

*COLLOQUIUM SERIES ON
NEUROGLIA IN BIOLOGY AND MEDICINE
FROM PHYSIOLOGY TO DISEASE*

Series Editors: Alexei Verkhratsky & Vladimir Parpura

Enteric Glia

Brian D. Gulbransen



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Enteric Glia



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Colloquium Series on Neuroglia in Biology and Medicine: From Physiology to Disease

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ABSTRACT

The enteric nervous system (ENS) is a complex neural network embedded in the gut wall that orchestrates the reflex behaviors of the intestine. The ENS is often referred to as the “little brain” in the gut because the ENS is more similar in size, complexity and autonomy to the central nervous system (CNS) than other components of the autonomic nervous system. Like the brain, the ENS is composed of neurons that are surrounded by glial cells. Enteric glia are a unique type of peripheral glia that are similar to astrocytes of the CNS. Yet enteric glial cells also differ from astrocytes in many important ways. The roles of enteric glial cell populations in the gut are beginning to come to light and recent evidence implicates enteric glia in almost every aspect of gastrointestinal physiology and pathophysiology. However, elucidating the exact mechanisms by which enteric glia influence gastrointestinal physiology and identifying how those roles are altered during gastrointestinal pathophysiology remain areas of intense research. The purpose of this e-book is to provide an introduction to enteric glial cells and to act as a resource for ongoing studies on this fascinating population of glia.

KEYWORDS

enteric nervous system, intestine, autonomic nervous system, myenteric, submucosal, neuron, peripheral nervous system, gut, intestinal nervous system, EG, ENS, astroglia

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CHAPTER 1

Introduction

“Well are you a brain guy or a gut guy?” is a question often posed to me by fellow scientists. My answer, “Both of course,” is usually met with some degree of confusion or skepticism. The truth is that even the most seasoned neuroscientists and gastroenterologists often overlook the fact that a “second brain,” known as the enteric nervous system (ENS), resides within the walls of the intestines and controls the ongoing activities of the gastrointestinal tract. The entire circuitry of the ENS is embedded in the gut wall and consists of aggregates of neurons and glia called enteric ganglia that are interconnected to form networks (plexuses) that extend the length of the intestine (Figure 1.1) (Furness, 2012). In total, approximately 100 million enteric neurons are housed in the gut wall and these neurons communicate among themselves using all known major classes of neurotransmitters found in the brain. Enteric neurons form integrative circuits that are capable of directly determining the moment-to-moment behaviors of the intestine in the absence of signals from central nervous system (CNS) command centers. The fine structure of the ENS and its capacity for integrative function are, in large, more similar to that of CNS, and in particular the spinal cord, than other elements of the autonomic nervous system (ANS). As a point of comparison, I like to tell students that their gut is as smart as their cat because the ENS has roughly the same number of neurons as a cat brain. But why should your digestive tract require the intelligence of a household pet? The answer becomes clear when we consider the special burdens imposed on the gut. The gut is our sole route of nutrient absorption and we depend on it to breakdown food, absorb the nutrients and ultimately excrete the waste. At the same time, the gut needs to protect itself from toxins, irritants, physical damage and the multitude of bacteria that reside within its length. Coordination of these complex and essential activities requires substantial neuronal processing. Rather than allocating significant space in the brain, it makes sense to allocate command to a local nervous system.

The ability of the intestine to exhibit reflex behaviors in the absence of CNS input was described as early as 1899 by Bayliss and Starling (Bayliss and Starling, 1900) and later confirmed in the elegant experiments by Trendelenburg (Trendelenburg, 1917). These pioneering scientists concluded that the reflex activity of the intestine was mediated by the “intrinsic nervous mechanism” of the bowel based on Auerbach’s prior work that demonstrated the presence of large numbers of neurons within the gut wall (Auerbach, 1862). Sir Johannes Newport Langley recognized that the intrinsic innervation of the gut was unique and independent from other branches of the ANS. He coined the term “enteric nervous system” to describe this third branch of the ANS in his classic book entitled *The Autonomic Nervous System* (Langley, 1921). Although

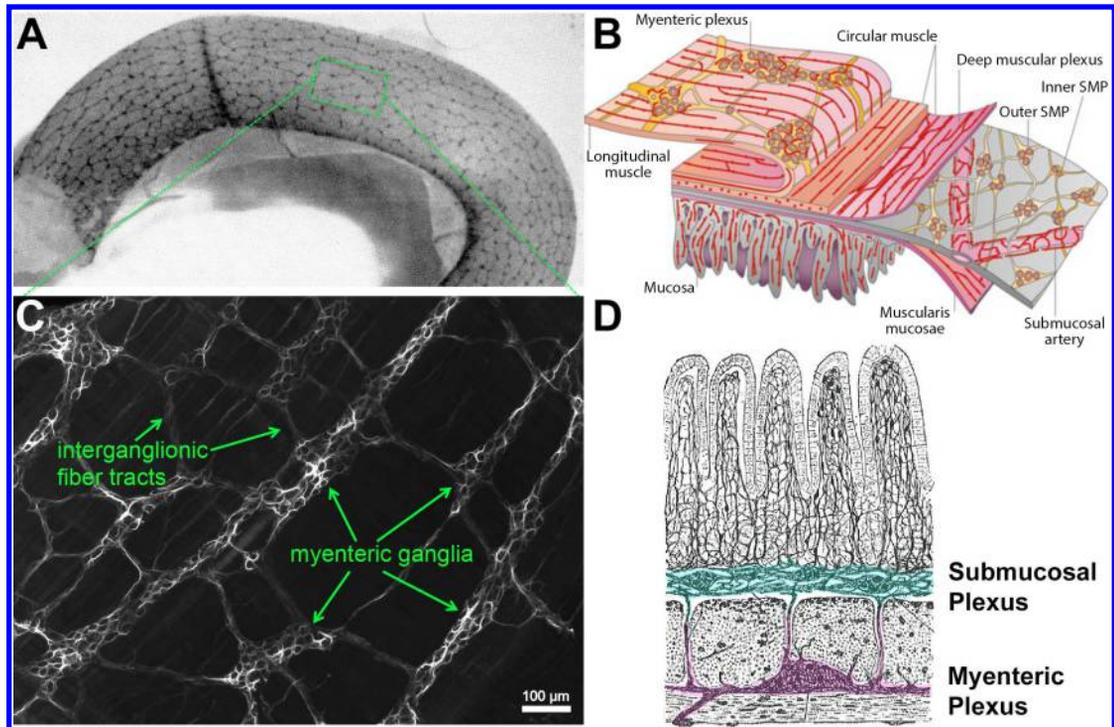


FIGURE 1.1: Organization of the enteric nervous system (ENS) in the gut wall. **A.** A segment of intestine from a chick stained histochemically to reveal the myenteric plexus *in toto* within the gut wall. Image from Gabella (1976). **B.** Diagrammatic view of the arrangement of the ENS in the gut wall. The ENS has two ganglionated plexuses—the submucosal plexus (SMP) located between the mucosa and circular muscle and the myenteric plexus located between circular and longitudinal smooth muscle coats. From Furness, J.B. (2012). **C.** Whole-mount preparation of the myenteric plexus and attached longitudinal muscle dissected from the mouse colon and labeled with antibodies against the glial cell marker, glial fibrillary acidic protein (GFAP). Note that ganglia (collections of neuron and glial cell bodies) are connected by nerve fiber tracts to form a mesh-like network. **D.** Diagram illustrating the arrangement of the plexuses and ganglia of the ENS as viewed in a cross section through the gut wall. Modified from an original drawing by Santiago Ramon y Cajal (Taken from the translation of Santiago Ramon y Cajal's *Les Nouvelles idées sur la structure du système nerveux chez l'homme et chez les vertébrés*,—New Ideas on the Structure of the Nervous System in Man and Vertebrates (1990)).

Langley emphasized the independence of the ENS, this concept fell out of favor as sympathetic and parasympathetic branches of the ANS were found to require central command. Until the middle of the 20th century, the ENS was erroneously considered to function as a relay between control centers in the brain and effectors in the gut wall. However, as multiple neurotransmitters were discovered in the ENS, renewed interest uncovered the enteric neuronal circuitry that underpins the reflex behaviors described by Bayliss and Starling and conclusively demonstrated that the ENS is capable of functioning as an independent integration center.

Because the ENS demonstrates the “brain-like” functions described above, it might not be surprising that the environment within enteric ganglia is strikingly similar to that of the brain with dense neuropil composed solely of neurons and glia (Figure 1.2). In the words of Giorgio Gabella, “At first glance an electron micrograph of the myenteric plexus might be taken for a section of an area of the central nervous system” (Gabella, 1976). In contrast to other autonomic ganglia, fibroblasts, macrophages, mast cells, collagen, blood vessels and chromaffin cells are not found within enteric ganglia under normal conditions. Further, enteric ganglia lack a perineural connective tissue capsule and a satellite cell sheath. The basic circuitry and function of the neuronal elements of the ENS is now well described and I will refer readers to the excellent e-books in the Integrated Systems Physiology Series by Dr. Jackie Wood (2011) and Drs. David Grundy and Simon Brookes (2011) for a thorough description of the neural control of gut functions. In contrast, relatively little is still known about the properties of glial cells that surround enteric neurons. Enteric glia are a unique population of peripheral glia that share many morphological, molecular and functional similarities with astrocytes in the brain. Recent evidence implicates enteric glial cell participation in virtually all essential gut processes but many questions remain regarding basic glial functions. The purpose of this e-book is to provide an overview of our current understanding of the function of enteric glia in gut physiology and how enteric glia are affected by, and contribute to gut pathophysiology. Yet, because enteric gliobiology as a field is still in its infancy, I also hope that this e-book highlights the need for a more comprehensive understanding of these fascinating cells.

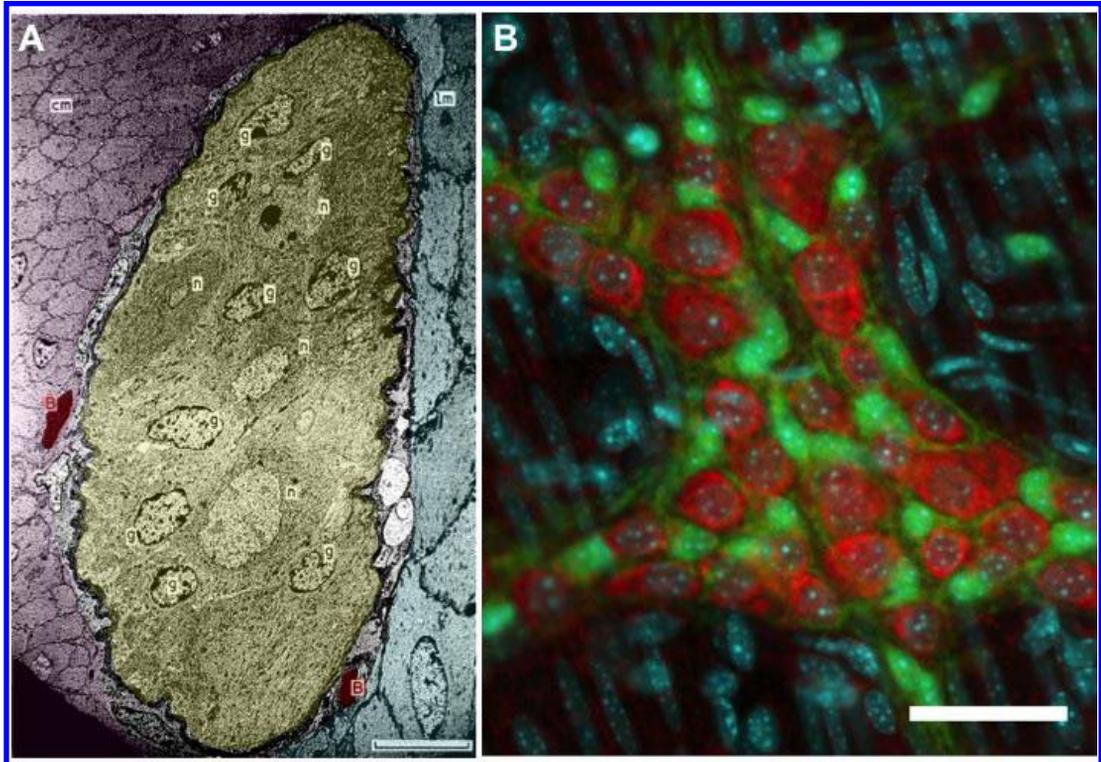


FIGURE 1.2: The neuropil within enteric ganglia is solely comprised of neurons and glia. **A.** Electron micrograph of a myenteric ganglion from the guinea pig ileum. The ganglion (shaded yellow) is sandwiched between layers of smooth muscle (Lm = longitudinal muscle, shaded light blue; cm = circular muscle, shaded light purple). Connective tissue coats the surface of the ganglion but does not penetrate its interior. Two blood vessels (B, shaded red) are just outside the ganglion. The dense neuropil within the ganglion is composed of the cell bodies and processes of neurons (n) and glial cells (g). Scale bar = 10 μ m. Image modified from Gabella, 1976. **B.** A myenteric ganglion from the mouse colon labeled with antibodies that recognize neurons (HuC/D, red) and enteric glia (S100, green). All nuclei are labeled with DAPI (blue). Note that all nuclei within the ganglion belong to neurons and glia. Scale bar = 20 μ m.

CHAPTER 2

A Historical Perspective on Enteric Glia

The first known description of enteric glia dates back over 115 years to Dogiel's 1899 landmark characterization of the construction of the ganglia in the plexus of the intestine (Dogiel, 1899). Yet, the concept of enteric glia as a unique class of cells has only emerged over the past 35 years with the term "enteric glial cell" being coined by Giorgio Gabella in 1981 (Gabella, 1981). For the most part, enteric glia were ignored or considered Schwann cells for the bulk of the 20th century (Gunn, 1951; Stohr, 1952; 1954; Hager and Tafuri, 1959; Baumgarten et al., 1970). The major factor that contributed to this oversight was a lack of techniques to adequately study glial cells. Dogiel's intra-vitam methylene blue technique allowed him to study the nerve plexuses in unprecedented detail. Although Dogiel was primarily concerned with classifying the various morphologies of enteric neurons, he also noted the presence of numerous nucleated satellite cells surrounding the nerve cell bodies within enteric ganglia. Unfortunately, technical difficulties associated with methylene blue labeling or silver impregnation prevented many subsequent investigators from observing enteric glia (Kuntz, 1913; Müller, 1920; Hill, 1927) and as a consequence, glia were largely ignored. Refined staining techniques and the incorporation of electron microscopy throughout the 1930s to 60s allowed investigators to consistently observe glial nuclei surrounding the ganglion cells of the ENS (Figure 2.1). Stöhr (Stohr, 1952; 1954) was able to study the structure of the ENS in great detail using the Bielschowsky silver stain method, a considerable improvement over the method developed by Ramon y Cajal. Using this technique, Stöhr consistently observed "Schwann cell" nuclei surrounding the neuronal elements of the gut and discovered that glia intervene between preganglionic nerve endings and enteric neurons (Stohr, 1954). Based on this histological evidence, Stöhr proposed a neurosecretory role for enteric glia, a concept that is only recently being tested.

Enteric glia were not fully appreciated as a unique cell type until the detailed analyses of enteric ganglia by Giorgio Gabella in the 1970s. Gabella was the first to specifically address the enteric glial cells, and his findings highlighted the complex structural relationship between enteric neurons and glia within the ENS (Gabella, 1972). Importantly, Gabella argued that enteric glia did not fulfill the requirements to be classified as Schwann cells because enteric glia have processes that branch extensively, contain bundles of gliofilaments and are surrounded by a single basement membrane (Gabella, 1972). Several years later, Cook and Burnstock (Cook and Burnstock, 1976) confirmed many of Gabella's observations but failed to appreciate the unique nature of the enteric

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glia. Rather, they argued that enteric glia should remain classified as Schwann cells and proposed the terms ganglionic Schwann cells and neuronal Schwann cells to distinguish between those within ganglia from those in nerve bundles, respectively. To clarify the issue, Gabella conducted an extensive analysis of the ultrastructural features of enteric glia (Gabella, 1981). His results clearly illustrated that the glial cells within enteric ganglia form a unique population of peripheral glial cells and proposed to label them “enteric glial cells.”

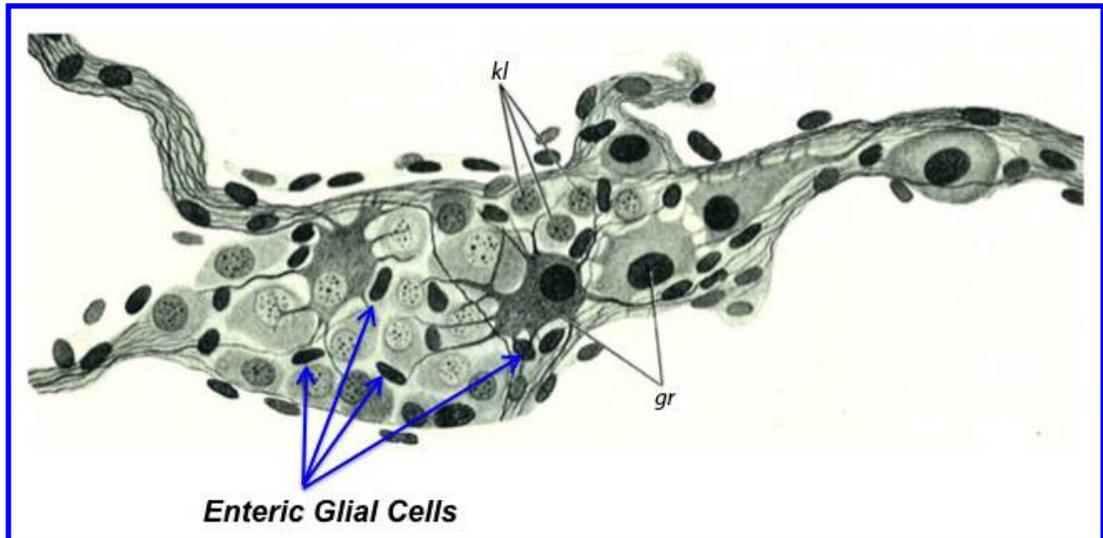


FIGURE 2.1: Cellular constituents of the enteric nervous system as depicted by Stöhr (1952). This drawing reproduces labeling with the silver impregnation (Beilschowsky) method in a submucosal ganglion from the rabbit small intestine. Large (gr = grobe ganglienzellen “coarse/large ganglion cells”) and small (kl = kleine ganglienzellen “small ganglion cells”) enteric neurons are labeled. The nuclei of many enteric glial cells are evident in this image surrounding enteric neurons (blue arrows point to four examples). Stöhr referred to these as Schwansche kerne or “Schwann cell nuclei.”

CHAPTER 3

Enteric Glia: The Astroglia of the Gut

Given the many “brain-like” attributes of the ENS, it is easy to speculate that enteric glia are the astroglia of the gut. Indeed, Gabella noted that, by comparison, enteric glial cells are more similar in ultrastructure, gross morphology and relationships with neuronal cell bodies and processes to the astrocytes of the CNS than to other peripheral glia. Like astrocytes, individual enteric glial cells display an irregular, stellate morphology dominated by an extensive array of highly branched processes (Figure 3.1). Enteric glial cells have small cell bodies and the majority of the cell body is filled by the nucleus, leaving very little cytoplasmic volume. The average nuclear diameter of enteric glial cells in the guinea pig myenteric plexus is between 2–3 μm —approximately 10 times smaller than that of enteric neurons. However, the smooth cell body of enteric glial cells accounts for merely 1/10 of the entire glial cell surface area (Hanani and Reichenbach, 1994). The rest is comprised of complex, branching processes that extend from the cell body and intercalate throughout the neuropil. Fractal dimension analysis (a measure of the complexity of a cell’s border) reveals that the complexity of glial cell projections in myenteric ganglia is comparable to that of protoplasmic astrocytes in the brain (Reichenbach et al., 1992). In both cases, astroglial processes are thought to perform important roles in regulating the neuronal microenvironment and enhancing the glial cell’s spatial buffering capabilities. Additionally, the processes of enteric glial cells and astrocytes often receive synaptic contacts from neurons, indicating that they are the primary points of neuron–glia communication (Gabella, 1972; 1981; Komuro et al., 1982).

Gabella introduced the idea of enteric glia as the astrocytes of the gut in the early 1970s based on ultrastructural observations but we now know that the similarities between enteric glia and astrocytes extend beyond morphology to the molecular level. In the early 1980s, Kristján Jessen and Rhona Mirsky found that enteric glia express an astrocytic-restricted panel of molecules (Jessen and Mirsky, 1983). Notably, the processes of enteric glia and astrocytes are rich in bundles of 10 nm gliofilaments composed of glial fibrillary acidic protein (GFAP) (Jessen and Mirsky, 1980) and vimentin (Jessen and Mirsky, 1983) (Figure 3.2). Like astrocytes, the composition of gliofilaments in enteric glia predominantly composed of vimentin during development and GFAP in adulthood. However, we typically find that adult myenteric glia in the mouse colon express robust immunoreactivity for both proteins (Figure 3.2). Although several subtypes of glial cells express GFAP,

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the form expressed by enteric glia is an astrocyte-associated determinant that is not expressed by Schwann cells (Jessen and Mirsky, 1985).

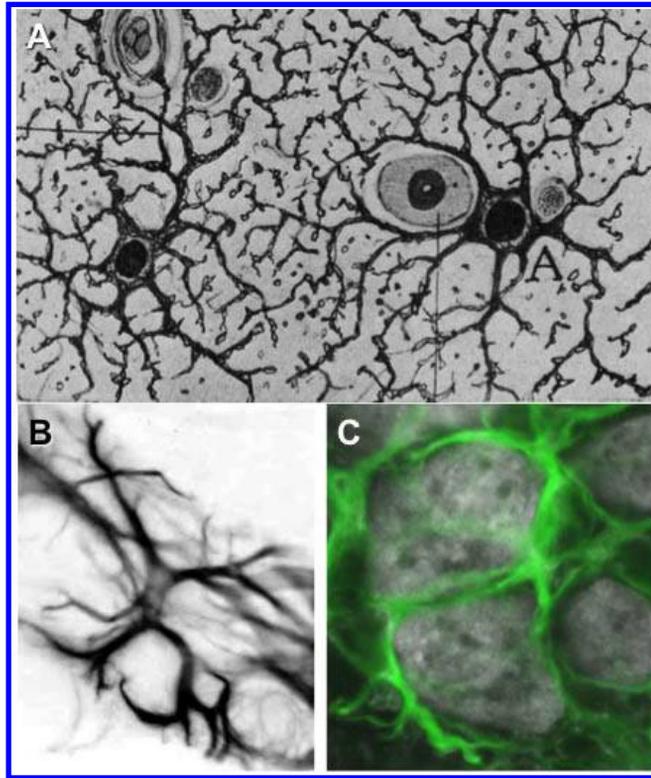


FIGURE 3.1: The morphology of enteric glia mirrors that of astrocytes. A. Classic drawing of astrocytes by Santiago Ramon y Cajal; reproduced from Somjen (1988). B and C. Two representative enteric glial cells in the myenteric plexus of the mouse colon labeled with antibodies against glial fibrillary acidic protein (GFAP, black in B and green in C). Note how the branching structure of the enteric glial cell in B is similar to that of the astrocyte on the left and how the enteric glial cell in C (green) embraces the enteric neuron (grayscale, HuC/D immunoreactivity) in a similar fashion as the astrocyte on the right.

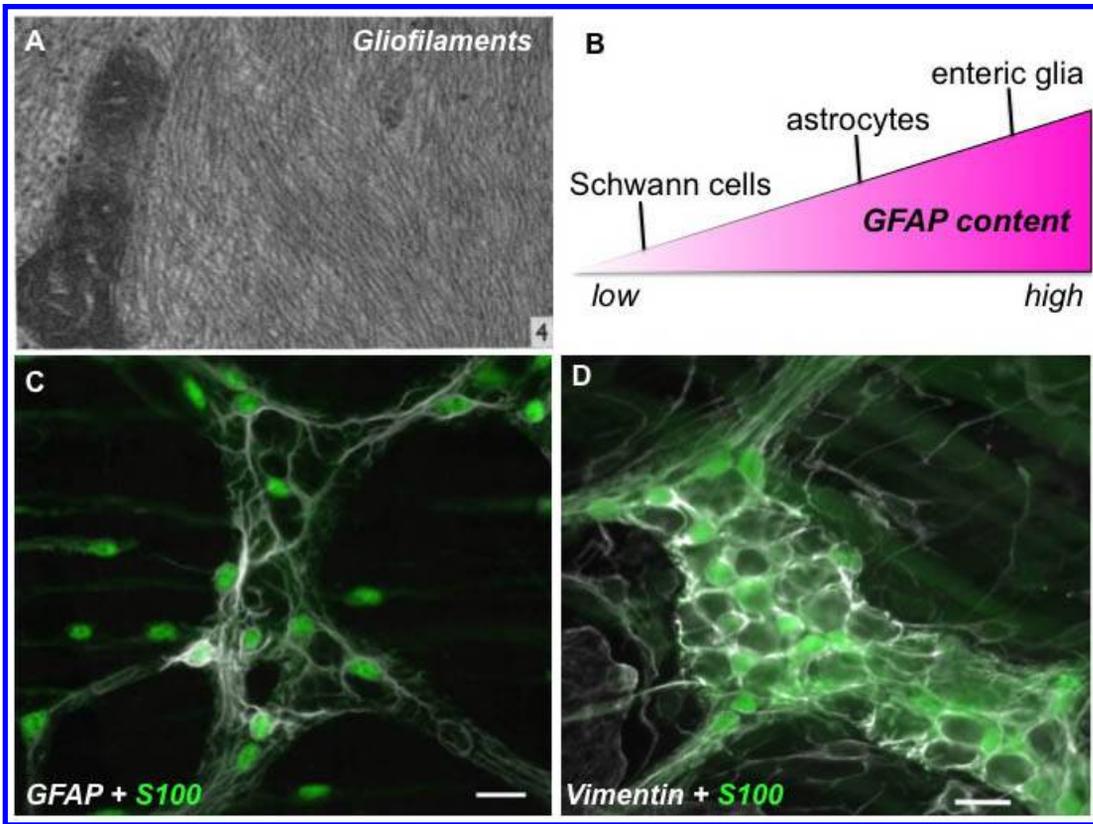


FIGURE 3.2: Gliofilaments in enteric glial cells. **A.** A defining feature of enteric glia is their high concentration and dense packing of 10 nm intermediate filaments called “gliofilaments.” This electron micrograph from Gabella’s original work in 1972 shows an example of the dense arrangement of gliofilaments within an enteric glial cell. **B.** Relative GFAP expression levels in enteric glia, astrocytes and Schwann cells. Enteric glia typically express higher levels of GFAP than astrocytes and much more than Schwann cells, presumably a consequence of cues from the gut microenvironment. **C.** Expression of GFAP (grayscale) and the Ca²⁺-binding protein, S100 (green), a marker of enteric glial cells, in the mouse colon myenteric plexus. Note how GFAP expression is restricted to glial cells and most prominently expressed by enteric glia within the myenteric plexus. GFAP is a much less reliable marker of extraganglionic enteric glia (seen in this image as S100-immunoreactive cells behind the ganglion within the smooth muscle coats). Extraganglionic glia do express GFAP, albeit at much lower levels than that of glia within the plexus. **D.** Expression of vimentin (grayscale) and S100 (green) in the mouse colon myenteric plexus. Note that vimentin is expressed by most myenteric glial cells in the adult mouse colon. Yet, vimentin expression is not restricted to glial cells and extraganglionic S100-negative cells, likely interstitial cells of Cajal, also express vimentin. Scale bars in C and D = 20 μ m.

Total GFAP expression levels are also similar in enteric glia and astrocytes (enteric glia express the highest levels) while Schwann cells express considerably less. The high level of GFAP expressed by enteric glia is likely a consequence of their unique microenvironment rather than an intrinsic difference between the glial cell types because intense GFAP immunoreactivity can be induced in Schwann cells and satellite cells of dorsal root ganglia when cocultured with bowel (Rothman et al., 1986). Further support for the notion that enteric glia are more similar to astrocytes than Schwann cells comes from evidence that shows expression of the cell surface antigen, Ran-1, and the membrane glycolipid, galactocerebroside, by Schwann cells but not by enteric glia or astrocytes and the fact that Schwann cells are surrounded by laminin while enteric glia and astrocytes are not (Jessen and Mirsky, 1985).

Finally, I would like to add a note of caution to not conclude that an enteric glial cell is an astrocyte and vice versa. The two cell types undoubtedly display many similarities but specific differences indicate that enteric glia are fundamentally different from astrocytes. First and foremost is that fact that enteric glia and astrocytes have different developmental origins: enteric glia are derived from the neural crest while astrocytes are derived from precursor cells that line the neural tube. This difference is highlighted by findings demonstrating that enteric glial cell development requires neuregulin signaling through the ErbB3 receptor while astrocytic development does not (Riethmacher et al., 1997). Further, mature enteric glia lack of expression of the astrocytic marker aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (Boesmans et al., 2014). Therefore, generalizing astrocytic properties to enteric glia for the purpose of modeling or developing hypotheses is a useful principle but one must keep in mind the unique nature of enteric glia.

3.1 RATIO OF ENTERIC GLIA TO NEURONS IN ENTERIC GANGLIA

A common misconception is that enteric glia vastly outnumber enteric neurons within enteric ganglia. Yet, the actual neuron to glia ratio varies substantially depending on the gut region and species (see Table 3.1) (Gabella, 1984). In general, the number of myenteric glia is equal to, or greater than, that of myenteric neurons while the reverse is true in the submucosal plexus. Also, myenteric glia tend to be larger than glial cells in the submucosal plexus (Hoff et al., 2008). Age and gender differences do not have a substantial influence on the ratio of glia to neurons in adult humans. However, gender and age differences have been observed in the intermediate submucosal plexus of the ileum and sigmoid colon myenteric plexus, respectively.

Another general rule seems to be that the ratio of glial cells to neurons increases with species size. For example, the ratio of glia to neurons in the myenteric plexus of the ileum increases from approximately 1:1 in mice to over 4:1 in sheep (Gabella and Trigg, 1984) and up to 7:1 in humans (Hoff et al., 2008). Why the ENS of larger animals would need additional glia is currently unclear

but it may reflect the high metabolic needs of the much larger neurons. Enteric glial cell body size remains relatively constant across species analyzed to date despite wide variability of neuron sizes. Thus, the amplified size mismatch in larger species may require additional glia.

TABLE 3.1: Glial index (the ratio of enteric glia to enteric neurons; Glia: Neurons) within myenteric and submucosal ganglia of the ileum in different species. Data adapted from Gabella and Trigg (1984) and Hoff et al. (2008).

Species	Myenteric Plexus	Submucosal Plexus
Mouse	1	0.64
Guinea pig	1.7–2.7	0.71–1
Rabbit	2.6	1
Sheep	4.5	1.5
Human	6–7	1.3–1.9

3.2 GLIAL HETEROGENEITY

As Gabella noted, the name “enteric glia” is rather noncommittal but sufficiently differentiates this unique group of cells from other varieties of glial cells in the central and peripheral nervous systems. Although “enteric glia” was originally coined to describe the glial cells associated with neuron cell bodies and processes in the enteric nerve plexuses, multiple diverse subpopulations of glial cells throughout the gastrointestinal tract currently fall under this definition. Major subpopulations of enteric glial cells reside within the myenteric and submucosal ganglia (intraganglionic), within interganglionic nerve fiber tracts, below the mucosal epithelial cells (subepithelial or mucosal) and associated with nerve fibers interspersed between smooth muscle cells (intramuscular) (Figure 3.3). All enteric glial cells appear to originate from a common pool of neural crest-derived progenitors (Laranjeira and Pachnis, 2009) and expression of a common set of biomarkers including S100 (Ferri et al., 1982), GFAP (Jessen and Mirsky, 1980) and the transcription factors Sox8, Sox9 or Sox10 (Hoff et al., 2008) reflects their common origin. Yet, the mature phenotype of an enteric glial cell is dictated by the unique microenvironment in which it resides. Therefore, the cell-cell interactions that occur within the various compartments of the gut wall produce extensive glial diversity in the intestines.

Unfortunately, a unifying system to classify the different types of enteric glial cells is still not in use. This lack of clarification is the source of apparent discrepancies among experimental findings because each population of enteric glia residing in microenvironmental compartments of the gut wall presumably represents a functionally distinct subtype of cell. In support, differences in

receptor expression (Nasser et al., 2006b), channel expression (Costagliola et al., 2009) and function (Maudlej and Hanani, 1992) suggest heterogeneities even among intraganglionic glial cells of the

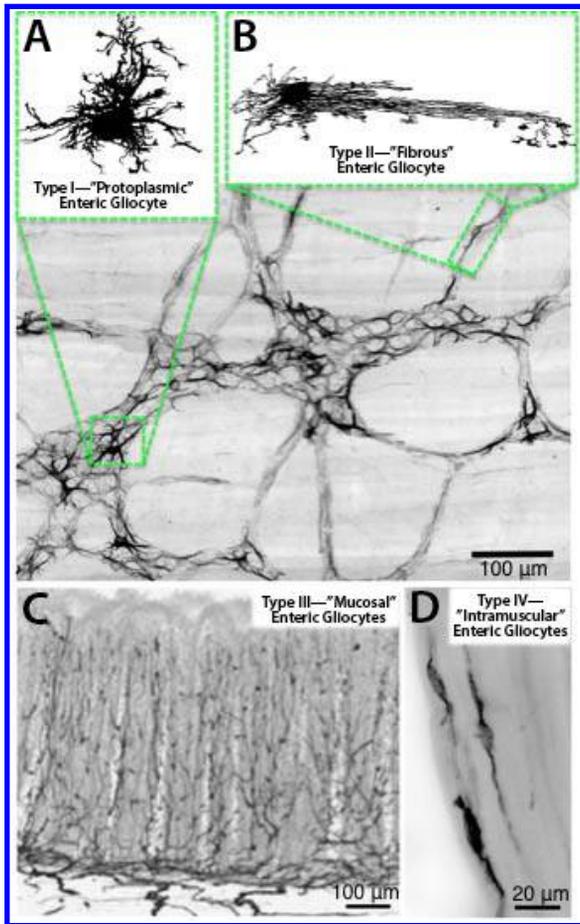


FIGURE 3.3: Four major subtypes of enteric glia. Image shows GFAP labeling in the myenteric plexus of the mouse colon. Boxed regions highlight examples of (A) type I “protoplasmic” enteric gliocyte associated with neurons in enteric ganglia and (B) type II “fibrous” enteric gliocyte found within nerve fiber bundles connecting enteric ganglia. C. Type III “mucosal” enteric gliocytes associated with nerve fibers and epithelial cells at the level of the mucosa. D. Type IV “intramuscular” enteric gliocytes are associated with nerve fibers in the smooth muscle coats. Images in A and B are modified from Hanani and Reichenbach (1994) and image in C is modified from Liu et al. (2013).

myenteric and submucosal plexuses from varying gut regions. Gabella noted similar glial heterogeneity in his early morphological analyses of myenteric glia and, as shown in the following quote, seems to have supported the need to classify enteric glia into subtypes.

In spite of obvious structural differences among the numerous glial cells of the intramural plexuses, these cells have not yet been classified in different types. Some glial cells are, so to speak, wrapped around a nerve cell and cover a large part of its surface. Other glial cells have so many processes (particularly rich in gliofilaments) that the shape of the cell body is ill-defined (Gabella 1976).

Following Gabella, attempts were made to classify the different types of enteric glial cells within the enteric plexuses based on morphological differences. Geoffrey Burnstock's group made the first attempt to classify the non-neuronal cells within the myenteric ganglia of the rabbit colon as type I, II or III glia based on size, gliofilament content and position within the ganglion (Komuro et al., 1982). Subsequently, Hanani proposed a system to classify enteric glia analogous to that in use for astrocytes (Hanani and Reichenbach, 1994). Based on morphological similarities with protoplasmic and fibrous subpopulations of astrocytes in the CNS, he suggested classifying the star-shaped enteric glia within enteric ganglia as “protoplasmic” or “type I” enteric gliocytes and the elongated glia within interganglionic fiber tracts as “fibrous” or “type II” enteric gliocytes. This is perhaps the most useful classification because it takes into account morphology, environment and presumed function. By extending this classification system to encompass extraganglionic glia, the subepithelial glia with several long-branching processes could be classified as “mucosal” or “type III” enteric gliocytes and the elongated glia running with nerve fibers in the musculature as “intra-muscular” or “type IV” enteric gliocytes (Figure 3.3). More specific classification schemes will likely evolve as our understanding of unique glial properties and functions improves but the adoption of a basic framework such as that outlined above would help when considering the diverse contributions of individual glial subpopulations in the regulation of gut function.

3.3 ENTERIC GLIA IN CULTURE

As noted above, the enteric glial cell phenotype is dictated by cues from their microenvironment. This is especially evident when enteric glial cells are removed from their native environment and cultured. In this setting, enteric glia dedifferentiate and adopt the phenotype of a non-myelinating Schwann cell. The first obvious change in culture is a complete restructuring of glial morphology. Enteric glial cells in culture have an unstable morphology and can alternate between a bipolar form, similar to that of Schwann cells in culture, to a flattened form with a central nucleus, to a form where the nucleus is offset in a thickened area of the cell and back to a bipolar form within a timespan of a few hours (Jessen et al., 1983). While this change in structure does not necessarily indicate an altered phenotype per se, the concomitant revamping of glial molecular machinery strongly supports the notion. For example, enteric glia acquire the expression of Schwann cell myelin protein, laminin and the surface antigen, Ran-1, in culture and but loose expression of the surface antigen, Ran-2, and express lower levels of the glial marker, GFAP (Jessen et al., 1983; Jessen and Mirsky, 1983). Further, enteric glia acquire neurogenic capabilities in culture that are not observed *in vivo* (Joseph et al., 2011; Laranjeira et al., 2011). These results indicate that cell-cell interactions in the gut microenvironment have a strong modifying effect on glial phenotype and that enteric glia revert to a default, Schwann cell-type of undifferentiated cell in the absence of

these cues. Therefore, extreme caution should be used when interpreting data from culture models as these are increasingly recognized as poor models of glia in their native environment.

3.4 STRUCTURAL RELATIONSHIPS BETWEEN ENTERIC GLIA AND OTHER CELLULAR ELEMENTS OF THE GUT

Enteric glia are present at all levels of the gut wall and are therefore in close proximity to nearly every cell type within the gut wall. Yet, glia only form specialized contacts with restricted populations of cells. These specialized interactions provide valuable insight into the potential roles of enteric glia and continue to guide current studies investigating the functional roles of glia.

The most intimate association is between enteric glia and the cell bodies and processes of neurons within the myenteric and submucosal plexuses. Within the plexus, enteric glia are molded over the surface of the adjacent neuronal structures (Figure 3.4A). Glial cell bodies are interposed between neurons and glial processes spread over the surface of neurons and between neuronal processes. Most, if not all, enteric glia have processes that extend out to the collagen capsule surrounding enteric ganglia and a single glial cell will often have processes contacting both longitudinal and circular muscle faces of the ganglion (Figure 3.4B). This does not, however, form a complete glial capsule surrounding the neurons and in fact, large areas of the surface of neuron cell bodies and processes have no glial coverage at all. Thus, enteric neuron membranes are often in direct contact with the collagenous sheath of the ganglion and direct membrane-to-membrane contact between neurons is common. Likewise, glial processes rarely isolate individual nerve processes and more often compartmentalize small bundles of axons leaving the neuronal processes within these bundles in direct membrane-to-membrane contact (Figure 3.4C) (Gabella, 1972; Komuro et al., 1982). Similar to astrocytes, a single enteric glial cell contacts many axons. In the guinea pig myenteric plexus, the ratio between axons and glial cells is estimated at 600 axons per enteric glial cell (Gabella, 1972).

Synapse-like junctions between enteric neurons and glial cells are very common and it is likely that every glial cell has at least one or more (Figure 3.4D) (Gabella, 1972; 1981). These junctions are found in all species analyzed to date and suggest specialized communication between neurons and glial cells in the ENS. Nerve terminals contacting enteric glial cells show presynaptic specializations that include a presynaptic density and a clustering of agranular synaptic vesicles. Yet, no obvious postsynaptic specializations are present in the enteric glial cell. Varicosities that form synapses on dendrites or perikarya or those that are located at the ganglionic surface do not form contacts with glia (Gabella, 1981). Nerve varicosities innervating glia are a specific type that primarily contains small agranular vesicles and a variable number of large granular vesicles. Other types of nerve varicosities such as those with small granular vesicles, heterogeneous granule vesicles or an almost pure population of small agranular vesicles do not appear to innervate glia (Gabella,

1981). Such a specialized interaction suggests that enteric glia are innervated by specific nerve pathways. In support, newer data show that enteric glia can discern activity in adjacent neural pathways and that glial responses are triggered by specific populations of neurons (Gulbransen et al., 2010). This is important because it suggests that glia are not indiscriminate detectors of neuronal activity and that neuron-glia communication is associated with defined synaptic events. Thus, the specificity of neuron-glia interactions suggests that glial cells participate in modulating distinct functions of the ENS.

Glial processes that extend to the surface of the ganglion contact non-neuronal elements that surround the ganglion including fibroblast-like cells, blood vessels and immune cells. Fibroblast-like cells coating the enteric ganglia and have processes that come within 20 nm of glial cells with no intervening basal lamina (Komuro et al., 1982). Junctional specializations between glia and fibroblast-like cells have not been observed to date. Likewise, glia come in close contact with capillaries and immune cells surrounding the ganglia but whether specialized points of communication exist is still unknown. Future work is needed to determine the exact nature of these relationships and elucidate whether glia bridge signals between the nervous system and non-neuronal elements surrounding the plexus.

Extraganglionic glia at the level of the mucosa contact blood vessels, lymphatics, myofibroblasts and epithelial cells (Figure 3.5A-D)(Liu et al., 2013). Mucosal glia are often interposed between myofibroblasts and the epithelial basement membrane in the crypt bases. These glia extend long, branching processes, some of which contact mucosal cells, while other contact capillaries or lymphatics. These multidirectional interactions raise the possibility that mucosal glia function to relay or integrate information between the three compartments. New data suggest that some mucosal glia have a specialized relationship with enteroendocrine cells (Figure 3.5E) (Bohórquez et al., 2014). Enteroendocrine cells have an axon-like basal process that contains the majority of synaptic vesicles. Glia contact and embrace these regions of enteroendocrine cells in a relationship that mirrors that of glial cell and neurons in the enteric plexuses.

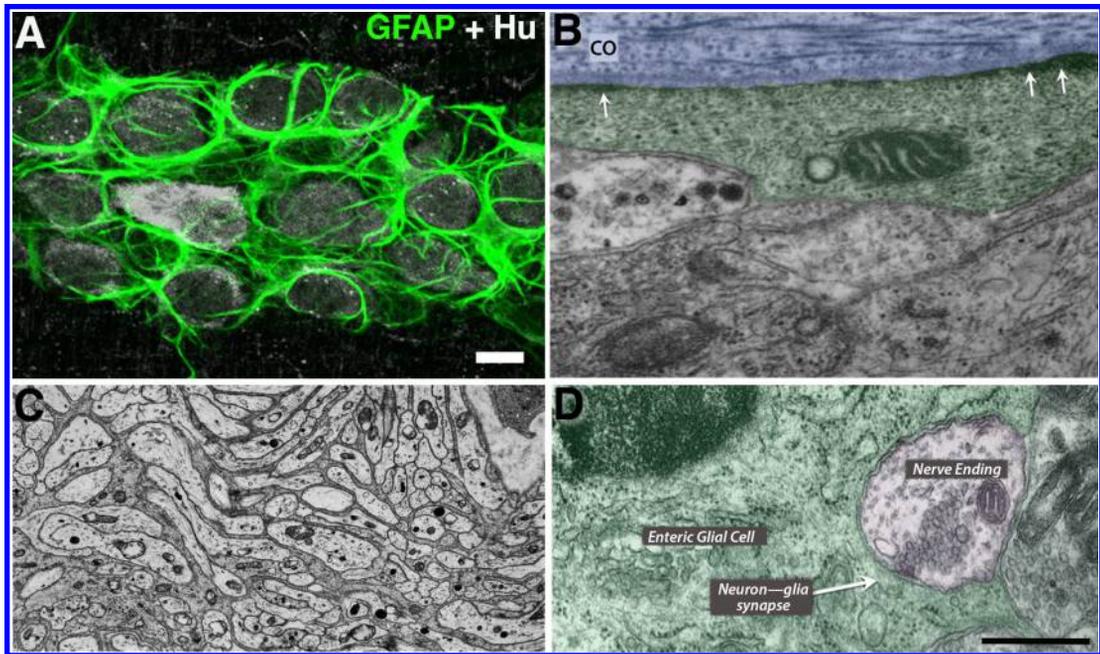


FIGURE 3.4: Representative examples of the structural relationship between enteric neurons and glia. **A.** Enteric glial cells (GFAP immunoreactive, green) surround enteric neurons (Hu immunoreactive, grayscale) within a myenteric ganglion in the myenteric plexus of the mouse colon. Scale bar = 20 μm . **B.** Electron micrograph modified from Gabella (1972) showing a glial processes (shaded green) extending to the surface of the ganglion. Glial processes lie under the basal lamina and collagen fibrils (shaded blue) coating the ganglion. Arrows point to patches of dense material on the inner side of the glial cell membrane where gliofilaments are attached. **C.** This electron micrograph from (Baumgarten et al., 1970) shows glial cells (identified by electron dense cytoplasm) surrounding unmyelinated nerve fibers in the myenteric plexus of the rhesus monkey large intestine. Note that enteric glia do not typically isolate individual nerve processes. Rather, enteric glia will compartmentalize groups of (up to 30 have been observed) nerve processes. **D.** Electron micrograph modified from Gabella (1972) showing an enteric neuron-to-enteric glia synapse. In this image, a nerve ending containing agranular vesicles shows a presynaptic specialization shaded purple and the glial cell is shaded green. These specialized neuro-glia junctions are very common and it is likely that each glial cell has one or more. Scale bar = 0.5 μm .

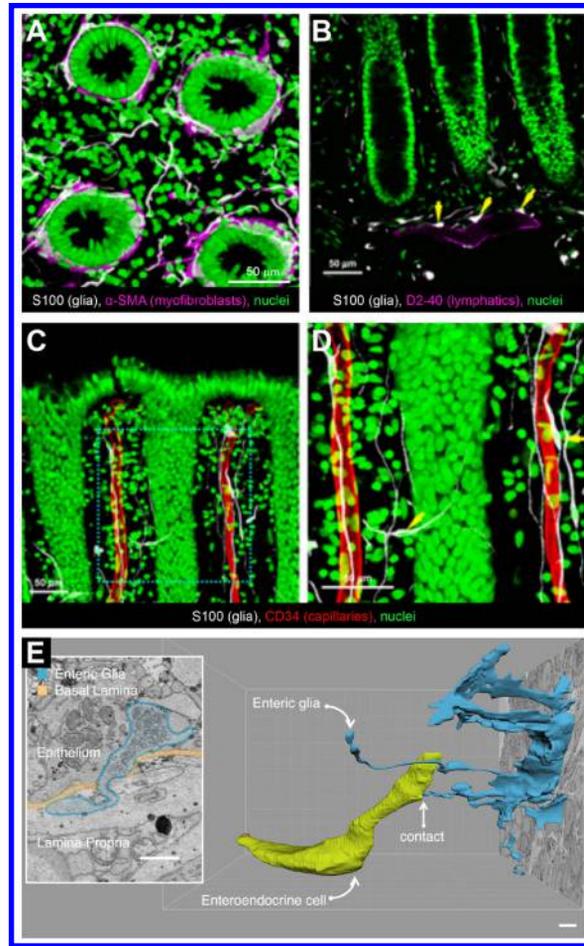


FIGURE 3.5: Enteric glia in the intestinal mucosa are associated with epithelial cells, myofibroblasts, lymphatics, blood vessels and enteroendocrine cells. **A.** S100 immunoreactive enteric glia (white) are interposed between myofibroblasts (α -smooth muscle actin (SMA) immunoreactive, magenta) and epithelial cells (green). **B.** Glia are associated with D2-40 immunoreactive lymphatics (magenta). Arrows indicate glia contacting lymphatics. **C.** Glia contact capillaries (CD34 immunoreactive, red) within the mucosal villi. Image in **(D)** shows enlarged view of boxed region in **(C)** Images in **A-D** modified from Liu et al. (2013). **E.** 3D reconstruction of serial block face scanning electron microscopy images reveals specialized contacts between mucosal enteric glial cells and the “neuropod” region of enteroendocrine cells. Image from Bohórquez et al. (2014).

CHAPTER 4

Molecular Composition of Enteric Glia

The molecular makeup of enteric glia is often cited as being analogous to that of astrocytes. This analogy is useful, superficially, because major astroglial markers are expressed by enteric glia and a similar collection of channels and transporters produce astrocyte-like electrophysiological properties in enteric glia. I will refer readers to the seminal work of Menachem Hanani ([Hanani et al., 2000](#)) for an excellent characterization of the electrophysiological characteristics of enteric glia. Yet as our knowledge of glial cells in the gut deepens, we are increasingly aware of the limits on generalizations between enteric glia and astrocytes. Specific differences between astrocytes and enteric glia and even among various populations of enteric glia are emerging ([Broussard et al., 1993](#); [Coscagliola et al., 2009](#)). Unfortunately, a genomic analysis of enteric glial cells is currently unavailable so that the two cell populations can be compared. Our current level of understanding of the breadth of glial expression of various proteins is mainly based on immunohistochemical localization, electrophysiology or Ca²⁺ imaging data. I have summarized the known breadth enteric glial receptors, channels, releasable factors and other signaling machinery in [Table 4.1](#).

4.1 COMMON MARKERS

Glial fibrillary acidic protein (GFAP; discussed in [Chapter 3](#)), the calcium binding protein S100 β and the transcription factor, SRY-related HMG-box (Sox) 10, are by far the most widely used markers of enteric glia. Each marker has specific advantages and disadvantages and usage varies depending on experimental needs. For example, GFAP is an excellent marker of glial processes and is useful for tracking changes in glial morphology. However, GFAP fails to label cell bodies or nuclei and quantification of glial cell numbers is usually not feasible with this marker. Sox10, on the other hand, labels glial nuclei and is the best marker for glial cell quantification but gives no information on glial morphology. S100 β is localized to the glial cytoplasm and is an effective marker of glial cell bodies. However, S100 β expression is not restricted to enteric glia in the gut and may also be extruded from glial cells under certain conditions.

4.2 RECEPTORS

Enteric glial cells express an impressive battery of receptors for almost every known neurotransmitter/neuromodulator in the gut. Most, if not all, of these receptors belong to the G-protein-coupled receptor (GPCR) superfamily. The most prominent among these in enteric glia are the receptors for nucleotides. At present, enteric glia are known to express receptors for adenosine diphosphate (ADP; P2Y1 receptor) (Gomes et al., 2009; Gulbransen et al., 2012; McClain et al., 2014), adenosine triphosphate (ATP) and uridine triphosphate (UTP) (both via P2Y4 receptors) (Kimball and Mulholland, 1996; Van Nassauw et al., 2006; Gulbransen and Sharkey, 2009) and adenosine (A2B receptors) (Christofi et al., 2001; Vieira et al., 2011). Thus, glial cells are well equipped to detect extracellular purines in the ENS. As I will discuss later, purines are important mediators of physiological and pathophysiological functions of enteric glia. The functional significance of other classes of neurotransmitter receptors expressed by enteric glia is less clear. These include those for norepinephrine (α 2a adrenergic receptors) (Nasser et al., 2006b), glutamate (mGluR5) (Nasser et al., 2007) and acetylcholine (mAChRs, unpublished personal observation). Norepinephrine and acetylcholine have well established signaling roles in the ENS but the functional significance of glutamate receptors in the gut is still unknown. Likewise, enteric glia express receptors for bioactive lipids such as the sphingosine-1-phosphate receptor (SP1R) (Segura et al., 2004b) and lysophosphatidic acid receptor 1 (LPA1) (Segura et al., 2004a), endothelin (likely ETB receptors) (Zhang et al., 1997), protease-activate receptors (PAR1 and PAR2) (Garrido et al., 2002) and bradykinin (B2 receptors) (Murakami et al., 2007) but the significance of these receptor types on enteric glia is largely unknown. Given what is known of these receptor subtypes in other systems, ETB receptors, B2 receptors and PARs likely mediate glial interactions with the vasculature but this hypothesis remains untested.

4.3 NEUROTRANSMITTER UPTAKE/DEGRADATION

Sequestration or degradation of neuroactive compounds is an important role of perisynaptic glial cells to sustain neurotransmission. Enteric glia express both neurotransmitter transporters and cell-surface enzymes to remove neuroactive compounds. Known neurotransmitter transporters on enteric glia include the peptide transporter, PEPT2 (Ruhl et al., 2005), and the *gamma*-Aminobutyric acid (GABA) transporter, GAT2 (Fletcher et al., 2002). The functional significance of these transporters is currently untested. In contrast, the enteric glial cell ectonucleotidase, eNTPDase2, clearly plays a role in degradation of extracellular ATP within the myenteric plexus (Braun et al., 2004; Lavoie et al., 2011). eNTPDase2 hydrolyses ATP release from enteric neurons to the enteric glial cell agonist, ADP (Gulbransen et al., 2012).

4.4 CHANNELS

Enteric glia express a number of cell surface ion channels. Electrophysiological and immunohistochemical data show that enteric glia express both voltage-gated sodium (Broussard et al., 1993; Hanani et al., 2000) and potassium channels (Broussard et al., 1993; Hanani et al., 2000; Costagliola et al., 2009). Delayed rectifier potassium channel expression in enteric glia varies between Kv1.1 and Kv1.2 depending on the different subpopulations of enteric glia. The subtypes of voltage-gated sodium and inwardly rectifying potassium channels expressed by enteric glia are currently unknown but electrophysiological data suggests that expression patterns vary between different subpopulations of enteric glia. Interestingly, the presence of inwardly rectifying potassium channels is hidden unless recordings are performed in the presence of gap junction modulators. Thus, this channel type is not thought to play a dominant role in normal glial function.

Gabella noted that a striking feature of enteric glia is the presence of numerous intramembrane particles on their surface (Gabella, 1981). Subsequent dye coupling experiments demonstrate that at least a portion of these intermembrane particles are true gap junctions (Hanani et al., 1989). However, the occurrence of gap junctions in enteric glia is very rare (Gabella, 1981) and the bulk of these intramembrane particles are thought to be hemichannels. We recently found that, like astrocytes, enteric glial hemichannels are composed of connexin-43 (Cx43) (McClain et al., 2014). Cx43 channels are currently of great interest as key mediators of enteric glial and astrocytic function and are implicated in functions ranging from potassium buffering to gliotransmitter release.

Enteric glia also exhibit immunoreactivity for aquaporin channels of the subtype aquaporin-4 (Jiang et al., 2013). Like sodium and potassium channels, aquaporin-4 expression varies between enteric glial subtypes and is expressed by glial cells within the submucosal and myenteric plexuses but not extraganglionic glia.

TABLE 4.1: Summary of known receptors, channels, transporters and releasable factors expressed by enteric glial cells

Channels		References
Voltage-gated sodium	Type unknown	Broussard et al. 1993; Hanani et al. 2000
Delayed rectifier potassium	Kv1.1 and Kv1.2	Broussard et al. 1993; Costagliola et al. 2009; Hanani et al. 2000
Inward rectifier potassium	Type unknown	Hanani et al. 2000
Aquaporins	Aquaporin-4	Jiang et al. 2013
Connexins	Cx43 hemichannels	McClain et al. 2014

Neurotransmitter receptors		
ATP	P2Y4	Gulbransen and Sharkey 2009; Van Nassauw et al. 2006; Kimball and Mulholland 1996
ADP	P2Y1	McClain et al. 2014; Gulbransen et al. 2012; Gomes et al. 2009
UTP	P2Y4	Gulbransen and Sharkey 2009; Van Nassauw et al. 2006; Kimball and Mulholland 1996
Adenosine	A2B	Vieira et al. 2011; Christofi et al. 2001
Glutamate	mGluR5	Nasser et al. 2007
Norepinephrine	α 2a	Nasser et al. 2006b
Bioactive lipids	SP1R, LPA1	Segura et al. 2004a; Segura et al. 2004b
Interaction with vasculature		
Endothelin	ETB	Zhang et al. 1997
Bradykinin	B2	Murakami et al. 2007
Protease-activated receptors	PAR1, PAR2	Garrido et al. 2002
Neurotransmitter uptake/degradation		
Small peptides	PEPT2	Rühl et al. 2005
GABA	GAT2	Fletcher et al. 2002
Ectonucleotidases	eNTPDase2	Lavoie et al. 2011; Braun et al. 2004
Trophic factors		
Receptors	TrkB	Levanti et al. 2009
Growth factors	Pro-epidermal growth factor (proEGF), Nerve Growth Factor (NGF)	Van Landeghem et al. 2011; von Boyen et al. 2006
Immune interactions		
Toll-like receptors	TLR4	Esposito et al. 2013

Major histocompatibility complex	HLA-DR class II complex (MHC-II)	da Silveira et al 2011; Koretz et al. 1987; Hirata et al. 1986
Releasable factors		
Purines	ATP	Zhang et al. 2003
Nitric oxide	iNOS	Esposito et al. 2013; MacEachern et al. 2011
Antioxidants	15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂ , S-nitrosoglutathione (GSNO)	Abdo et al. 2012; Bach-Ngohou et al. 2010; Abdo et al. 2010; Savidge et al. 2007
Prostaglandins	Prostaglandin E(2)	Murakami et al. 2007
Cytokines	Interleukin-1 β , TGF- β , Interleukin-6	Murakami et al. 2009; Neunlist et al. 2007; Rühl et al. 2001

4.5 TROPHIC FACTORS

Enteric glia are dependent upon expression of TrkB, the high affinity receptor for the neurotrophins brain-derived neurotrophic factor (BDNF) and NT-4, for normal development (Levanti et al., 2009). Whether enteric glial cells maintain this requirement in the adult animal is unknown. Enteric glia can, in turn, produce neurotrophins such as nerve growth factor (NGF) and the precursor form of epidermal growth factor (proEGF) that affect neuron and epithelial cell health, survival and proliferation (Boyen et al., 2006; Van Landeghem et al., 2011). Although glial cell-derived neurotrophic factor (GDNF) expression in the gut is often attributed to enteric glia, current data show that GDNF expression in the gut is confined to smooth muscle cells, not glia (Korsak et al., 2012).

4.6 MEDIATORS OF IMMUNE INTERACTIONS

Enteric glial cells are increasingly recognized as playing key roles in gut pathophysiology. At least a portion of this revelation is based on the ability of enteric glia to interact with both innate and adaptive branches of the immune system. Expression of toll-like receptors (TLRs) by enteric glia suggests that glial cells may serve a sentinel role in the gut and detect invading microbes (Esposito et al., 2013). Glial cells also up-regulate expression of major histocompatibility complex class II in disease states, suggesting that glia bridge adaptive immune responses with dysfunction in the ENS (Hirata et al., 1986; Koretz et al., 1987; da Silveira et al., 2011).

4.7 RELEASABLE FACTORS

Enteric glial cells were classically viewed as passive supporting cells but this view is being replaced by a new vision in which glial cells release a host of compounds that can modify surrounding cell types. Gliotransmission is becoming an accepted concept in the brain but in the gut, the concept of enteric gliotransmission is still in its infancy. As with neurotransmitter receptor expression, the most well characterized substance released from enteric glia is ATP (Zhang et al., 2003). ATP release from enteric glia *in situ* and in cultured cells requires connexin-43 hemichannel opening (Zhang et al., 2003; McClain et al., 2014). Glial-derived ATP has the potential to modify adjacent glia, triggering intercellular Ca^{2+} waves, and to influence adjacent neurons. Selectively attenuating the ability of enteric glia to release ATP by deleting connexin-43 hemichannels in a glial-specific and inducible mouse knockout model alters gastrointestinal motility (McClain et al., 2014). This finding suggests that glial ATP release plays an important role in modulating enteric neurotransmission.

Enteric glia are also capable of producing nitric oxide (NO); a key inhibitory neurotransmitter in the ENS and factor driving oxidative stress in disease (MacEachern et al., 2011; Esposito et al., 2013). Glial NO is produced by enzyme, inducible nitric oxide synthase (iNOS). Traditionally, induction of glial iNOS is thought to only occur in response to pathological stimuli and large amounts of NO produced by iNOS may be protective or deleterious depending on the circumstances. Some evidence now suggests that enteric glial cells may constitutively express iNOS and that glial-derived NO plays a physiological role by modulating epithelial ion transport (MacEachern et al., 2011).

Like NO, prostaglandins produced by enteric glia such as prostaglandin E(2) (PGE2) may serve diverse roles (Murakami et al., 2007). Glial-derived PGE2 may act to modulate enteric neurotransmission, as a vasodilator or as a contributor to oxidative stressor in disease but the role of this compound in the intact ENS is currently unknown.

In contrast to NO and PGE2, glia also produce neuroprotective compounds such as antioxidants. Known glial antioxidants are 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 and S-nitrosoglutathione (GSNO) (Savidge et al., 2007; Abdo et al., 2010; Bach-Ngohou et al., 2010; Abdo et al., 2012). Current data support the notion that glia produce these compounds under physiological conditions both *in situ* and *in vitro*. Thus, glial-derived compounds likely promote neuron health or modify function under normal circumstances but pathological stimuli have the potential to drive the release of toxic compounds from glia.

CHAPTER 5

Development of Enteric Glia

Enteric neurons and glia arise from a common population of neural crest precursor cells that migrate to the gut from the vagal and sacral portions of the neuraxis (Le Douarin and Teillet, 1973). The majority of the ENS is formed from vagal neural crest cells that emigrate from the level of somites 1–7. Sacral neural crest precursors originating from somite 24 contribute to the later colonization of the hindgut. In mice, migratory precursor cells colonize the foregut by E9 (Rothman et al., 1986) and progress distally until the entire gut is colonized by E13.5 (Kapur et al., 1992). These precursor cells do not express markers of neurons or glia when they arrive in the gut, and progenitor differentiation follows the initial colonization of the bowel in a rostral to caudal gradient with markers of myenteric neurons appearing soon after colonization at E9.5–10 in the foregut and E14.5 in the rectum (Baetge and Gershon, 1989).

Glial cell differentiation lags well behind the initial wave front of colonization, and glial precursors are not detected in the foregut until E11.5 (Young et al., 2002). Thus, enteric glia are not thought to play a role in the migration of neural crest cells or the initial differentiation of enteric neurons. Commitment to a glial fate is initially recognized in progenitor cells by expression of a panel of markers that includes the transcription factor, *Sox10*, the glial cell precursor-specific marker, brain-specific fatty acid binding protein (B-FABP) and high expression levels of the p75 neurotrophin receptor (Young et al., 2002). *Sox10* is a member of the high-mobility (HMG) group gene family that is expressed by all undifferentiated neural crest-derived cells just as they migrate from the dorsal neural tube. *Sox10* maintains multipotency of precursor cells by inhibition of neural cell fates (Kim et al., 2003) and its expression is downregulated in differentiating neurons but maintained in glia. S100 β and GFAP, markers of terminally differentiated glia, appear several days after the appearance of the glial precursors with S100 β expression appearing in differentiating glia in mice at E14.4, three days after the first detection of glial precursors (Young et al., 2002), and GFAP only appearing in fully differentiated glia at E16, a full six days after neuronal differentiation (Rothman et al., 1986).

Gene knockout studies are beginning to provide insight into the key signaling pathways and molecules that control the differentiation of enteric glia from neural crest precursor cells. What these studies show is that complex and precisely timed interactions between cell-intrinsic mechanisms regulated by *Sox10* (Paratore et al., 2002) and *Foxd3* (Mundell et al., 2012) with extracellular cues impinging on Notch signaling pathways (Taylor et al., 2007; Okamura and Saga, 2008) and the neuregulin receptor ErbB3 (Riethmacher et al., 1997; Chalazonitis et al., 2011) are necessary

for gliogenesis. Cell-extrinsic factors such as Notch ligands and neuregulin (Nrg) interact with these pathways and are necessary for normal gliogenesis and can instruct precursor cells to commit to a glial fate (Morrison et al., 2000; Taylor et al., 2007; Chalazonitis et al., 2011). Similarly, bone morphogenic proteins (BMPs) participate in enteric glial specification through interactions with ErbB3 (Chalazonitis et al., 2011). BMPs induce glial differentiation from precursor cells *in vitro* and these developing glia become dependent on glial growth factor 2 (Ggf2, a neuregulin-1 isoform) signaling through ErbB3 for survival. In addition, autocrine gliogenic-modifying factors such as leucine-rich glioma inactivated 4 (Lgi4) are produced by migrating enteric neural crest-derived cells and glia themselves. Mutations in *Lgi4* or its receptor *ADAM22* reduce the number of enteric glia and alter ENS structure, suggesting that these autocrine factors are key regulators of gliogenesis (Nishino et al., 2010).

Like astrocytes, enteric glial cells undergo substantial postnatal development. Although the specific mechanisms and extent of postnatal modifications are currently unknown, one of the most prominent changes is an age-dependent shift in glial metabolism. This shift is uncovered by the susceptibility of enteric glia to two metabolic toxins: 6-aminonicotinamide (6-AN) and fluorocitrate (fluoroacetate). Both toxins are primarily taken up by astroglia and block glucose utilization but do so via different pathways. 6-AN is an antagonist of niacin that inhibits hexose monophosphate pathway (HMP)-dependent glucose metabolism by interfering with the HMP enzyme 6-phosphogluconate dehydrogenase. In contrast, fluorocitrate blocks tricarboxylic acid cycle (TCA)-dependent metabolism by inhibition of the TCA enzyme aconitase. Administration of 6-AN to early postnatal mice causes severe degeneration of enteric glia and diarrhea (Aikawa and Suzuki, 1985; 1986). However, 6-AN has no obvious effect on enteric glial cell survival or function when administered to adult mice (my unpublished observations). In contrast, these adult glia become susceptible to the toxic effects of fluorocitrate (Nasser et al., 2006a). This metabolic switch mirrors that of astroglia in the CNS where 6-AN causes widespread degeneration of astrocytes when administered prior to the second week of postnatal development in rats while only very restricted populations of astrocytes remain susceptible in adult animals (Krum, 1995). To my knowledge, no study has assessed the effects of fluorocitrate on early postnatal enteric glia so the extent to which they rely on TCA glycolysis is unknown. Yet, the above data strongly suggest that enteric glia primarily rely on HMP-dependent glycolysis in very young animals and shift to TCA-dependent metabolism in the adult.

CHAPTER 6

Functional Roles of Enteric Glia

The perceived importance of glial cells in the nervous system has undergone a drastic revision within the past 15 years. During this time, intense focus on glial cell physiology has uncovered important roles of glial cells in virtually all nervous system functions. Likewise, findings over the past five years have led to the revelation that enteric glial cells are actively involved in modulating gut processes. These findings challenge the previous neurocentric view of ENS-regulated gut functions and suggest that the diverse populations of glial cells in the gut wall are integral for intestinal function.

6.1 APPROACHES TO STUDYING ENTERIC GLIA

The primary reason that the functional roles of enteric glial cells were historically underappreciated was the lack of adequate methodology to probe glial functions. Throughout the 1980s and 1990s, electrophysiology (predominantly intracellular recordings with sharp electrodes) was the mainstay method to study the underpinnings of enteric neurophysiology. However, enteric glial cells are incredibly unremarkable in this experimental paradigm because enteric glia are electrically inexcitable and display mostly passive currents (Hanani *et al.*, 2000). Thus, the advent of new techniques that permitted the study of glial cells in the gut and the brain allowed investigators to ask questions that were previously not feasible. Such rapidly evolving methodologies continue to propel the field and allow researchers to probe deeper into glial functions. The techniques I describe below are currently the primary methods to study enteric glia and are intended to complement one another. In this regard, histological identification of a signaling pathway would lead to validation with calcium (Ca^{2+}) imaging and then to conditional and inducible gene manipulation experiments to test the role in physiological gut processes.

6.1.1 HISTOLOGICAL ASSAYS

Histological assays have been the most widely employed methods to study enteric glia by far. In particular, electron microscopy has been used for the study of the fine structure of glial cells and association with other cellular elements in the gut and immunohistochemistry to assay the expression of various proteins in glia. Results from these assays provided the basic framework for our understanding of glial interactions with other cellular elements in the gut and potential mediators involved. However, these assays are limited by the fact that they do not directly test the function-

ality of the proposed signaling mechanism of interest. For example, Gabella essentially discovered neuron-to-glia transmission using electron microscopy 1970s but the functionality of neuron-glia junctions was not tested until 2009 (Gabella, 1972; Gulbransen and Sharkey, 2009). Similarly, expression of a receptor or signaling molecule by glial cells does not necessarily indicate any clear functional relevance. Unfortunately, many immunohistochemical results reporting the expression of various receptors by enteric glia are still in question because the original works did not take into consideration how intertwined neuron and glial processes are within the enteric plexuses and adequately delineate expression between the two. Although histological assays continue to be an important tool to investigate enteric glia, great care should be employed to correctly localize various markers. Histological assays are particularly useful when combined with the functional assays and gene manipulation experiments described below.

6.1.2 CALCIUM (Ca^{2+}) IMAGING

Enteric glia, like astrocytes, display a form of excitability mediated by elevations in intracellular Ca^{2+} ions (Figure 6.1). These intracellular Ca^{2+} responses are largely considered central to many glial functions but specific details surrounding this link continue to be a matter of great debate. As described earlier in Section 4.2 and Table 4.1, most enteric glial receptors for neuroactive compounds are GPCRs and a majority of these couple to Gq and downstream intracellular pathways that elevate intracellular Ca^{2+} . The advent of Ca^{2+} imaging is likely the most significant recent advancement with regard to glial physiology because this technique permits glial responses to be monitored in real time. This ability opens the door to testing a host of glial properties including interactions with other cell types, responsiveness to various mediators and the involvement of glial responses in physiological gut processes. Ca^{2+} imaging is easily adaptable to glial cells *in situ*, and μ and is now the primary method to monitor glial cell activation. The employment of Ca^{2+} imaging has led to significant leaps in our understanding of the role of glial cells in the ENS. Specifically, Ca^{2+} was essential to establish that neuron-to-glia communication occurs in the ENS and to identify the mediators involved (Gulbransen and Sharkey, 2009; Gulbransen et al., 2010; 2012). Of course, Ca^{2+} imaging does have its limitations and is at best an observational technique. However, Ca^{2+} imaging is an important technique that will be essential to unravel the roles of glia in the gut.

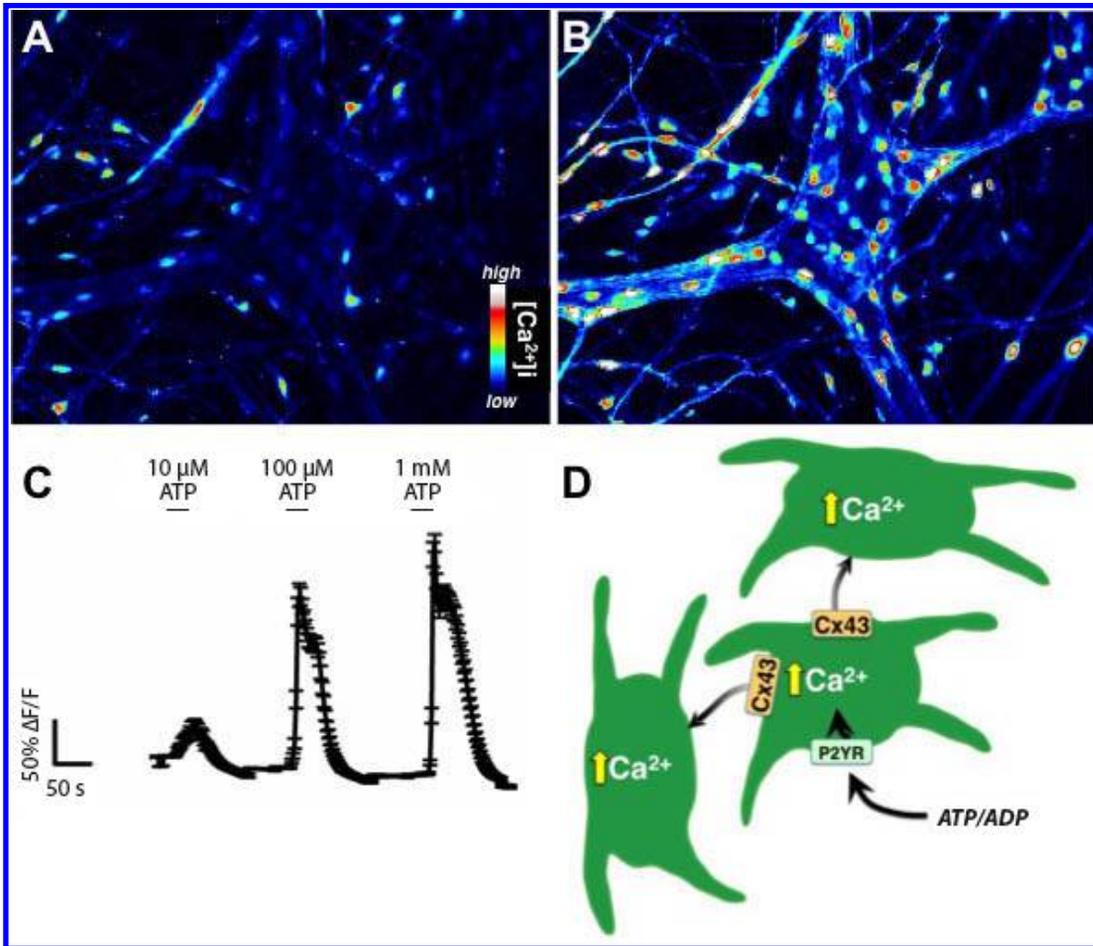
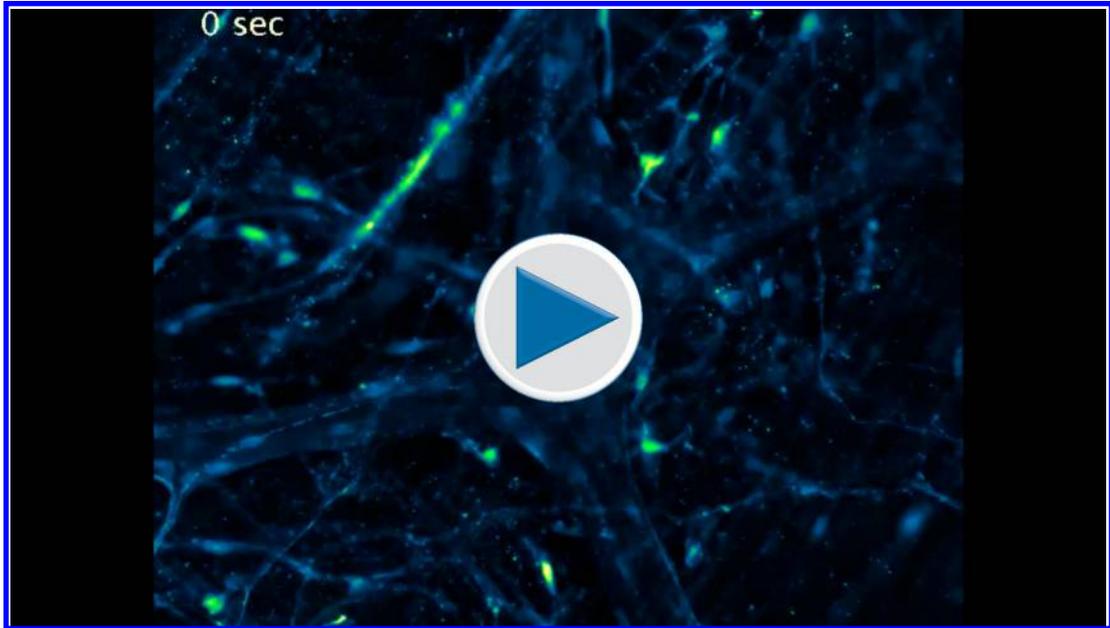


FIGURE 6.1: Ca^{2+} imaging of enteric glial cell activity. **A–B.** Representative images of agonist-evoked Ca^{2+} responses in enteric glial cells *in situ*. Enteric glial cells in a wholemount of ENS from the guinea pig distal colon were bulk loaded with the Ca^{2+} indicator dye, Fluo4-AM and stimulated with the agonist, ATP at various concentrations. Images are pseudocolored on a heatmap scale where blues and blacks represent low Ca^{2+} levels and reds and whites are high Ca^{2+} levels. For complete video, see [Supplementary Video 1](#). **A.** Baseline cytoplasmic Ca^{2+} levels are low in enteric glial cells. **B.** Exposure to ATP initiates large intracellular Ca^{2+} responses in myenteric glial cells. **C.** Trace showing the averaged Ca^{2+} response in all glial cells in the same experiment as **A–B**. **D.** Schematic depicting how GPCR agonists trigger Ca^{2+} responses through the enteric glial network. **A–C** are adapted from Gulbransen and Sharkey (2009) and **D** is adapted from McClain et al. (2014).



Supplementary Video 1: Ca²⁺ imaging of enteric glial cell activity.

6.1.3 GENETIC APPROACHES

The newest, and perhaps most powerful, approaches to studying enteric glial cell function are conditional and inducible gene manipulation models such as *cre-lox* and Tet expression systems. These models allow for expression or deletion of specific genes in a targeted population of cells on demand, and several glial cell driver lines are now commercially available. Both *cre-lox* and Tet expression systems have several distinct advantages over other methods. First, these models allow for specific modulation of signaling pathways in glial cells *in vivo*. Traditional pharmacology lacks this degree of selectivity because no known receptor or signaling pathway is selectively confined to glial cells. Thus, diffuse effects of drugs on many cell populations will confound any interpretation of specific glial roles. Second, genetic models selectively remove a targeted glial function. This is in contrast to gliotoxin models that are relatively coarse, sledgehammer approaches that lack the ability to selectively remove any one glial function. Application of gliotoxins such as fluorocitrate or fluoroacetate has become a popular method to assess glial function *in vivo*. These chemicals are metabolic poisons that rely on preferential glial uptake to kill glial cells. At best, one might be able to draw the conclusion that glial cells are involved in a given process using this technique but the specific role of the glial cells will remain in question. Finally, gene deletion or expression can be in-

duced on demand. The inducible aspect of genetic models is particularly important when targeting glial cells because of their propensity for compensation and possible developmental effects. Glial cells are experts at compensating for the loss of channels and receptors by upregulating expression of another subtype if gene knockouts are constitutive. Likewise, glia play essential roles in the development and maintenance of neural circuits, and developmental effects of constitutive knockout models could confound interpretations.

6.2 BARRIER MAINTENANCE

As mentioned earlier, glial cells in the intestinal mucosa (type III, mucosal enteric gliocytes) are sandwiched between epithelial cells, nerve fibers and blood vessels (Neunlist et al., 2012). This population of glial cells has emerged as a key regulator of gut barrier function. The intestinal barrier is required to limit invasion by the multitude of bacteria within the gut lumen as well as viruses or other foreign substances. Indeed, an increase in epithelial permeability is associated with many gastrointestinal diseases and is regarded as a contributing event to the onset of disease pathology in the gut. Current data support the notion that enteric glial cells regulate gut barrier function by releasing factors that modulate the transcriptome of gut epithelial cells. Cultured enteric glia release a number of factors including S-nitrosoglutathione (GSNO) (Savidge et al., 2007), 15-deoxy- Δ 12,14-prostaglandin J2 (15d-PGJ2) (Bach-Ngohou et al., 2010), transforming growth factor (TGF)- β 1 (Neunlist et al., 2007) and pro-epidermal growth factor (proEGF) (Van Landeghem et al., 2011) that influence the differentiation, adhesion, migration and proliferation of epithelial cell monolayers. Unfortunately, these pathways are currently untested *in vivo* and, given the propensity of glia to change under culture conditions, whether mucosal glia fulfill the same roles *in vivo* is still in question. Current *in vivo* support for glial regulation of barrier function comes mainly from models of conditional ablation of enteric glia (Bush et al., 1998; Cornet et al., 2001; Aube et al., 2006; Savidge et al., 2007). In this paradigm, epithelial permeability does increase following the destruction of enteric glia. However, glial ablation is also accompanied by inflammation. Determining whether changes in intestinal permeability reflect an inflammatory environment fostered by infected and dying glia or if increased permeability is the result of ablated glial homeostatic functions remains a challenge. Experimental findings showing that GSNO has a pro-barrier effect after ileitis (Savidge et al., 2007) and burn injury-mediated increases in intestinal permeability (Costantini et al., 2010) support a role for glial in the restitution of epithelial barrier function after injury. Yet, future work will be required to directly test the roles of enteric glia in the regulation of the intestinal mucosa.

6.3 SUPPORT OF ENTERIC NEURONS

Glia within enteric ganglia (type I and II enteric gliocytes) are actively involved in homeostatic mechanisms that maintain enteric neurotransmission (Gulbransen and Sharkey, 2012). Intraganglionic glia supply enteric neurons with essential precursors for the synthesis of neurotransmitters including nitric oxide (NO) (Aoki et al., 1991; Nagahama et al., 2001), glutamate and γ -aminobutyric acid (GABA) (Jessen and Mirsky, 1983). NO has well characterized functions in both the physiology and pathophysiology of the ENS but the physiological significance of glutamate and GABA remain unclear. Interestingly, dysregulation of either glutamate or NO participates in disease by promoting the death of enteric neurons (Kirchgessner et al., 1997). In health, enteric glia supply neurons with antioxidants (Abdo et al., 2010; 2012) and growth factors (Boyen et al., 2006). In addition, mounting evidence suggests that enteric glia normally function to sustain enteric neurotransmission by regulating the bioavailability of neuroactive substances in the extracellular environment. Enteric glial cell surface enzymes such as the ectonucleotidase, nucleoside triphosphate diphosphohydrolase 2 (eNTPDase2) (Braun et al., 2004; Lavoie et al., 2011) and transporters including those for peptides (Ruhl et al., 2005) and GABA (Fletcher et al., 2002) are essential for the removal of neuroactive compounds surrounding enteric neurons. Likewise, glial channels maintain neurotransmission and prevent excitotoxic neuron death by regulating, and buffering extracellular potassium (Hanani et al., 2000; Costagliola et al., 2009). Taken together, this evidence suggests that glia are essential points of homeostatic control in enteric ganglia and that disruption of glial functions could alter gastrointestinal physiology by altering enteric neurotransmission or permitting enteric neuron death.

6.4 REGULATION OF NEUROTRANSMISSION/ GLIOTRANSMISSION

In addition to the passive, homeostatic mechanisms discussed above, recent evidence suggests that enteric glia actively participate in enteric neurotransmission and are capable of detecting and in turn, modulating the activity of enteric neurons (Figure 6.2). Enteric glia express a diverse assortment of receptors (see Table 4.1) that permits glia to “listen” to neuronal conversations and initiate intracellular signaling mechanisms in response to neurotransmitters released from enteric neurons (Gomes et al., 2009; Gulbransen and Sharkey, 2009; Gulbransen et al., 2010; Broadhead et al., 2012; Gulbransen et al., 2012; McClain et al., 2014). ATP and related purines are the most ubiquitous signaling molecules involved in enteric neuron-to-glia transmission *in vitro* (Kimball and Mulholland, 1996; Gomes et al., 2009) and *in situ* (Gulbransen and Sharkey, 2009; Gulbransen et al., 2010; Broadhead et al., 2012; Gulbransen et al., 2012; McClain et al., 2014) but enteric glia have the potential to detect a number of neuroactive substances including norepinephrine (NE) (Nasser et al., 2006b), glutamate (Nasser et al., 2007), thrombin (Garrido et al., 2002), lipid signal-

ing molecules (Segura et al., 2004a; 2004b), serotonin (Kimball and Mulholland, 1996; Boesmans et al., 2013), bradykinin (Kimball and Mulholland, 1996), histamine (Kimball and Mulholland, 1996) and endothelins (Zhang et al., 1997). Precisely how glial signaling mechanisms affect neurotransmission is still debated but recent evidence demonstrates that glial responses are associated with neuron activity during patterns of activity that underlie physiological gut function (Broadhead et al., 2012).

The tight association of glial responses with neuron activity raises the possibility that enteric glia function as a feedback loop to modulate enteric neurotransmission. Conceivably, enteric glia could modulate enteric neurotransmission by either utilizing the mechanisms described above to regulate the availability of neurotransmitters or by releasing neuroactive substances upon stimulation in a process called gliotransmission. Although few studies have addressed the potential of enteric glia to release neuroactive substances, the available evidence does support the notion of enteric gliotransmission. Indeed, Ca^{2+} responses in enteric glia can initiate the release of ATP *in vitro* (Zhang et al., 2003) and ATP and its purine metabolites are well known neurotransmitters/neuromodulators in the ENS (Galligan and Bertrand, 1994). *In vitro*, propagation of Ca^{2+} responses between enteric glia depends on their release of ATP through hemichannels (Zhang et al., 2003). We found that this process is maintained *in situ* and *in vivo* and that substances released through connexin-43 hemichannels mediate intercellular communication between enteric glia (McClain et al., 2014). Importantly, we found that gastrointestinal transit is delayed following the conditional ablation of connexin-43 in glia. These results provide strong evidence that enteric glia have the potential to release gliotransmitters and that gliotransmitters influence gut function through actions on enteric neurons.

How other populations of enteric glia outside enteric ganglia interact with enteric neurons is currently unknown. Enteric glia are associated with nerve fibers within the smooth muscle coats of the gut wall but, unfortunately, very little is known about this class of cells except that they are morphologically similar to nonmyelinating Schwann cells. Intramuscular enteric glia do express receptors for neuroactive substances (Vanderwinden et al., 2003) but the interactions between nerve fibers and enteric glia in the muscle coats are still unexplored.

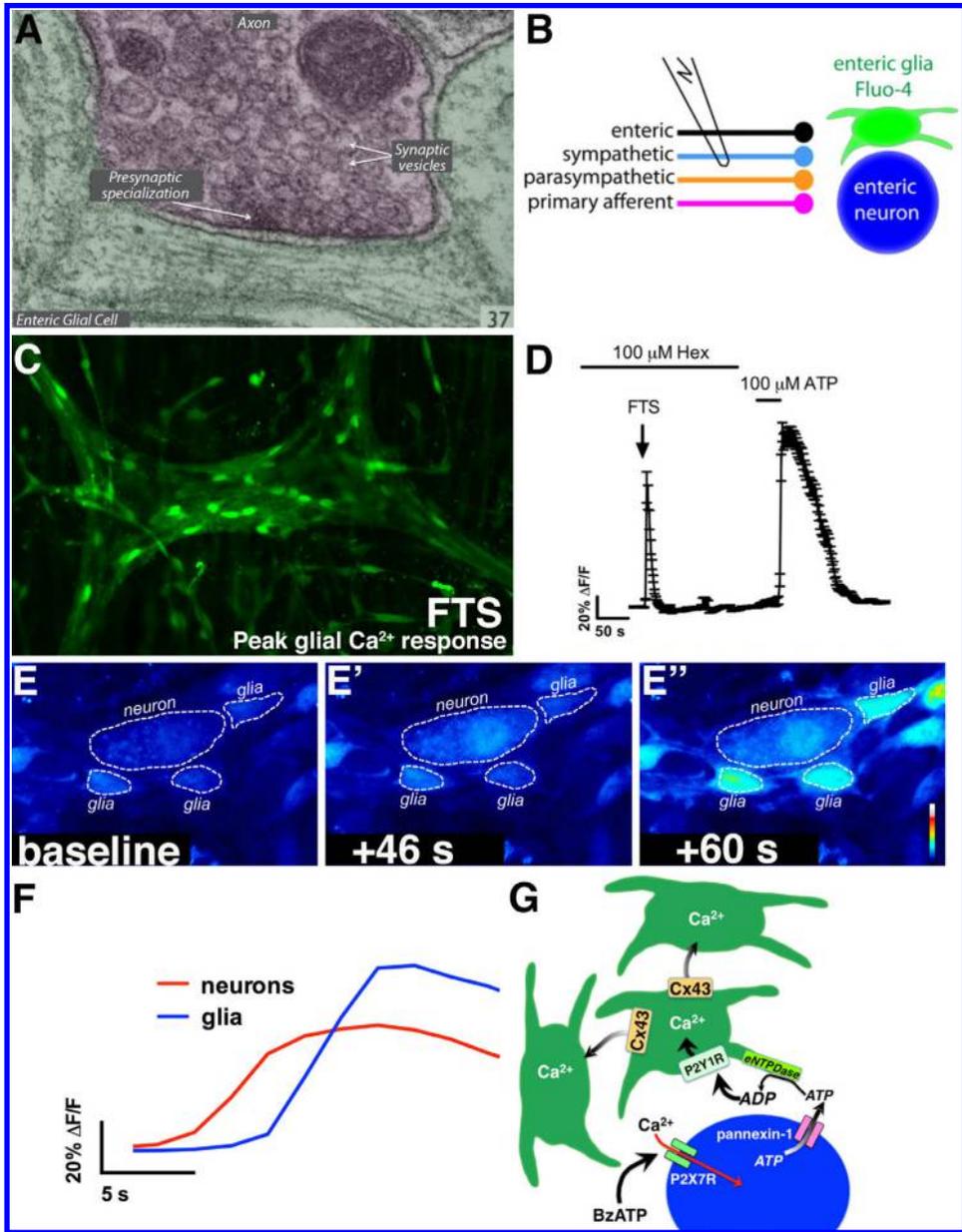
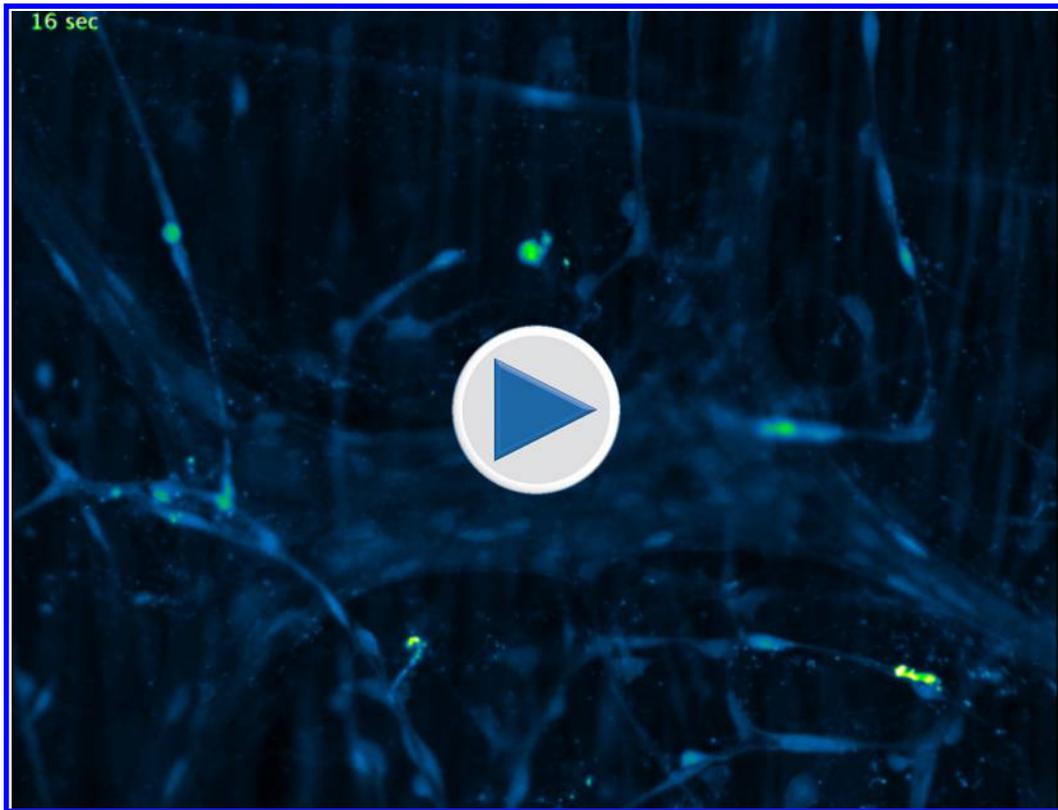
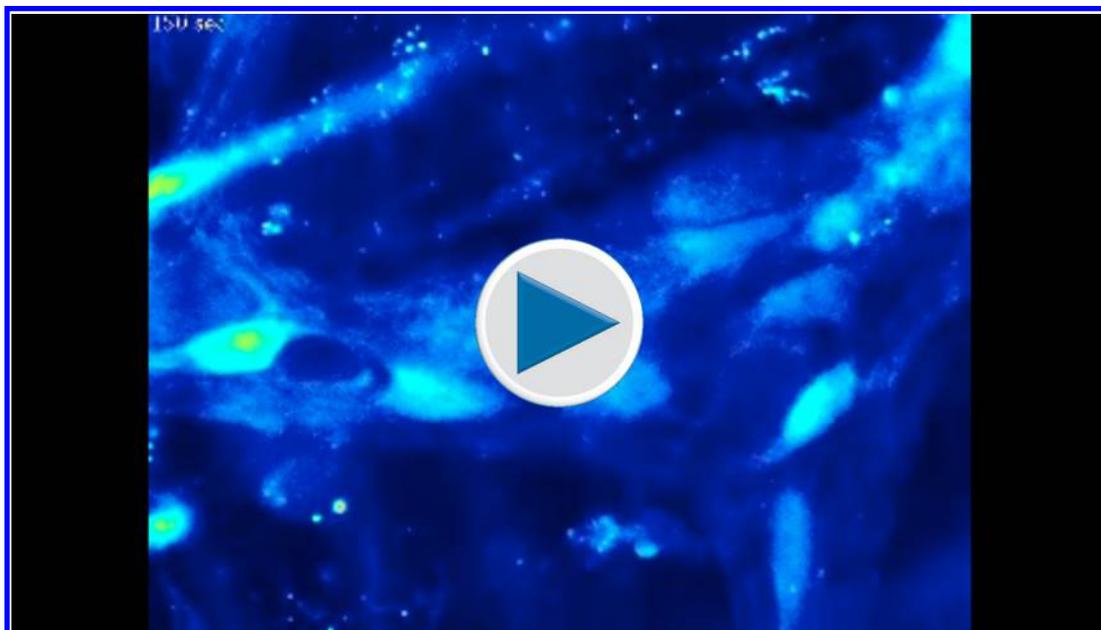


FIGURE 6.2: Enteric neurons “talk” to enteric glial cells. **A.** Electron micrograph modified from Ga-bella (1972) showing a neuron-glia “synapse.” Note the presynaptic specialization in the nerve process (shaded purple) abutting an enteric glial cell (shaded green). **B.** Schematic depicting experimental paradigm to stimulate neuron-glia communication in the ENS. Interganglionic nerve fiber bundles containing the axons of enteric, sympathetic, parasympathetic and primary afferent neurons are acti-

vated with an electrical stimulus (fiber tract stimulation, FTS), and Ca^{2+} responses are monitored in enteric glia within an adjacent ganglion. **C.** Peak Ca^{2+} response of glial cells following depolarization of nerve fiber bundles (FTS). For full video, see [Supplementary Video 2](#). **D.** Averaged response of all glia within the ganglion shown in **C**. Note that glial cells respond to neuron activity (FTS) and the agonist, ATP. **B–D** are from Gulbransen et al. (2010). **E–E’.** Representative responses in enteric glial cells following stimulation of enteric neurons with the P2X7 receptor agonist, BzATP. For full video, see [Supplementary video 3](#). **F.** Traces showing neuron and glial responses in the experiment shown in **E–E’**. Note that neuron responses to BzATP precede glial responses. **G.** Schematic showing the mechanisms involved in enteric neuron-to-enteric glia communication with purines. For details, see Gulbransen et al. (2012). **E–G** are adapted from Gulbransen et al. (2012) and McClain et al. (2014).



Supplementary Video 2: Enteric neurons “talk” to enteric glial cells. Video of Ca^{2+} imaging results shown in [Figure 6.2C–D](#). The first response in glial cells is activated by neuronal depolarization with an electrical stimulus. The second glial response is activated by application of the agonist, ATP.



Supplementary Video 3: Enteric neurons “talk” to enteric glial cells. Video of Ca²⁺ imaging results shown in Figure 6.2E-F. Application of the neuronal P2X7 receptor agonist, BzATP, triggers a response in neurons that recruits Ca²⁺ responses in the surrounding glial cells.

CHAPTER 7

Enteric Glia and Disease Processes in the Gut

Glial cells are emerging as key mediators of gut pathophysiology. Data now show that glial alterations are associated with a wide range of gut pathologies and that glia have the potential to contribute to disease progression. Inflammation, in particular, is a key-contributing factor to intestinal dysfunction in many gastrointestinal diseases, and enteric glia are increasingly implicated in inflammatory processes within the ENS. Our current understanding of how gut disease alters enteric glia stems mainly from histological studies but functional analyses are beginning to uncover the specifics of how glia are affected by and contribute to gastrointestinal disease and dysfunction.

7.1 MORPHOLOGICAL CHANGES

Changes in glial cell number or content of GFAP, S100 or Sox10 are traditionally the main read-outs used to indicate glial alterations in diseased tissue. To date, alterations in the number of enteric glial cells are linked with the inflammatory bowel diseases (IBDs; Crohn's and ulcerative colitis) (Cornet et al., 2001; Boyen et al., 2011), chronic idiopathic intestinal pseudo-obstruction (CIIP) (Selgrad et al., 2009), intractable slow-transit constipation (STC) (Bassotti et al., 2006), diverticular disease (DD) (Wedel et al., 2010), necrotizing enterocolitis (NEC) (Wedel et al., 1998), Chagas disease (da Silveira et al., 2009), type II diabetes (Stenkamp-Strahm et al., 2013) and Parkinson's disease (Devos et al., 2013). Low glial cell density is the most commonly reported abnormality in these diseases but the nature of the glial changes varies quite widely, even within a single disease. For example, GFAP content is low in non-inflamed regions in Crohn's disease but not in ulcerative colitis (Cornet et al., 2001; Boyen et al., 2011). In contrast, GFAP content is elevated in areas of active inflammation in both diseases, albeit to a lesser extent in Crohn's. Likewise, the number of S100 immunoreactive enteric glial cells decreases in Chagas disease while GFAP content increases (da Silveira et al., 2009). Clearly, glial alterations are associated with a broad range of intestinal pathologies but exactly how enteric glia respond to disease processed in the gut is still not understood. However, the current data do suggest several important features of glial cells in disease. First, glia are differentially affected depending on proximity to active inflammation. Second, changes in GFAP or S100 expression do not necessarily indicate alterations in glial cell numbers. Rather, glial expression of these markers may vary widely across the disease process as these proteins are

up- or downregulated. Further, expression of GFAP and S100 may vary independently because GFAP expression reflects glial intermediate filament density and S100 is a diffusible factor that can be released from glial cells. Finally, the significance of glial cell morphological changes is not straightforward and future studies will be necessary to determine the causative factors that drive glial alterations and the downstream consequences.

7.2 FUNCTIONAL CHANGES

Although the aforementioned morphological changes do not necessarily indicate a shift in glial function in disease, evidence is beginning to emerge that supports this notion. Inflammation, in particular, alters glial cell expression of receptors (Nasser et al., 2007) and enzymes (Green et al., 2004) and increases glial proliferation (Joseph et al., 2011). Pro-inflammatory cytokines including IL-1 β and TNF- α seem to be the primary driving force for glial alterations and directly activate enteric glial cells. *In vivo* treatment with the pro-inflammatory cytokine, IL-1 β upregulates expression of the immediate early gene, *c-Fos*, in both myenteric and submucosal glia (Tjwa et al., 2003). Likewise, application of TNF- α to cultured rat enteric ganglia causes translocation of the signal transducer and activator of transcription 5 (STAT5) protein, a measure of cellular activation in response to TNF- α signaling, in glia (Rehn et al., 2004). GFAP expression is at least one downstream effector of the cytokine response in enteric glia and *in vitro* treatment with either IL-1 β or TNF- α induce an increase in GFAP expression in glial cells (Boyen, 2004).

Glia are also sites of action for non-cytokine inflammatory mediators such as thrombin and ATP. Thrombin and other proteases activate a class of receptors known as protease-activated receptors (PARs). As mentioned earlier, PARs are expressed by enteric glial cells and PAR agonists trigger enteric glial cell Ca²⁺ responses (Garrido et al., 2002). Likewise, purines are key-contributing factors to the pathophysiology of gut inflammation in both humans and experimental models. Increased purine release (Wynn et al., 2004; Lomax et al., 2005) and decreased hydrolysis (Wynn et al., 2004; Friedman et al., 2009) elevate extracellular purine levels during inflammation. Enteric glia express purine receptors and are highly responsive to extracellular purines (Kimball and Mulholland, 1996; Gomes et al., 2009; Gulbransen and Sharkey, 2009; Gulbransen et al., 2010; Broadhead et al., 2012; Gulbransen et al., 2012; McClain et al., 2014). The functional consequences of activation of either PAR or purine receptor pathways in glia are still unresolved but may involve the reciprocal release of pro-inflammatory substances from glia. In support, PAR activation in astrocytes elicits the secretion of pro-inflammatory cytokines (Zeng et al., 2013) and cultured enteric glia release IL-6 upon incubation with the pro-inflammatory cytokine, IL-1 β (Rühl et al., 2001). Similarly, purine-activated intracellular Ca²⁺ responses in enteric glia trigger ATP release from enteric glia (Zhang et al., 2003; McClain et al., 2014).

One prominent downstream effect of glial alterations in disease is enteric neuron death. Enteric neuron death is associated with, and predictive of, many gastrointestinal diseases, and the loss of ENS control produces intractable intestinal dysfunction. Alterations to enteric glial functions including a loss of neuroprotective functions or an induction of pro-inflammatory mediators are now considered key factors in enteric neuropathies. Enteric glia play an essential role in neuroprotection in the ENS and glial ablation results in a substantial decrease in enteric neuron density (Bush et al., 1998). Cultured enteric glial cells secrete the neurotrophic factors (Boyen et al., 2006) that protect enteric neurons during inflammation (Liu et al., 2014). Cultured enteric glial cells also secrete potent antioxidants including glutathione (Abdo et al., 2010) and 15d-PGJ2 (Bach-Ngohou et al., 2010). Both are integral in regulating oxidative stress in enteric neurons and are capable of protecting neurons from oxidative stress-mediated death (Abdo et al., 2010; 2012). Further, enteric glia are essential sites of purine regulation within the ENS and are responsible for both purine catabolism (Braun et al., 2004; Lavoie et al., 2011), and purine production (Zhang et al., 2003; McClain et al., 2014). Disruption of either purine catabolism or production has the potential to promote neuron death during disease by elevating extracellular purines and driving the neurotoxic activation of P2X7 receptors (Gulbransen et al., 2012). Thus, current evidence supports the hypothesis that enteric glia protect neuron survival in health but that glial alterations in disease promote neuron death.

7.3 GLIA-IMMUNE INTERACTIONS

The idea that glial cells act as a bridge between immunity and ENS dysfunction is currently gaining great traction and interest. Relatively little is still known about how enteric glial cells interact with immune cells but several lines of evidence suggest that glial cells mediate neuro-immune crosstalk. As mentioned above, glial cells respond to inflammatory mediators and are important sites of action for pro-inflammatory cues. Glial cells also act as a key component of the innate immune response to invading bacteria and detect lipopolysaccharide through TLR4 receptors (Esposito et al., 2013). What actions glia take in response to bacteria are still unknown. One hypothesis is that glial cells engulf the bacteria and subsequently present engulfed material to T-cells through expression of major histocompatibility complex (MHC) proteins. In Chagas disease, both the human leukocyte antigen (HLA)-DR class II peptide and the costimulatory proteins CD80 and CD86 are expressed on the surface of enteric glia (da Silveira et al., 2011). Similarly, enteric glial cell HLA-DR expression is upregulated in Crohn's disease (Koretz et al., 1987). Importantly, glial HLA-DR expression correlates with the density of lymphocyte infiltrate present. Thus, the ability of enteric glia to act as antigen-presenting cells may attract immune cells to the enteric nerve plexuses during inflammation. In support, mast cells are recruited to enteric ganglia during inflammatory conditions and appear to contact enteric glia (Bassotti et al., 2012). In summary, current data support the notion

that glial cells are important mediators of interaction between the ENS and gut immune system but additional research will be necessary to parse out the details of these interactions.

Concluding Remarks

Enteric glial cells are a fascinating population of cells and we are only beginning to uncover their diverse roles in the gut. Glial cells are likely involved in most physiological and pathophysiological processes in the gut but elucidating their precise roles has, to this point, remained technologically challenging. Now, technical capabilities have improved to the point where we are able to directly interrogate specific glial functions and I anticipate that a wealth of new knowledge on enteric glial cells will emerge over the next several years. My personal feeling is that understanding the physiological and pathophysiological functions of enteric glial cells holds great promise for the development of new therapeutics for gastrointestinal disorders. I sincerely hope that you have enjoyed reading this book and find it a useful resource. I also hope that the information in this book serves as stimulus for future studies addressing glial cell physiology in the gut.

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Neuroglia in Development and Neurogenesis

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Neuroglia in Functional Brain Imaging

Neuroglia in *Drosophila*

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Activation of Microglia: General Concepts

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Neuroglia in Chronic Pain

Demyelinating Diseases

Astrocytes in Psychiatric Disorders (schizophrenia, bipolar disorder, autism, etc.)