100 or 1:200, compared to serum from healthy control subjects (Figure 2.10 C&D, p<0.01). Contrary to our hypothesis that MS patients with constipation would exhibit increased immunoreactivity compared to MS patients without constipation, there was no significant difference between the binding intensity of serum in these two groups. In the MS groups, 15.7% (3/19) of MS subjects without constipation, and 19.0% (4/21) of subjects with constipation, exhibited immunoreactivity intensity greater than 3 S.D. from the mean of the control group.

**MS and EAE serum targets include neurons and glia of the ENS**

The cellular targets of human serum from MS patients included both enteric glia, that co-localized with S100β antibodies (Figure 2.11A), and enteric neurons, that co-localized with HuD antibodies (Figure 2.11B). In some cases, serum appeared to target both neurons and glia, and also included nuclear staining (data not shown). Serum isolated from B6 naïve control or vehicle control mice did not exhibit positive immunoreactivity against LMMP tissue (Figure 2.12 A&B). Serum isolated from individual B6 + MOG EAE mice within the same cohort differentially targeted cells of the ENS, with some samples binding only to neurons (Figure 2.12 C) and other samples binding to both neurons and glia (Figure 2.12 D).

**Discussion**

This study was designed to test whether EAE mice develop GI dysmotility that replicates symptoms observed in clinical MS and to determine whether EAE causes functional and structural changes in the ENS. We also tested whether antibodies from MS
patients and EAE mice exhibit immunoreactivity against cells of the ENS. Constipation in the EAE model has not been well-established, and the characteristics of this symptom have not been described in regards to mouse strains (Wunsch et al., 2017). For this reason, we tested constipation in both SJL and B6 mice, immunized against PLP, MOG, or MSCH. Our findings demonstrate that all EAE mice that we tested develop aberrant GI motility in vivo. In SJL + MSCH mice, we found that in vivo changes are associated with altered slow waves and CMMCs, as well as changes in the GFAP-positive glial cell population of the ENS. Our data indicated that EAE mice have slower whole GI transit, and motility is differentially affected throughout regions of the GI tract, causing increased rates of gastric emptying and decreased colonic motility. Ex vivo spatiotemporal analysis of slow waves and CMMCs demonstrated that EAE mice have longer intervals between events in both types of contractile activity. Because the spatiotemporal mapping protocol requires that the colon be removed from the body, these data suggest that the observed colonic dysmotility is due to functional changes at the level of the ENS. Histological investigation of the ENS of EAE mice revealed decreased GFAP distribution in myenteric ganglia. Indirect immunohistochemistry demonstrated that MS patients have increased serum immunoreactivity against calls of the ENS compared to control patients, and this is most prominent in serum samples from patients with MS who do not experience constipation. There was no significant difference in the serum immunoreactivity against myenteric ganglia between MS patients with constipation versus those without constipation, suggesting that this metric does not predict constipation. Both MS and EAE serum samples exhibited heterogeneous patterns
of immune staining between individual subjects, with targeted structures including neurons, glia, or a combination of both cell types.

Collectively, these studies contribute to our knowledge of GI involvement in MS by demonstrating that EAE mice develop gut dysfunction similar to that observed in MS, which could serve as a model for future studies of these symptoms. Importantly, we found that the EAE causes functional changes of the intrinsic enteric circuitry that persist when isolated from extrinsic autonomic innervation, and may be related to alterations in the ENS glia population. Antibody targeting of the ENS could be one such mechanism through which MS pathogenesis exerts these effects on gut motility, but future studies are needed to further investigate the pathogenicity of these antibodies.

We interpreted that altered GI motility in EAE mice represents pathology specific to EAE, but one limitation of these studies is that we did not test whether the changes are a result of a generalized sickness response that is not disease specific. EAE mice lose a significant percentage of bodyweight during the time course that these analyses took place. Anorexia and dehydration could both affect gastrointestinal motility, as well as increased inflammatory cytokines associated with the sickness response. Future studies will be designed to test the influence of these factors, and are described in chapter 3.

**Gastric Motility in MS**

The primary symptom of gastric motility reported in patients with MS is delayed gastric emptying, termed gastroparesis. We hypothesized that EAE mice would experience slower rates of gastric emptying than control mice. Interestingly, our data showed that all models of EAE mice had a faster rate of gastric emptying compared to...
controls. In the clinical literature, one study of gastric emptying scintigraphy in MS patients found that approximately 50% of patients experienced slower, and 20% experienced faster gastric emptying than control subjects, exhibiting that this symptom is experienced by some MS patients (el-Maghraby et al., 2005). Alternatively, idiopathic slow transit constipation has been associated with gastric, gallbladder, and small intestinal dysmotility suggesting that dysfunction at one site of the GI tract could affect motility in other regions (Gunay et al., 2004; van der Sijp et al., 1993). However, in most cases, the directionality of dysmotility is affected similarly across regions, so that slower transit in one region is associated with slower transit in other regions. Our data differ from those reported in the functional constipation literature because we found that in EAE, gastric motility and colonic transit are differentially affected. Few studies have analyzed gastric motility in MS, but a significant relationship between rates of gastric emptying and constipation have not been shown (el-Maghraby et al., 2005). The discrepancy between our experimental finding and the patterns of motility reported clinically could be related to the small sample sizes in the clinical literature, and relatively few studies of gut dysfunction in MS. Alternatively, this finding could represent one way in which the EAE model does not perfectly replicate features of the clinical disease.

Colonic Motility in MS

Constipation and fecal incontinence are both widely reported by MS patients, but few studies have investigated the etiology of these symptoms. Our data demonstrate that EAE mice have slower whole GI transit, slower colonic motility, and decreased fecal water
content compared to control mice. This is consistent with the slower GI transit reported by Wunsch and colleagues in EAE mice (2017). Similarly, one clinical report found that patients with MS have slow transit constipation, including the ascending and transverse colon, suggesting that the cause of the dysmotility is within the neurocircuitry of the gut itself (Chia et al., 1996). Often, patients with MS exhibit paradoxical constipation combined with fecal incontinence, and it is not likely for pelvic floor dysfunction to cause both patterns of bowel dysmotility simultaneously.

There is some evidence that GI involvement in MS is related to spinal cord pathology because it is sometimes associated with both urinary bladder dysfunction and clinical motor symptoms as measured by the expanded disease disability scale (EDSS) that reflects spinal cord involvement (Hinds et al., 1990; Preziosi et al., 2013). However, patients present with patterns of constipation that are inconsistent with pelvic floor dysfunction, a primary mechanism of constipation in spinal cord disease. In order to study these functions independently of both the pelvic floor and innervation derived from the spinal cord, we used ex vivo spatiotemporal analysis, as described below.

**CMMCs and Slow Waves**

Neuronally-mediated, rhythmic electrical activity termed CMMCs generate regular contractions that propagate along the length of the colon. CMMCs are thought to be the major impetus that generates propulsive motility within the GI tract and requires cooperation of both inhibitory and excitatory motor neurons (Dickson et al., 2010). In contrast, small, fast oscillations referred to as slow wave contractions are generated by ICC and reflect the pacemaker activity of these cells (Dickson et al., 2010). Slow wave
activity can regulate CMMCs, but are not necessary for their generation (Spencer et al., 2003). In the present study, we were able to investigate characteristics of CMMCs and slow waves, and found that there is a longer interval between both events in EAE mice compared to control mice. Importantly, in our preparation the colons were removed from the body and therefore the abnormal activity that we observed was independent of extrinsic spinal cord innervation or pelvic floor dysfunction. Our findings suggest that pathogenesis in EAE and MS result in altered function at the level of the intrinsic neuromuscular circuitry of the gut.

In Wunsch and colleagues’ investigation of constipation in EAE mice, myography recordings of circular muscle demonstrated decreased smooth muscle contraction (2017). Clinically, colonic myoelectrical activity has been compared between patients with MS and healthy controls both at rest and following meal consumption, and MS patients were found to have reduced amplitude of myoelectrical spike potentials in both digestive states (Glick et al., 1982). In MS patients, the frequency of myoelectrical events was not different from controls at baseline, but was significantly reduced in the post-prandial period. Our results are consistent with other reports in the literature, and together support that constipation in MS and EAE involves dysfunction within ENS neuro-muscular circuitry.

Cellular Changes within Myenteric Ganglia

Enteric glial cells are increasingly recognized for their heterogeneous phenotypes, anatomical locations, and roles in gut homeostasis. Similar to CNS astrocytes, enteric glia can undergo reactive gliosis and phenotypic plasticity in response to injury or
inflammation. Here, we report decreased GFAP-positive area in myenteric ganglia of EAE colons.

Interestingly, the only other study of the ENS in EAE mice reports increased GFAP expression in small intestinal myenteric ganglia (Wunsch et al., 2017). The discrepant findings between our study and the Wunsch study could be related to EAE induction methods, or could represent differential effects of GFAP expression throughout different regions. For example, in Crohn’s disease, submucosal plexus enteric glial cells near sites of inflammation have increased GFAP expression, but those in non-inflamed sites have reduced GFAP expression (von Boyen et al., 2011). Consistent with our finding of reduced GFAP distribution, the association between loss of enteric glia and irregular GI motility has been widely reported in the literature. Clinically, patients with slow transit constipation have fewer enteric glial cells in their ileum than non-constipated controls (Bassotti et al., 2006a). Experimental antagonism of Ca^{2+} hemichannels used by these cells causes impaired GI motility in mice (McClain et al., 2014).

In the present study, we did not observe a loss of colonic myenteric neurons or HuD expression. However, disruption of GFAP-expressing enteric glia has been shown to induce phenotypic changes in neurons without changing neuron numbers, leading to altered GI motility (Aube et al., 2006). Although we did not characterize neurochemical coding in neurons of our EAE mice, we did observe a loss of GFAP expression without a change in neuron number similar to the results of the Aubé study. Whether or not EAE mice exhibit myenteric neurochemical plasticity could contribute to altered motility, and warrants further investigation.
Antibodies in MS

AGID is an increasingly recognized clinical phenomenon that occurs when autoimmune processes, especially antibodies, target the GI tract idiopathically or secondary to another autoimmune disease. Here, we report that the serum of MS patients and EAE mice contains antibodies that have increased immunoreactivity against enteric neurons and glia compared to control serum. We tested whether MS patients who experience constipation develop elevated serum antibodies against myenteric ganglia compared to MS patients without constipation. Contrary to our hypothesis, there was no significant difference in serum immunoreactivity between these two groups. This finding suggests that serum immunoreactivity does not reflect clinical constipation in MS patients. One limitation of our approach is the sensitivity of using immunohistochemistry as a quantitative assay, and future studies should implement more quantitative methods.

Further, it is possible that if antibodies do play a role in constipation in MS, they could act as functional autoantibodies that stimulate or inhibit a neurotransmitter receptor or ion channel. Functional antibodies may not bind tightly to these targets, complicating their detection ex vivo. An approach to test this hypothesis is to study the function of autoantibodies on GI motility by passive transfer into a naïve animal. Similar to the functional approach, is whether autoantibodies reach myenteric ganglia in vivo, and the permeability of ganglia to these antibodies could vary between patients. Another possibility is that only some targets of serum immunoreactivity, such as specificity against glia or neurons, predict GI dysfunction in MS patients, and we did not test this hypothesis in the current study. One result is that we could observe null findings in these
additional approaches, which would suggest that autoantibody immunoreactivity against targets of the ENS is an epiphenomenon in MS patients of no clinical significance.

Although we did not observe a difference between MS patients with and without constipation, the results of MS serum immunoreactivity compared to that of control patients is consistent with Wunsch and colleagues’ report that EAE and MS serum have increased immunoreactivity against ENS targets compared to controls (2017). AGID has been described extensively in Chagas’ disease (Sterin-Borda et al., 2001), paraneoplastic syndrome (Sodhi et al., 1989), Sjögren’s syndrome (Gordon et al., 2001), and type I diabetes (Jackson et al., 2004). Over twenty autoantibodies have been identified in MS (Schirmer et al., 2014) and several of these protein targets, including proteolipid protein 1 (PLP-1) and myelin basic protein (MBP) are also widely expressed in the ENS (Rao et al., 2015). In one study of GI antibodies in MS patients, 28% of patients were found to have at least one antibody against mucosal antigens, including tissue transglutaminase, intrinsic factor, and parietal cells, supporting the possibility of further enteric targets of the disease (Banati et al., 2013). Together with our findings, these data suggest that MS could represent an additional autoimmune disease in which autoantibodies contribute to constipation, and future studies are warranted to better characterize whether these antibodies play a significant role in GI dysmotility in MS.

Concluding Remarks

In this study, we have conducted a broad analysis of GI motility in the EAE mouse model to improve our understanding of constipation and GI dysfunction in patients with MS, a symptom that is often overlooked in the MS literature. Our models replicate many
features consistent with clinical constipation, and suggest that MS is associated with functional and potentially structural changes of the ENS. This finding is important because it demonstrates that the observed GI dysfunction extends beyond spinal cord demyelination; ex vivo CMMC and slow wave dysmotility, and reduced GFAP distribution, represent alterations in the ENS itself. Our finding of increased MS serum immunoreactivity against myenteric ganglia, with MS and EAE sera targeting neurons and glia, suggests that pathogenic autoantibodies could target the ENS in vivo. However, the absence of significant differences between immunoreactivity of serum from constipated versus non-constipated MS patients limits our interpretation of antibody pathogenicity in this context because it suggests that serum immunoreactivity does not predict constipation. Future studies are warranted to characterize altered neurocircuitry or phenotypic plasticity in the ENS of EAE, including glia, and to test the pathogenicity of MS or EAE autoantibodies in constipation. Collectively, these studies advance our knowledge of enteric pathophysiology associated with MS that could lead to improved treatment of constipation in these patients.
References for Chapter 2


Figure 2.1
Figure Legend

Figure 2.1 Whole GI transit time is measured as the time in minutes it takes for carmine red dye administered via oral gavage to be passed in the fecal pellets of mice. Data represented as mean ± SEM. Transit time is significantly slower in EAE mice induced under the B6 + MOG, SJL + PLP, and SJL + MSCH induction protocols. There was no significant difference in the B6 + MSCH protocol. (A Control n=7, EAE n=9, *p<0.05; B Control n=8, EAE n=14, n.s.; C Control n=8, EAE n=7, *p<0.05; D Control n=8, EAE n=8, *p<0.05). Student’s t-test with (B) or without (A, C, D) Welch’s correction.
Figure 2.2
**Figure Legend**

Figure 2.2 Fecal water content is lower in EAE mice compared to controls. Graphs show the percentage of water in fecal pellets collected from EAE and control mice over one hour. Data represented as mean ± SEM. (A Control n=7, EAE n=9, *p<0.05; B Control n=8, EAE n=14, **p<0.01; C Control n=10, EAE n=10, **p<0.01; D Control n=5, EAE n=6, ****p<0.0001). Student’s t-test with (C) or without (A, B, D) Welch’s correction.
Figure 2.3
Figure Legend

Figure 2.3 Colonic motility is slower in EAE mice compared to controls. Graph shows the time in minutes until expulsion of a glass bead inserted 2.0 cm into the distal colon. Data represented as mean ± SEM *p<0.05, ***p<0.0001 by Student’s t-test.
Figure 2.4
Figure Legend

Figure 2.4 To measure gastric emptying, mice were administered a fluorescent meal by oral gavage the percentage of fluorescence that had emptied the stomach after 20 minutes (A/B) or 24 minutes (C/D). A-D) EAE mice demonstrated significantly increased gastric emptying compared to control mice. (A Control n=5, EAE n=5, *p<0.05.; B Control n=7, EAE n=9, * p<0.05; C Control n=6, EAE n=5, ***p<0.01; D Control n=14, EAE n=12, * p<0.05) Unpaired, two-tailed t-test.
Figure 2.5

Leading Edge

A

B

C

D

Geometric Center

E

F

G

H

Figure 2.5
Figure Legend

Figure 2.5. To measure small intestinal motility, mice were administered a fluorescent meal by oral gavage, and the distribution of fluorescence in the small intestine was measured as leading edge (A-D) and geometric center (E-H). Small intestinal motility is not significantly different between EAE mice and controls.
Figure 2.6

A. Slow Wave Interval Distribution

B. CMMC Interval Distribution

****p<0.0001

Percent of Events (%)

Time (s)

0 20 40 60 80 100

0 50 100 150 200 250 300 350 400

Control

EAE

0 20 40 60 80 100

0 20 40 60 80 100

****p<0.0001

Control

EAE

Figure 2.6
**Figure Legend**

Figure 2.6 The interval between slow wave contractions and CMMCs was measured between EAE SJL + MSCH mice (n=4) and control mice (n=5). Graphs demonstrate the cumulative frequency distribution of each interval duration. A) EAE mice have an altered distribution of slow wave intervals compared to controls, measured by the Kolmogrov-Smirnoff test (p<0.0001). A non-linear regression demonstrates that there is a significantly different curve for each data set (p<0.0001). In slow wave data, every 10\textsuperscript{th} data point is plotted for visibility. B) EAE mice have an altered distribution of CMMC intervals compared to control mice, measured by the Kolmogrov-Smirnoff test (p<0.0001). A non-linear regression demonstrates there is a significantly different curve for each data set (p<0.0001). In CMMC data, every 20\textsuperscript{th} data point is plotted for visibility.
Figure 2.7
Figure Legend

Figure 2.7 Ganglia from EAE SJL + MSCH mice (n=5) and control mice (n=5) were stained with anti-HuD antibody and assessed for morphological changes in A) neuron number, B) ganglion area, and C) neuronal density within ganglia. There were no differences found in either metric. Data represented as mean ± SEM and analyzed as student’s t-test. Approximately 20 ganglia were counted per mouse.
Figure 2.8
Figure Legend

Figure 2.8 The area of cells in the ENS was calculated in EAE mice induced under the SJL + MSCH protocol. Three different cell markers were used. A) There was no difference in the percentage of pixels per ganglion that expressed neuron HuD. B) There was no difference in the percentage of pixels per ganglion that expressed the glial protein S100β. C) There was a significant reduction in the distribution of GFAP within ENS ganglia of EAE mice compared to control mice (p<0.05, student’s t-test). Representative images demonstrate the distribution of HuD, S100β and GFAP in control mice (D, E, F) and EAE mice (G, H, I), respectively.
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<th>Years since Diagnosis</th>
<th>EDSS</th>
<th>Years of Constipation</th>
<th>Constipation Precede Diagnosis?</th>
<th>Bowel Movements per Week</th>
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<td>12.2 ± 9.1</td>
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Figure Legend

Figure 2.9 Table of clinical subjects. MS subjects with constipation have a significantly fewer bowel movements per week compared to controls or MS patients without constipation (one-way ANOVA, $p<0.0001$, Bonferroni post-hoc). Data reported as percent, or as mean ± SD.
Figure 2.10
Figure Legend

Figure 2.10 Human serum immunoreactivity against ENS ganglia of guinea pig LMMP preparations. A) Serum isolated from MS patients (n=40) has more intense immunoreactivity against cells of the guinea pig ENS than does serum from healthy control patients (n=20) without GI symptoms when diluted to 1:100 or B) 1:200. 17.5% of MS patient serum (7/40) binds with an intensity greater than 3 S.D. from the control mean. C) Serum from MS patients without constipation (MS no GI) has more intense immunoreactivity against cells of the guinea pig ENS than does serum from healthy control patients (n=20) at 1:100 and D) 1:200 dilution (p<0.05, one-way ANOVA, Bonferroni post-hoc). There is no significant difference between serum immunoreactivity of MS patients with constipation compared to those without constipation. 15.8% (3/19) of serum from MS patients without constipation, and 19.0% (4/21) of serum from MS patients with constipation binds to ENS cells with greater than 3 S.D. from the control mean.
Figure 2.11
Figure Legend

Figure 2.11 Patterns of serum immunoreactivity of MS patients who experience constipation include glial-predominant and neuronal-predominant staining. A) Example of an MS patient serum sample that co-localizes with S100β antibodies. B) Example of an MS patient serum sample that co-localizes with HuD antibodies.
Figure 2.12
Figure Legend

Figure 2.12 Immunoreactivity of mouse serum against guinea pig LMMP. A) Naïve B6 control mice exhibit no positive immunoreactivity against cells of the ENS. B) CFA-treated B6 control mice exhibit no positive immunoreactivity against cells of the ENS. C) EAE B6 + MOG treated mice demonstrated immunoreactivity against ENS neurons, or D) against neurons and glia.
CHAPTER 3: FINAL CONCLUSIONS AND FUTURE DIRECTIONS

I. Gastrointestinal Dysmotility in EAE

*Summary and Conclusions*

Multiple sclerosis (MS) is an autoimmune disease that causes central nervous system (CNS) demyelination and axonal degeneration, resulting in motor, visual, sensory and cognitive dysfunction. Gastrointestinal (GI) dysmotility, including constipation, is a frequent complication of MS, and the patterns of constipation suggest that dysfunction within the enteric nervous system (ENS) itself could occur in the disease. Patients with MS frequently exhibit slow transit constipation, suggesting dysfunction of the ENS (Chia et al., 1996). Decreased amplitude and frequency of colonic myoelectrical events following meal consumption in MS patients is additional evidence for ENS involvement, as these patterns of motor activity are generated by the ENS (Glick et al., 1982). To date, only one other study has assessed GI motility in an animal model of MS, and found that mice induced with experimental autoimmune encephalomyelitis (EAE) develop slower GI transit and decreased smooth muscle contractility, and increased GFAP expression in the small intestine (Wunsch et al., 2017). This dissertation provides a comprehensive analysis of whole GI transit, gastric emptying, small intestinal transit, colonic motility, and fecal water content in several models of EAE. Our analysis of colonic migrating myoelectric complexes (CMMCs) and slow wave contractions is a valuable measure of ENS activity independent of extrinsic innervation. Further, we report immunohistochemical evaluation of neurons and glia of the EAE myenteric plexus to demonstrate cellular changes of the ENS.
The data presented in this dissertation demonstrate that whole GI motility is significantly slower in EAE mice compared to controls, and colonic motility may be particularly susceptible to these effects. Interestingly, EAE mice exhibited increased rates of gastric emptying. The intervals of CMMCs and slow wave contractions were longer in EAE mice compared to controls in *ex vivo* studies during which the colon is separated from extrinsic innervation and influence of pelvic floor dysregulation. This suggests that GI dysmotility in EAE occurs as a result of altered network function in the ENS. Results from immunohistochemistry of the myenteric plexus of EAE neurons demonstrated a decrease in the area of glial GFAP expression with no changes in S100β expression or enteric neurons.

*Alternative Approaches and Future Directions*

1. *Sickness Response*

   One limitation of the studies described in this dissertation is that we did not test whether the GI dysmotility is a result of sickness response in the EAE mice. The generalized sickness response is a result of immune activation during infection and was first identified when it was found that cytokine administration to treat cancer patients resulted in non-specific symptoms including fever, malaise, fatigue, and anorexia (Kelley et al., 2003). This has also been demonstrated experimentally, as intraperitoneal administration of interleukin-1 in rats results in fever, social withdrawal and anorexia (Kent et al., 1992). EAE mice undergo a sickness response, best recognized by their initial weight loss. Similar behaviors likely develop in MS. In addition to the stark loss of mobility that characterizes the disease, MS patients also experience depression, fatigue,
and malaise. IL-1β along with other inflammatory cytokines, is elevated in patients with MS and may represent one component of clinical sickness response in these patients (Tsukada et al., 1991). While no group has experimentally studied the effect of the sickness response on gastrointestinal motility, it is possible that both generalized inflammation and the associated symptoms including lethargy, anorexia, and decreased water intake, could contribute to constipation. For example, IL-1β results in decreased GI motility in rats, and could be related to its effects on the CNS including corticotropin release (Fargeas et al., 1993) or due to direct effects of inflammatory cytokines on cells of the enteric nervous system (Kelles et al., 2000). Decreased food intake as a result of the sickness response could also contribute to constipation. For example, clinical patients with anorexia experience altered gastrointestinal motility (Norris et al., 2016), including constipation that is associated with altered colonic transit time and anorectal dysfunction (Chiaroni et al., 2000). Both MS patients and EAE mice experience loss of mobility and paralysis that could further contribute to constipation - physical activity has been shown to increase intestinal transit time and alleviate constipation (De Schryver et al., 2005).

In the studies described in this dissertation, we did not comprehensively test whether the altered GI motility observed in EAE mice is related to a sickness response. This will be important to rule out because EAE mice do undergo a sickness response, which includes weight loss likely related to reduced food and water consumption. The maximum weight loss experienced by these mice within a 28-day window is approximately 10-15% of body weight, whereas controls on average gain about 5% body weight (data not shown). Because EAE mice do in fact lose weight, they must be consuming less food and water at some point in the disease course, but it is unknown
whether this loss in body weight affects GI motility. In order to assess food and water intake, we planned to use metabolic chambers, but those available to us were not adequately sensitive for our needs. An alternative approach to assess food consumption is to weigh the contents of the colon to determine whether the mass differs between EAE and control mice. A more sensitive approach to measure hydration status could be to calculate serum osmolality at the time of euthanasia. These measures should be calculated in parallel with animals used for motility assays, and unfortunately were not done in the studies described in this dissertation.

In our studies, we used control mice that were administered complete Freund’s adjuvant and pertussis toxin to control for some aspects of inflammation in EAE mice. Because the control mice do not lose as much weight as the EAE mice and do not undergo a highly active immune response, they likely do not develop a strong sickness response. It is important to specifically study whether the sickness response contributes to altered GI motility. Future studies could involve an additional control group that is administered IL-1β, TNF-α and other cytokines that mediate the sickness response to determine whether there is a significant difference between GI motility in these mice compared to EAE.

2. Changes in myenteric neurochemical coding

In this dissertation, we hypothesized that neuronal numbers would be reduced in EAE mice, because a loss of enteric neurons is associated with some forms of constipation. This has been demonstrated in slow transit constipation (Bassotti et al., 2006b), constipation in aging populations (Coulombe et al., 2014), and Chagasic megacolon
(Iantorno et al., 2007). In the current studies, we quantified neurons, neuronal density, and the area of HuD expression in myenteric ganglia, and found no significant differences in these metrics. This finding does not eliminate the possibility that ENS neurons are changing in EAE. One alternative hypothesis could be that the phenotype of neuronal subpopulations is changing independent of total neuron numbers.

The ENS contains functionally distinct subpopulations of neurons with unique neurochemical coding, and one limitation of our approach is that we did not quantify these subpopulations. This is important to evaluate, because neurochemical plasticity of neurons has been associated with constipation independent of total neuron numbers. In one study of enteric glial ablation in mice, the researchers found no change in small intestinal neuron numbers, but instead identified phenotypic plasticity within neurons (Aube et al., 2006). Specifically, they found a reduction of neurons that positively express vasoactive intestinal peptide (VIP) and substance P (SP) in the submucosal plexus. In myenteric plexus neurons, these changes were represented as increased choline acetyltransferase (ChAT) and reduced nitric oxide synthase (NOS) immunoreactivity. Functionally, these changes were associated with delayed GI transit that was caused by a failure of muscle relaxation, an integral component of coordinated propulsive motility. Loss of neuronal NOS, similar to the observations of the Aubé study, is known to reduce the frequency of CMMCs (Dickson et al., 2010). Neurochemical plasticity of enteric neurons is also reported in diabetic gastroparesis (as reviewed by Chandrasekharan et al., 2007), intestinal inflammation (Lomax et al., 2005) and experimental models of Parkinson’s disease (Chaumette et al., 2009).
Assessing whether enteric neurons in EAE demonstrate neurochemical plasticity is an important addition to the studies that we have already completed. The EAE model shares many characteristics with studies of altered neuronal phenotype, including the loss of GFAP and delayed whole GI and colonic transit (Aube et al., 2006), and delayed CMMC and slow wave motor patterns (Dickson et al., 2010). The addition of phenotypic analysis would complement our understanding of mechanisms associated with constipation in EAE by allowing identification of specific cellular changes.

3. **Significance of decreased GFAP distribution**

Enteric glial cells have been increasingly recognized for their significant role in GI homeostasis, which includes regulation of motility, host defense, synaptic transmission, and potentially barrier function. Recently, researchers have begun to classify enteric glia based on anatomical localization and phenotype. Enteric glia are canonically recognized for their localization within ganglia of the myenteric and submucosal plexus, and these cells are also located in the mucosa and the muscularis propria (Gulbransen et al., 2012). Proteolipid protein 1 (PLP1) is the most ubiquitously expressed glial protein in the gut (Rao et al., 2015), and other markers including SOX10, GFAP, and S100β are more differentially expressed in subpopulations of glia (Boesmans et al., 2015; Gulbransen et al., 2012). The distribution of these cell markers varies substantially within the different glial compartments of the gut. One study that characterized glia within myenteric ganglia classified the cells into four subtypes based on their morphology, and determined that the ratio of S100β, GFAP, and SOX10 expression was unique in each morphological subtype (Boesmans et al., 2015).
In the current study, we did not observe a change in the area of myenteric ganglia that expressed S100β, but did observe decreased GFAP. Altered populations of S100β and GFAP have each been associated with constipation independently. Decreased S100β expression occurs in the myenteric and submucosal ganglia of patients with slow transit constipation (Bassotti et al., 2006b), and selective ablation of GFAP expressing glia in mice resulted in slower GI motility (Aube et al., 2006). This suggests that both populations are important for normal GI motility, but studies have shown that morphological and phenotypic subtypes of glia within a myenteric ganglion have unique sensitivities to purinergic signaling Ca\(^{2+}\) transients (Boesmans et al., 2015). Ca\(^{2+}\) propagates throughout the glial syncytium via connexin 43 hemichannels, and disruption of this communication results in symptoms consistent with constipation (McClain et al., 2014). Therefore, alterations within certain subpopulations of glia may have a greater effect on motility than others.

To better understand the significance of decreased GFAP distribution in enteric glia in EAE, a meaningful approach would be to analyze S100β and GFAP co-localization to identify whether a specific morphological sub-population of glia is affected. We should also test whether altered GFAP represents a shift in glial phenotype or cell death of GFAP glia. We do not expect to observe glial cell death at the time points we tested because we did not see any change in S100β-expressing neurons, and GFAP is rarely expressed in the absence of S100β. If cell death of GFAP positive cells was occurring, we would likely detect differences in the expression of both proteins. Further studies could investigate the function of glia in EAE mice by using Ca\(^{2+}\) imaging to determine whether altered Ca\(^{2+}\) signaling contributes to altered GI function in EAE.
4. Mucosal serotonin and melatonin signaling

5-hydroxytryptamine (5-HT), more commonly known as serotonin, is a neurotransmitter and paracrine signaling molecule of the gut that is released from enterochromaffin cells. Most of the body’s serotonin, over of 90%, is located in the gut, and only 3% is found in the CNS. Serotonin cannot cross the blood brain barrier, and thus gut-derived serotonin does not enter the CNS (Spohn et al., 2017). Serotonin is generated from tryptophan under the rate-limiting enzyme tryptophan hydroxylase 1 (TpH1) or TpH2, which are differentially used by neurons and enterochromaffin cells, respectively (Cote et al., 2003; Walther et al., 2003). Serotonin that is released into a synapse or the gut parenchyma is cleared by neurons or enterocytes via the serotonin reuptake transporter (SERT) (Gershon, 2003). Once it is transported into nerve terminals or epithelial cells, it is degraded by monoamine oxidase A (MAO-A) into 5-hydroxindoleacetic acid (5-HIAA), which is then excreted in urine (Mawe et al., 2013; Spohn et al., 2017).

Serotonin release from enterochromaffin cells stimulates 5-HT receptors on intrinsic primary afferent neurons (IPANs) whose cell bodies are located in the submucosal or myenteric plexus. Alternately, there are 5-HT receptors on enterochromaffin cells themselves, enterocytes, goblet cells, smooth muscle cells, ICC, interneurons and motor neurons (Mawe et al., 2013). Serotonin has many regulatory roles in the gut, including motility, secretion, sensation and inflammation. Generally, it is thought to be a pro-kinetic molecule, or one that increases GI motility. This is supported by findings from our lab showing increased motility by stimulation of 5-HT₄ receptors (Hoffman et al., 2012),
and other groups have reported pro-kinetic effects of 5-HT3 and 5-HT7 receptors (Kendig et al., 2015). Heredia and colleagues’ data demonstrated that mice deficient in TpH1 generated CMMCs at a lower frequency than control mice, supporting the role of serotonin in propulsive motility (Heredia et al., 2013).

Alternatively, serotonin has also been shown to cause constipation. Our lab and others have demonstrated this effect by administering high levels of selective serotonin reuptake inhibitors (SSRIs), such as paroxetine or fluoxetine, to mice, and observing a decrease in GI motility (Coates et al., 2006; Wade et al., 1996). The discrepancy between these contradictory findings is likely related to a dose effect. At high levels, increased levels of extracellular serotonin cause receptor desensitization, resulting in less stimulation of IPANs and decreased motility.

The relationship between serotonin and gut inflammation has been studied extensively, and the molecule can have both pro-inflammatory and anti-inflammatory effects. There are seven serotonin receptors expressed in the gut, and 5-HT7 receptor is thought to promote inflammation (Bischoff et al., 2009; Ghia et al., 2009) whereas data from our lab supports anti-inflammatory effects of 5-HT4 receptor activation (Spohn et al., 2016). Interestingly, our lab has demonstrated that SERT levels are decreased in Caco-2 enterocyte cells that are incubated with pro-inflammatory cytokines IFN-γ and TNF-α, which in turn causes increased extracellular serotonin (Foley et al., 2007). Furthermore, inflammation has been shown to result in decreased colonic epithelial SERT expression in ulcerative colitis, diverticulitis, and a variety of animal models (Coates et al., 2017)
Serotonin is the precursor to another molecule named melatonin, that, is converted by the enzymes arylalkylamine N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT). Unlike serotonin, melatonin is readily lipid-soluble and thus can freely diffuse through cell membranes, including the blood-brain barrier and does not rely on a transporter for reuptake. Like serotonin, melatonin is also found in much higher concentrations in the gut than in the brain and has differential effects on motility. At low doses, melatonin has pro-kinetic effects, and at high doses, results in decreased GI motility (Drago et al., 2002). A reduction of melatonin release in the colon is associated with constipation in aging that can be remedied with melatonin supplementation (Diss et al., 2013). Melatonin is thought to have anti-inflammatory actions in the gut, and among other responses, increases Th2 lymphocyte activity (Chen et al., 2011; Shaji et al., 1998). Treatment of experimental colitis with melatonin supports its protective role in the disease (as reviewed by (Shaji et al., 1998)).

Because of the intimate relationship between serotonin and melatonin with GI motility and inflammation, we hypothesize that either of these molecules could be associated with constipation in EAE. To test whether release of serotonin or melatonin is altered in EAE mice, we used an electrochemistry approach, called amperometry (Diss et al., 2013; Patel, 2011). Electrochemistry relies on the abilities of molecules to undergo an oxidation reaction when a voltage is applied, releasing a free electron. Collectively, the release of electrons from these molecules is detected by a recording electrode and the current is measured by an amp meter. Importantly, different molecules are oxidized by different voltages, which allows for the specificity of the measurement to the molecule of
interest. For example, serotonin is oxidized at 650 mV, and melatonin is oxidized at 800 mV.

EAE was induced in B6 mice under the MSCH protocol. Control mice received CFA and PTX injections as described previously, but no MSCH. Naïve control mice received no injections. After EAE motor symptoms had developed, mice were euthanized and the colons were removed, cut open longitudinally, and pinned in a sylgard-coated organ bath circulated with 37°C Krebs solution. The amperometry apparatus is arranged so that the tips of a Ag|AgCl reference electrode and stainless-steel auxiliary electrode are submerged in the bath adjacent to the tissue, and a boron-doped diamond (BDD) microelectrode referred to as the working sensor is directly opposed to the intestine, just touching the mucosal surface. A potentiostat (BioStat multichannel potentiostat, ESA Biosciences, Inc.) allows for communication between the reference electrode and working sensor to apply a voltage, and between the working sensory and auxiliary electrode to measure the current. The current was measured in each tissue preparation by applying a voltage of 650 mV for 40s to measure serotonin release. The value reported for each animal is an average of five recordings. The voltage was then increased to 800 mV for 40s intervals to measure melatonin, and again repeated five times. To assess SERT integrity, we conducted an additional recording at 650 mV in the presence of the SERT inhibitor, fluoxetine. Measurements were recorded in amps, and calibration with reference solutions of serotonin and melatonin allowed for conversion into micromoles.

In the colons of EAE mice (n=8), there was a significantly higher concentration of serotonin at baseline compared to naïve control (n=6) or control mice (n=6) (p<0.0001, two-way ANOVA, Figure 3.1 A). To test whether elevated serotonin was related to
decreased SERT activity, 1μM fluoxetine was added to the bath and recordings were continued at the 650 mV holding potential (Figure 3.1 A). In the presence of fluoxetine, the serotonin concentration did not differ amongst the groups. However, the increase in serotonin concentration elicited by fluoxetine was lower in the EAE group than both the naïve control (p<0.0001) or the control group (p<0.001, Figure 3.1B), indicating that SERT function is suppressed in the colonic mucosa of EAE mice.

To test whether melatonin is altered in EAE, we applied a voltage of 800 mV to the tissue, and subtracted the current generated from serotonin oxidation at baseline. Melatonin was significantly decreased in EAE mice compared to both control groups (p<0.0001, one-way ANOVA, Figure 3.1 C). Next, we measured whether the ratio of the two molecules was abnormal in EAE, and found that the ratio of melatonin to serotonin was significantly decreased from both control groups (p<0.0001, one-way ANOVA, Figure 3.1 D).

Together, these data suggest that constipation in EAE could involve dysfunctional serotonin and/or melatonin signaling. Increased serotonin observed in EAE could cause receptor desensitization and result in constipation. Similarly, a loss of melatonin could fail to sufficiently stimulate melatonin receptors that help to regulate normal propulsive motility. This has been shown in aging mice that develop constipation related to loss of mucosal melatonin, and motility can be restored following melatonin administration (Diss et al., 2013). Further, Nouri and colleagues have reported that the mucosa of EAE intestines develops elevated inflammation compared to controls (Nouri et al., 2014), and this could be potentiated by inflammatory effects of serotonin and the loss of anti-inflammatory effects of melatonin.
One interpretation of altered serotonin availability in EAE colonic mucosa is related to release and reuptake mediated by gut epithelial cells, including enterochromaffin cells and enterocytes. However, serotonin synthesis and reuptake in the periphery is not limited to these cells but can also be synthesized by mast cells. Therefore, because we did not specifically test the source of the serotonin in the electrochemistry experiments, we do not know with certainty that it is enterochromaffin-derived. Further, this molecule has broad interactions with other systems including immune, bone, pulmonary, cardiac, metabolic, and vascular, expanding the possible interpretations of altered serotonin availability (Spohn et al., 2017). Several alternative implications of serotonin will be discussed here and could serve as the justification for future experiments regarding this molecule in the context of EAE.

Mast cells are likely a source of serotonin in rats and mice (Weitzman et al., 1985), and possibly in humans (Kushnir-Sukhov et al., 2007). This molecule is a potent mediator of inflammation and immune responses. For example, serotonin is released following mast cell degranulation in response to topical capsaicin application in mice, and acts through 5-HT\textsubscript{2} receptors to promote edema (Inoue et al., 1995). In mice, T-cell dependent mast cell degranulation and serotonin release have been implicated in the delayed-type hypersensitivity following immunization by injection of sheep erythrocytes into the footpad (Askenase et al., 1980). In humans, the serotonin production of mast cells in less clear. Our lab has previously demonstrated positive serotonin immunoreactivity of enterochromaffin cells in human colon, with no positive labeling of mast cells (Mawe et al., 2013). Buhner and colleagues also report a lack of serotonin expression in human intestinal mast cells (Buhner et al., 2012). Serotonin has been identified in mast cells
isolated from human blood (Kushnir-Sukhov et al., 2007). These subjects had mastocytosis and their cells were grown in culture which both could affect their mast cell phenotype. There is evidence of serotonin-positive mast cells in the intestines of healthy rats (Fujimiya et al., 1997). In rats induced with the dextran sodium sulfate model of colitis, serotonin immunoreactivity is significantly increased in both mucosal and connective tissue mast cells of the colon (Nagata et al., 2001). In human tissue, one paper reports immunohistochemical evidence that intestinal mast cells are in close proximity to enteric neurons and enterochromaffin cells, but do not positively express serotonin (Buhner et al., 2012). Others report a significant increase in the number of serotonin-expressing mast cells in the mucosa, submucosa, and muscularis of human colon from patients with ulcerative colitis (Stoyanova et al., 2002). Few studies have specifically investigated serotonin release from intestinal mast cells in mice, although it is known that the number of mast cells does increase in the mucosa following infection (Wheatcroft et al., 2005). It is likely that mouse intestinal mast cells do secrete amount of serotonin, and this serotonin release could contribute to local inflammation and neuronal signaling, and could affect tissues distal to the gut by reuptake onto circulating platelets. While mast cell serotonin secretion has not been specifically studied in the intestines of EAE mice, researchers have demonstrated intestinal inflammation in these animals, and it is probable that mast cells also play a role in this pathology and could involve serotonin release (Nouri et al., 2014).

In addition to local effects, the functions of intestinal-derived serotonin that is absorbed into the bloodstream could promote pathology throughout the body. This has been demonstrated in our lab by studying serotonin-mediated changes in bone density in
animals induced with experimental colitis (Lavoie et al., unpublished data). Serotonin promotes vascular permeability by increasing local nitric oxide synthesis, which could increase immune cell extravasation (Fujii et al., 1994). Local increases in serotonin can also increase the permeability of the blood brain barrier, and this has been demonstrated by dynamic serotonin release by the brainstem raphe nucleus (Cohen et al., 1996). An interesting question is whether increased serotonin availability in the intestines could increase blood-brain barrier permeability, representing a mechanism by which gastrointestinal disease could increase central nervous system pathology. One paper reports that experimental intravenous administration of serotonin in rats increased blood brain barrier permeability (Sharma et al., 1990). Rats with experimentally induced colitis show evidence of increased blood brain-barrier permeability, although the effect of serotonin in this context was not specifically studied (Natah et al., 2005). More thorough investigation of the role of serotonin in barrier function could have major implications in the relationship between gastrointestinal and nervous system diseases.

An additional confound in our interpretation of increased serotonin availability in the colons of EAE mice is the role of the microbiome. The microbiome contributes to serotonin availability in the gut, because bacteria can secrete metabolites that regulate serotonin synthesis from enterochromaffin cells (Yano et al., 2015). Altered intestinal microbiota, such as following antibiotic usage, has been shown to alter both serotonin availability and intestinal motility (Ge et al., 2017). The microbiome is altered in patients with multiple sclerosis (Jangi et al., 2016). It is possible that the altered microbiome in MS mediates serotonin released by both the mucosal immune system and epithelium to together promote inflammation, vascular permeability, and altered ENS sensitivity.
Similarly, our finding of decreased melatonin availability in the gut cannot be limited to its synthesis by enterochromaffin cells. Research using cultured rat mast cells supports that melatonin is synthesized and released from these cells (Maldonado et al., 2010). Consistent with its anti-inflammatory properties, melatonin is protective against blood brain barrier permeability, and similar to serotonin, the implications of altered melatonin synthesis in the gut could have global effects on the body’s homeostasis (Chen et al., 2006). The breadth of research regarding melatonin is limited compared to serotonin, but future studies in our lab should investigate whether the decreased melatonin in EAE colons is related to its release and reuptake from the gut epithelium or alternately, mast cells and other immune cells.

Our future studies should aim to identify the source of altered melatonin and serotonin levels in the colonic mucosa as it related to gut epithelium and immune cells. Using our amperometry approach, we could for example, study serotonin availability in EAE mice in the presence and absence of mast cell stabilizers. Further studies in our lab should investigate the molecular pathway that is altered to cause of elevated mucosal serotonin in EAE. Our data suggest that SERT is still present and functional in EAE mice, but that SERT function is suppressed, possibly due to decreased SERT expression. Future investigations should measure SERT protein by ELISA and RNA by qPCR, as well as investigating SERT function by measuring uptake of $^3$H-5-HT, as has previously been performed in the Mawe laboratory (Foley et al., 2007; Linden et al., 2005). It is possible that protein levels are not different, but the effects that we observe are related to a loss of function and could relate to SERT genetic polymorphisms (Colucci et al., 2008; Kim et al., 2004), dysfunctional transport, or other mechanisms. Alternately, there could
be no change in SERT levels, but an upregulation of serotonin synthesis and release. This finding would be interesting because if serotonin were upregulated, we would expect to see increased melatonin, as opposed to the decrease that we observe. The finding that 5-HT levels did not differ amongst the groups in the presence of fluoxetine indicate that serotonin synthesis/release is not altered in EAE, but rather that the higher concentrations of serotonin measured in basal conditions are due to attenuated SERT function. The results of these findings could have major implications into the design of clinical therapies that target serotonin or melatonin for treatment of constipation in MS.

5. **Interstitial cells of Cajal**

Interstitial cells of Cajal (ICC) are a c-kit expressing mesodermal cell that are located within the muscularis propria and plexus layers of the gut. ICC are referred to as the pacemaker cell of the GI tract, and generate regular Ca\(^{2+}\) dependent oscillations that, during the depolarization phase, activate a smooth muscle contraction named the slow wave (Torihashi et al., 2002). While the mechanisms involved in ICC pacemaker activity are incompletely understood, Ano1, a Ca\(^{2+}\) activated Cl\(^{-}\) channel, has been identified as an important component of this process (Mawe, 2009; Singh et al., 2014). Genetic knockout of Ano1 in mice results in reduced frequency and irregularity of small intestinal slow waves and ICC Ca\(^{2+}\) transients (Singh et al., 2014). In another study, neonatal mouse intestines exposed to c-kit antibodies in culture developed abnormal slow waves that were reduced in both amplitude and frequency (Ward et al., 1997). Clinically, loss of ICC has been observed in colons of patients with slow transit constipation (Kashyap et al., 2011; Lyford et al., 2002).
The finding of decreased frequency of slow wave contractions in our EAE mice is consistent with clinical and experimental loss of ICC, suggesting that these cells could contribute to GI dysmotility in EAE. EAE mice developed decreased slow wave frequency, but my measure represents the average interval time, and we did not consider the regularity or amplitude of these contractions. These measures could be readily completed by analyzing data that we have already acquired. Future studies involving EAE should quantify c-kit or Ano1 positive ICC in the colons of EAE mice, with the hypothesis that these cells may be decreased in EAE.

6. **Sexual dimorphism of gastrointestinal dysmotility in EAE**

Multiple sclerosis is a sexually dimorphic disease, with women developing MS approximately three times more frequently than men (Koch-Henriksen et al., 2010). Similarly, EAE is sexually dimorphic and may relate to genetic contributions of the Y chromosome (Spach et al., 2009) in addition to sex hormones (Fillmore et al., 2004). Sexual dimorphism of constipation in MS or in EAE have not been specifically studied.

A role of sex on GI motility has been reported in a number of studies. Sex hormones are thought to contribute to the increased prevalence of both irritable bowel syndrome (IBS) (Meleine et al., 2014) and idiopathic constipation in females compared to males (Kamm et al., 1991). Conversely, in one study that administered progesterone and/or estradiol to post-menopausal women, the hormones increased rates of colonic transit (Gonenne et al., 2006). Recently, it was reported that PLP1 glial deficiency differentially affects female mice. Females were shown to have decreased whole GI transit time, with no change in gastric emptying or small intestinal transit, in addition to increased CMMC.
frequency (Rao et al., 2017). There was no effect of PLP1 glial deficiency on GI transit or CMMC frequency in male mice.

The only currently published report of GI motility in EAE was conducted in female mice, and the described delay in GI transit is consistent with our observations in male EAE mice (Wunsch et al., 2017). Given the sexual dimorphism of MS, EAE, and GI motility, combined with recent reports of enteric glia sexual dimorphism, future studies in our lab should involve both male and female mice so that there are sufficient animal numbers to allow for comparison. Currently, our report of decreased colonic GFAP in EAE contrasts with Wunsch and colleagues’ report of increased small intestinal GFAP, although this finding could be related to one of many factors, including sex, anatomical location, and local inflammation.

7. Relationship between EAE and barrier function

An additional contribution to constipation in EAE could be that a leaky intestinal barrier promotes inflammation and dysmotility in EAE intestines. An investigation of the intestinal epithelium of EAE mice demonstrated a loss of small intestinal barrier integrity compared to controls, as shown by increased serum levels of macromolecules administered by oral gavage (Nouri et al., 2014).

Until recently, it was thought that enteric glia were important regulators of epithelial barrier integrity in the gut. An original study to test the role of glia in intestinal barrier function used transgenic mice that expressed the herpes simplex virus thymidine kinase gene (HSVtk) under the GFAP promoter and administered an antiviral drug to ablate GFAP+ glial cells (Savidge et al., 2007). Animals developed increased intestinal
permeability, myenteric neuronal degeneration and altered GI motility. However, another report used the same GFAP-HSVtk, and found that the GFAP-HSVtk mouse had many off-target effects, including GFAP$^+$ epithelial cells, suggesting that the observed barrier permeability was independent of glia (Rao et al., 2017). Rao and colleagues also tested motility and intestinal permeability in a similar transgenic mouse that allowed for ablation of PLP1$^+$ glial cells, and found that while PLP1$^+$ glia were involved in regulation of motility, ablation of these cells had no effect on permeability. This is supported by study of Gulbransen and colleagues involving two transgenic models to knock out connexin-43 hemichannels (Cx43-igKO), preventing the flux of Ca$^{2+}$ between cells, or to express an engineered $G_q$ receptor activated by clozapine-N-oxide (hM3Dq mice) to stimulate activation of GFAP$^+$ cells (Grubisic et al., 2017). Neither approach of glial modulation had an effect on colonic epithelial permeability in Ussing chamber preparations. Together, it can be concluded from these studies that our observed loss in GFAP expression is not a likely cause of increased barrier permeability in EAE mice.

However, independent of glia, the increased barrier permeability reported in EAE could contribute to our observation of GI dysmotility. Future studies in our lab should investigate the relationship between intestinal barrier function in EAE and the development of GI symptoms. For example, we could determine whether increased translocation of orally-administered macromolecules relates to measures of GI motility. Increased gut permeability in EAE is associated with higher levels of inflammatory cytokines and infiltration of CD$^{4+}$ T cells, and it is possible that these factors contribute to the development of constipation in EAE
II. Autoimmune Gastrointestinal Dysmotility in Multiple Sclerosis and EAE

Summary and Conclusions

MS is an autoimmune disease that involves autoreactive T and B lymphocyte homing to the CNS (Frohman et al., 2006). During this process, autoantibodies against CNS proteins are generated by plasma cells and can be detected in both the cerebrospinal fluid as oligoclonal bands (Link et al., 2006), and in the serum of MS patients (Schirmer et al., 2014). The pathogenicity of these antibodies is unclear, but plasma exchange is reported to improve symptoms in some patients with MS, supporting the role of serum antibodies in the disease (Keegan et al., 2005). Antibodies have been the focus of studies that have attempted to define the causative antigen of MS, but the heterogeneity of the disease is reflected in these findings, as over 20 autoantibodies have been identified in MS (Schirmer et al., 2014).

Constipation is a common symptom of MS that is poorly understood, and it is possible that autoimmune processes, including autoantibodies, could contribute to the development of constipation. Among the antigens that have been identified in MS are components of CNS myelin including proteolipid protein 1 (PLP-1) and myelin basic protein (MBP) (Schirmer et al., 2014), which are also enriched in the ENS (Rao et al., 2015). This knowledge leads to the question of whether ENS proteins could be a target of autoimmunity in MS that leads to constipation. Autoimmune gastrointestinal dysmotility (AGID) is a clinically recognized entity in which constipation occurs secondary to an autoimmune disease (Dhamija et al., 2008), and has been widely reported in Chagas’ disease (Girones et al., 2005), paraneoplastic syndrome (Sodhi et al., 1989), and Type I
Diabetes (Jackson et al., 2004), among others. A recent study supports both MS and EAE as diseases that can result in AGID (Wunsch et al., 2017).

In this dissertation, we used indirect immunohistochemistry to test immunoreactivity of human serum against guinea pig longitudinal muscle / myenteric plexus preparations (LMMP). We demonstrate that serum from MS patients exhibits increased immunoreactivity against cells of the ENS compared to serum from healthy controls. Interestingly, the effect was most prominent in MS patients who did not exhibit constipation, compared to those who did. This finding could reflect the process of antibodies binding to their antigens in vivo, and therefore being present at lower levels in the serum. There was no significant difference between the immunoreactivity intensity of serum from MS patients with constipation versus those without constipation. This limits our interpretation of autoantibodies because it suggests that their immunoreactivity against ENS targets is not a predictor of constipation in MS. However, other disease processes could affect the pathogenicity of these antibodies in vivo. For example, the permeability of myenteric ganglia to serum autoantibodies could be different in MS patients who do develop autoantibodies versus those who do not. MS serum samples, regardless of constipation, targeted either neurons or glia, and sometimes immunoreactivity was demonstrated against both cell types. EAE serum also exhibited immunoreactivity against neurons and glia of the myenteric plexus that was not observed in control animals. Together, these findings demonstrate the immunoreactivity of antibodies against structures in the ENS and supports the possibility of autoantibodies as a cause of constipation in MS. Further studies will test whether these autoantibodies elicit a functional role in vivo.
Alternative Approaches and Future Directions

1. Antibody deficient mice

The studies described in chapter 2 of this dissertation demonstrate that wild-type (WT) mice induced with EAE develop GI dysmotility. Our data and others’ (Wunsch et al., 2017) support that autoantibodies target the ENS and may represent one component of constipation in MS. Because of the complexity of the disease, there are likely to be multiple mechanisms that contribute to constipation in MS. Therefore, we hypothesize that antibody deficiency will protect against constipation in EAE, but GI function may not be completely returned to that of control animals. To further investigate the pathogenicity of auto-antibodies in constipation associated with MS and EAE, we have begun to analyze GI motility in B-cell deficient EAE mice. We induced EAE in B-cell deficient muMt – mice (B6.129S2 – Ighm<sup>tm1Cgn</sup>/J, Jackson Laboratories, Bar Harbor, Maine) using the same protocol described in chapter 2 for B6 + MSCH EAE induction. Mice were immunized by S.Q. injection of 3.75 mg mouse spinal cord homogenate and complete Freund’s adjuvant (CFA) containing 300µg <i>M. tuberculosis</i> divided between three sites on day 0. Intraperitoneal injections containing 200ng of Pertussis Toxin (PTX, List Biological Laboratories, Inc.) were administered on day 0 and day 2. Control muMt – mice received injections containing CFA and PTX, but no MSCH.

Beginning at day 14, mice underwent analysis of fecal water content and colonic propulsive motility on subsequent days (Figure 3.1). Fecal water content was not significantly different (p<0.85) in muMt – EAE mice (n=18) compared to muMt – controls.
Colonic propulsive motility was also not significantly different between the groups (n=16 both groups, p<0.1182, t-test with Welch’s correction).

These data suggest that antibody deficiency may be protective against GI dysmotility in EAE mice, and support our hypothesis of MS as a disease of AGID. However, there are several limitations of the data shown here. Most importantly, we are unable to directly compare the motility of B6 EAE mice to that of the muMt′ EAE mice because we tested each group of animals independently against CFA control mice of the same strain. To conclusively interpret that B cells play a role in constipation in EAE, we need to demonstrate that muMt′ mice have significantly different motility compared to B6 mice. Further studies will directly compare these two groups, and will include additional motility assays, such as whole GI transit, gastric emptying, small intestinal transit, as well as CMMC and slow wave analysis. One limitation of the in vivo studies that we have analyzed is their sensitivity. Because we do not anticipate that autoantibodies are the only contributing factor to constipation in MS, it is possible that our in vivo metrics lack the sensitivity to observe changes. Spatiotemporal mapping techniques or other ex vivo approaches such as Ca\(^{2+}\) imaging, have excellent spatial and temporal resolution and may be better suited to identify differences between these groups.

One possible outcome of future experiments is that antibodies partially attenuate the GI changes that we observe in EAE, or alternately, antibodies could play no role. If our data ultimately support these alternative outcomes, this would suggest that constipation is a combination of immunological and neurological processes, or that serum autoantibodies in MS lack pathogenicity against the ENS. We have reported the immunoreactivity of human serum antibodies against guinea pig LMMP tissue, and are beginning studies to
test their cross-reactivity with autologous tissue from human colon. One possibility is that pathogenic autoantibodies circulate in the serum but do not reach their targets due to impermeability of myenteric ganglia to large molecules, but we know that antibodies reach the ENS in other diseases. Alternately, Nouri and colleagues report infiltration of Th1 and Th17 cells in the small intestine of EAE mice, in addition to increased barrier permeability, and both of these processes could be involved in constipation and act independent of autoantibodies (Nouri et al., 2014).

2. ELISA of MS and EAE serum against ENS proteins

In chapter 2, we describe quantification of MS serum immunoreactivity against myenteric ganglia using guinea pig longitudinal muscle-myenteric plexus preparations (LMMP). An additional approach that we tested was enzyme-linked immunosorbent assay (ELISA) against myenteric plexus proteins.

In order to isolate myenteric plexus proteins, we removed the small intestine of a healthy guinea pig and peeled away the outer layer of tissue, which only contains LMMP. This tissue was dissected into small sections approximately 1cm in length in cold Ca$^{2+}$/Mg$^{2+}$ free HBSS (Gibco). To dissociate smooth muscle and enrich for myenteric ganglia, sections were transferred to a digestion solution containing 3.75 U Collagenase A (Sigma Aldrich), 22 U neutral protease (Worthington Biochemical Corporation), 7ug choline TEA, 25 µg DNase I, and 3mg BSA (Sigma Aldrich) in 10mL HBSS. Following 20-minute digestion in a 37°C water bath with intermittent vortex by hand, the solution was passed with HBSS over a 200 µm nylon filter (pluriSelect) that had been incubated in BSA. The flow-through was centrifuged for 10 minutes at 1200rpm, and the pellet re-
suspended in lysis buffer containing 10 µL 100x protease inhibitor (Calbiochem) and 100 
µL 10x cell lysis buffer (Cell Signaling Technology) in 900 µL nanopure water. The 
suspension was sonicated for 10-20 seconds and centrifuged again at 4º C and 900g for 1 
minute. The supernatant was removed at stored at -80º C until further use.

Nunc MaxiSorp™ 96-well ELISA plates (ThermoFisher) were incubated overnight 
with 10µg/mL of guinea pig myenteric plexus proteins in NahCO₃ buffer. Each plate was 
washed 3 x 5 minutes with 0.2% Tween 20 (Sigma) in 1X PBS and blocked overnight in 
5% BSA. The next day, duplicate MS or EAE serum samples were diluted and pre- 
absorbed with the guinea pig ENS proteins to quench non-specific binding and, incubated 
at room temperature for two hours. Excess primaries were removed by wash, and 
secondary anti-human HRP or anti-mouse HRP antibodies were diluted at 1:5000 and 
incubated for 2 hours. Following a final wash, plates were incubated with TMB (Sigma) 
for 15 minutes at 37ºC and a stop solution was added to the wells (Abcam). The level of 
fluorescence was measured on a BioTek Synergy H4 Hybrid Microplate Reader using 
Gen5 software (BioTek, Winooski, Vermont).

This approach did not yield significant results in either MS or EAE serum. However, 
there are several limitations to this ELISA method that could have affected the strength of 
the assay. Although we enriched the samples for myenteric ganglia, it is possible that 
through our isolation protocol, the target antigen is diluted relative to release of other 
proteins and there is not enough antigen to yield significant results. Another possibility is 
that the processes involved in this protocol alter the antigen conformation so that it is no 
longer recognized by the antibodies. One approach to increasing specificity of this assay 
is to use same-species proteins opposed to testing human or mouse serum.
immunoreactivity against guinea pig ENS proteins. However, the same limitations as described above would still apply to this method unless cytoplasmic and membrane proteins were separated to increase the ratio of target antigen. In chapter 2, our data demonstrated that MS serum exhibits increased immunoreactivity against myenteric ganglia compared to control serum. A more reliable measure of immunoreactivity that eliminates the limitations described above would be whole-cell ELISA (Erdile et al., 2001). This approach would allow antigens to maintain their biological conformation and would allow for better comparison between serum samples than that acquired through immunohistochemistry.

3. Autologous immunoreactivity

In chapter 2 of this dissertation, we describe that serum from patients with MS exhibits increased immunoreactivity against LMMP guinea pig preparations compared to serum from control subjects. This is an established method in the field that has been used to identify autoantibodies in idiopathic achalasia and irritable bowel syndrome (Moses et al., 2003; Wood et al., 2012). The size and easy dissection of guinea pig intestine lends itself to en face preparations of myenteric ganglia, and is the best approach for visualizing the orientation of the cells within ganglia. Human tissue is larger and cannot be dissected in layers, and therefore the ganglia must be viewed in cross-section. However, due to uncertainty regarding homology with guinea pig proteins, it is important to test the immunoreactivity of human antibodies against autologous tissue.

Preliminary studies in our lab have begun to assess the reactivity of human serum samples collected in chapter 2 against human colon specimens, using a similar
immunohistochemistry approach to that described in chapter 2. In brief, our protocol begins with paraffin embedded colon sections from healthy human tissue removed at the margins of colon cancer. After deparaffinization, tissue is blocked in 4% goat serum, 0.5% triton X 100 in 1X PBS, with the addition of unlabeled goat human anti-IgG to block endogenous antibodies already present in the tissue. Following the blocking step, sera are diluted at 1:100 in normal block overnight. The next day, tissue is washed and incubated for two hours in goat anti-human IgG coupled to Cy3. Secondary is removed and slides visualized to determine positive binding.

Our preliminary results testing autologous immunoreactivity demonstrate the cross-reactivity of serum from a patient with MS and constipation against neurons of the myenteric plexus (Figure 3.4 A). No positive staining is present in secondary control samples (Figure 3.4 B). As we continue to optimize this method in lab, and to avoid autofluorescence, we have switched to using bright field microscopy with goat anti-human HRP secondary antibodies and a 3,3' -diaminobenzidine tetrahydrochloride (DAB) substrate reaction with hematoxylin counterstain. Our preliminary trials using this approach results in lower background (data not shown). We are continuing to test additional serum samples, and if successful, we intend to repeat the intensity analysis described in chapter 2, as well as to better characterize the cellular targets of autoantibodies.

4. Disease specificity of serum staining and ELISA

One limitation of the serum immunoreactivity that we have measured both by immunohistochemistry in chapter 2, and by ELISA in this chapter, is that we have not tested the disease specificity of the serum. In chapter 2, we found that MS serum is more
immunoreactive against guinea pig myenteric ganglia compared to control serum. However, these results could be a function of heightened immune activity in patients with autoimmune disease compared to healthy control subjects, and may not be specific to MS. We believe that if we compared the immunoreactivity of MS serum to other autoimmune diseases that exhibit a GI component, we would see equal immunoreactivity between these groups because many diseases are associated with autoimmune gastrointestinal dysmotility (AGID). Therefore, the immunoreactivity observed in MS could be a feature of AGID and not unique to MS. However, the specific cellular targets could be different between individual autoimmune diseases based on the antibodies produced in each disease. An important control group that should be included in our analyses is an autoimmune disease that is not associated with AGID, such as Rheumatoid arthritis. Alternately, we should test the immunoreactivity of serum from a group with a neurological disease and GI involvement that does not have a major autoimmune component, such as Parkinson’s disease. Finally, it is important to test whether this immunoreactivity is a feature of functional constipation in an otherwise healthy individual. These control groups would help to elucidate whether the immunoreactivity observed with MS serum is unique, or whether it is a shared feature of all autoimmune diseases or alternately, of neurological or GI diseases.

5. **Passive transfer of antibodies**

Testing the immunoreactivity of MS and EAE antibodies using immunohistochemistry or ELISA are useful approaches to determine whether the antibodies interact with cells of the ENS. However, it is ultimately crucial to test whether
the autoantibodies identified in serum elicit a functional effect in living tissue. In addition to the muMt` EAE experiments described above, another approach to test the activity of autoantibodies is passive transfer.

Previous studies have used passive transfer to administer purified IgG from human serum of patients with type I diabetes these antibodies to naïve mice in vivo, and have also applied them to tissue in ex vivo motility preparations (Jackson et al., 2004; Wan et al., 2008). These techniques were sufficient to identify a functional autoantibody against voltage-gated calcium channels in type I diabetes (Jackson et al., 2004; Wan et al., 2008). The significance of this finding is that functional autoantibodies can act as agonists or antagonists to ion channels and may be difficult to detect using standard immunoreactivity approaches such as immunohistochemistry or ELISA.

Using similar methods to those described by Jackson and colleagues, future experiments in our lab should administer MS or EAE antibodies to naïve mice and test the mice for altered GI motility. The acute antibody effect could be tested by incubating naïve mouse colons with purified IgG for a designated period of time before conducting ex vivo motility analyses including spatiotemporal mapping to evaluate CMMCs and slow wave contractions. Results acquired through these methods would increase the interpretability of our current findings of serum immunoreactivity against structures of the ENS.

6. Role of other immune cells in constipation associated with MS and EAE

The study of B lymphocytes and pathogenic antibodies in MS is an expanding field, as MS has classically been considered a T lymphocyte predominant disease (Bielekova et
In our studies, we demonstrated that MS and EAE sera contain autoantibodies that bind to cells of the ENS, but we did not characterize immune cell localization to the ENS. Studies conducted by Wunsch and colleagues report increased numbers of infiltrating T lymphocytes, B lymphocytes, and macrophages to the myenteric plexus of EAE (2017). Their report was conducted in the small intestine, whereas our histological analysis was completed in the colon. It would be interesting to determine whether immunological infiltrates are differentially distributed through the GI tract of EAE. Preliminarily, our data support this hypothesis because the studies reported by Wunsch and colleagues describe increased GFAP in the small intestines, whereas we report decreased GFAP in colons. These contrasting results could relate to local inflammatory levels, increased expression of GFAP has been reported in inflamed tissues of Crohn’s disease patients, but is decreased in non-inflamed regions relative to controls (von Boyen et al., 2011).

A study of intestinal permeability in EAE reports that passive transfer of MOG-reactive CD4+ T cells is sufficient to induce morphological changes in the intestinal epithelium and increased permeability in naïve mice (Nouri et al., 2014). Again, this study was conducted in three different regions of small intestine, but was not repeated in colon samples. Interestingly, in our motility analyses, we observed no change in small intestinal motility, and the cause of constipation was likely due to changes in the colon. Nouri and colleagues did not assess GI motility associated with the adoptive transfer of T cells, and our use of this induction model would allow for us to test the pathogenicity of these cells in the development of constipation.
7. Myenteric ganglion permeability

In this set of studies, we have demonstrated that serum from MS patients has more intense immunoreactivity against cells of the ENS than serum from control patients. Targets of both MS and EAE serum include enteric neurons and glia. However, we did not observe differences in the immunoreactivity of MS patients with constipation compared to MS patients without constipation. One major question raised by these studies is the proximity of antibodies to the ENS in vivo, an important future experiment would be to test the permeability of ENS ganglia to serum antibodies.

The permeability of the ENS ganglia to serum proteins has been studied in the past, but rarely under pathology. One study tested the permeability of different neurological tissues in healthy rats to macromolecules including horseradish peroxidase (HRP, 33,890 MW), endogenous albumin (64,000 MW), and rhodamine-B conjugated bovine albumin (67,000 MW) (Allen et al., 1994). Both HRP and endogenous albumin were found within the myenteric ganglia. The ENS was thought to be less permeable than CNS, sensory, or sympathetic ganglia, because rhodamine-B conjugated bovine albumin surrounded the myenteric ganglia, but did not enter them. The authors noted that non-ionic bonds between rhodamine-B could have increased the actual size of these molecules and prevented their entry. Human IgG are approximately 150,000 kDa in size, which is more than double the size of albumin (Janeway et al., 2001). Dorsal root ganglia of rats are permeable to IgG, where the molecule exits fenestrated capillaries and localizes to the interstitial space but does not permeate the connective tissue capsule around cells (Azzi et al., 1990). Within the gut, most fenestrated capillaries are localized to the mucosa and submucosa, whereas those in the muscularis externa contain tight junctions (Gershon et
al., 1978). Following injection with HRP or serum albumin, the molecules permeated mucosa and submucosa within minutes and was minimally detected in the myenteric plexus layer after an hour, and similar to other studies, was found at the perimeter of the ganglia and not within them. However, other groups do report HRP localizing within myenteric ganglia (Jacobs, 1977). Studies that find macromolecules to be limited to the perimeters of ganglia, acknowledge their close proximity to the encapsulating glia and inter-ganglionic connectives, which could be sufficient to interact with cells (Allen et al., 1994).

Changes in the permeability of myenteric ganglia have not been extensively studied in disease states. It can be reasonably hypothesized that there is increased permeability of the ganglia to macromolecules during inflammation. Importantly, Gershon and colleagues (1978) did not detect barriers between layers of the gut that would prevent movement of molecules from the mucosa and submucosa to the muscularis externa, demonstrating that macromolecules released from fenestrated capillaries, or those that cross a leaky gut barrier, could diffuse through the intestinal layers and reach the myenteric plexus (1978). Studies testing HRP and albumin localization, such as those performed by Gershon (1978) and Allen (1994) to myenteric ganglia should be reproduced in EAE mice. Alternatively, the permeability of submucosal ganglia is also poorly characterized, although their closer proximity to fenestrated capillaries and immune-rich regions of the gut could lend them more permeable to IgG, and perhaps these ganglia are a more likely target of autoimmune pathologies of the ENS.
III. Final Summary and Conclusions

The results of these dissertation studies validate EAE as an animal model of constipation in MS. EAE mice develop symptoms consistent with constipation, including slower whole GI transit, delayed colonic propulsive motility, and drier fecal water content. EAE mice continue to exhibit GI pathology in the absence of extrinsic innervation or pelvic floor influences, as demonstrated by prolonged CMMC and slow wave intervals and decreased myenteric GFAP. One mechanism involved could be autoimmune targeting of myenteric ganglia, because serum from MS patients and EAE mice contains autoantibodies that bind to cells of the ENS. Preliminary data implicates that abnormal serotonin and melatonin signaling may also be involved in the pathogenesis of constipation. The data presented here support that the mechanisms responsible for constipation in EAE and MS are generated by pathology of the ENS, and may be a separate entity from CNS demyelination that characterizes the diseases.
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A Naive Control
B Control
C EAE

Concentration (µM)

Baseline Fluoxetine

Serotonin

**** **** **** ****

Change in Serotonin

Fold Change in Serotonin

**** ****

Ratio of Melatonin:Serotonin

**** * ****

Ratio of Melatonin:Serotonin
Figure 3.1

Figure Legend

Figure 3.1 Amperometry was used to measure mucosal serotonin and melatonin. A) At baseline, EAE (n=8) mice have significantly higher levels of extracellular serotonin than naïve control (n=6) or control mice (n=6, p<0.0001). Following fluoxetine administration, serotonin significantly increases in both EAE and all control mice (p<0.0001). This demonstrates that SERT is still at least partially functional in EAE mice. B) The increase in serotonin availability following fluoxetine administration is smaller in EAE mice compared to naïve controls (p<0.0001) and controls (p<0.001). C) Melatonin in EAE mice is significantly lower than either control group (p<0.0001). D) The ratio of melatonin:serotonin is significantly decreased in control mice administered CFA and PTX adjuvants compared to naïve control mice (p<0.05). The ratio is significantly lower in EAE mice compared to either control group (p<0.0001). All graphs analyzed by one- or two-way ANOVA with Bonferroni post-hoc test.
Figure 3.2
**Figure Legend**

Figure 3.2 A) The fecal water content is not different in antibody-deficient control mice (n=20) compared to those induced with EAE (n=18) (p<0.8530, two-tailed t-test). B) Colonic motility is not different in antibody-deficient control mice (n=16) compared to those induced with EAE (n=16) (p<0.1280, two-tailed t-test with Welch’s correction).

Data represented as mean ± SEM.
Figure 3.3
Figure Legend

Figure 3.3 Results from the ELISA assay testing the immunoreactivity of A) human and B) mouse serum against protein samples isolated from guinea pig myenteric ganglia. A) Serum from MS patients (n=40) does not exhibit increased immunoreactivity against ENS proteins compared to control serum (n=20) (two-way ANOVA, Bonferroni post-hoc). B) Serum from EAE B6 + MOG mice (n=8) does not exhibit increased immunoreactivity against ENS proteins compared to control serum (n=8) (two-way ANOVA, Bonferroni post-hoc).
Figure 3.4

A  Human MS serum on a human colon section

B  Secondary Control
Figure Legend

Figure 3.4 Human serum diluted at 1:100 from an MS patient with constipation exhibits immunoreactivity against human myenteric ganglia. There is no positive staining observed in secondary control tissue.
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