



HHS Public Access

Author manuscript

Brain Res Bull. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

Brain Res Bull. 2018 January ; 136: 109–117. doi:10.1016/j.brainresbull.2017.03.011.

Enteric glia regulate gut motility in health and disease

Vladimir Grubišić^{a,b}, Alexei Verkhratsky^{c,d}, Robert Zorec^{e,f}, and Vladimir Parpura^a

^aDepartment of Neurobiology, University of Alabama at Birmingham, Birmingham, AL, USA

^bNeuroscience Program, Department of Physiology, Michigan State University, 567 Wilson Road, East Lansing, MI, 48824 USA

^cThe University of Manchester, Manchester, UK

^dAchucarro Center for Neuroscience, IKERBASQUE, Basque Foundation for Science, 48011, Bilbao, Spain

^eLaboratory of Neuroendocrinology-Molecular Cell Physiology, Faculty of Medicine, Institute of Pathophysiology University of Ljubljana, Ljubljana, Slovenia

^fCelica BIOMEDICAL, Ljubljana, Slovenia

Abstract

The enteric nervous system, often referred to as the second brain, is the largest assembly of neurons and glia outside the central nervous system. The enteric nervous system resides within the wall of the digestive tract and regulates local gut reflexes involved in gastrointestinal motility, blood flow and fluid transport; all these functions can be accomplished in the absence of the extrinsic innervation from the central nervous system. It is neurons and their circuitry within the enteric nervous system that govern the gut reflexes. However, it is becoming clear that enteric glial cells are also actively involved in this process through the bidirectional signaling with neurons and other cells in the gut wall. We synthesize the recently discovered modulatory roles of enteric gliotransmission in gut motility and provide our perspective for future lines of research.

Keywords

bidirectional purinergic signaling; enteric glial cells; enteric neurons; enteric nervous system; gliotransmission

Correspondence to: Vladimir Parpura.

University of Alabama at Birmingham, Department of Neurobiology, 1719 6th Avenue South, CIRC 429, Birmingham AL 35294, USA

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Disclosures: The authors declare no competing financial interests.

Introduction

The digestive tract has both intrinsic and extrinsic innervations (reviewed in [27]). The intrinsic innervation is provided by the enteric nervous system (ENS), which resides within the gut wall [3] and can control basic gastrointestinal (GI) functions, such as motility and secretion/absorption (reviewed in [25]), in the complete absence of the extrinsic innervation [4, 85]. Consequently, the ENS has been recognized as the third division, (the other two being sympathetic and parasympathetic branches) of the autonomic nervous system [56]. Even though local ENS circuits collectively operate as an independent integration center, they normally work alongside with the extrinsic innervation, i.e., reflex loops at the level of sympathetic ganglia and the central nervous system (CNS) (reviewed in [27, 28]). The ENS is organized in two networks, myenteric (Auerbach's) and submucosal (Meissner's) plexuses, which in human contains ~500 million neurons, the number comparable to that of neurons in the spinal cord. The population of enteric neurons is rather diverse with approximately 20 types of functionally defined cells utilizing various transmitters common to the CNS [25]. In addition to neurons, the ENS also houses extended population of enteric glial cells (EGCs), whose number exceeds that of neurons by 6–7 times in the myenteric plexus and 1.3–1.9 times in the submucosal plexus of the human gut [32, 48]; the glia to neuron ratio, however, differs between various species. Unlike the ganglia of peripheral and autonomic nervous systems but similar to the CNS, the ENS lacks collagen and fibroblasts under normal conditions [30]. Due to its size, complexity, structure and the ability to independently integrate information, the ENS is often called “the second brain” [33], or “the brain in the gut” [95]. Undeniably, the gut reflexes are controlled by local neuronal networks. However, relatively recent studies indicate the role of glia-neuron signaling in gut functions in health and disease (reviewed in [38, 72, 81]). In this brief review we focus on the (patho)physiological roles of enteric gliotransmission in gut motility.

Enteric glial cells: A historic perspective on this distinct and heterogeneous cell population

The concept of neuroglia was introduced in 1858 by Rudolf Virchow as a “substance... which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree” ([91], English translation in [52]). At the end of the same century, Alexandr Dogiel discovered the glial cells that reside in the intestinal wall [21], which were subsequently and temporarily classified as Schwann cells [84] due to their ability to partially separate, albeit not ensheath, nerve fibers. It was not until Giorgio Gabella that these glial cells within the gut wall were recognized as a distinct cell type [29]; subsequently, Gabella termed them enteric glial cells (EGCs) [31]. In parallel, due to their morphology (with “protoplasmic” and “fibrous” appearances) and expression of some known astrocytic markers, such as glial fibrillary acidic protein (GFAP, Figure 1A) and Ca²⁺ binding protein S100β [24, 50], EGCs were also temporarily considered as an astrocyte subtype, termed “intestinal astrocytes”.

However, there are important distinctions between EGCs of the ENS and astrocytes of the CNS. For instance, aldehyde dehydrogenase 1 family member L1, an astrocytic marker, is

not expressed in EGCs [8]. Additionally, EGCs have significant transcriptional similarities not only to astrocytes, but also to oligodendrocytes and neurons of the CNS [77]. Moreover, EGCs have different embryonic being derived from the neural crest whereas astrocytes develop from the neuroectoderm. Consequently, the contemporary view is well aligned with Gabella's ideas. Not only that EGCs are now considered as a distinct glial cell type, but are also recognized as a diverse population of cells, whose four major types are distinguished by their morphology and location within the gut wall (reviewed in [44]). Thus, (i) "protoplasmic" star-shaped cells reside in the enteric plexuses, (ii) "fibrous" elongated cells are found within fiber tracts connecting plexuses, while (iii) "intermuscular" and (iv) "mucosal" glia reflect the residential sites within the gut wall. The microbiota of the gut lumen can have a major effect on the development of the mucosal glia [51]. Nonetheless, beside specific location and variance in morphology, these EGC types differentially and variably express some of the key cell markers, such as already mentioned GFAP, S100B, as well as Sox10 [7], a neural crest transcription factor crucial for specification, maturation, and maintenance of Schwann cells and melanocytes (reviewed in [71]). Such dynamic protein expression patterns likely indicate functional diversity and plasticity of various EGCs types [7].

In summary, EGCs are juxtapositioned to enteric neurons, smooth muscle and intestinal epithelium of the gut wall, being thus strategically positioned to affect neural networks controlling gut motility and intestinal epithelial functions, as well as contribute to GI disorders (reviewed in [17, 38, 70, 72, 81]).

Cytosolic Ca²⁺ dynamics as a hallmark of enteric glial cell excitability

In contrast to enteric neurons capable of generating action potentials, EGCs only exhibit large "passive" currents [45] being thus electrically non-excitabile cells. It was the pioneering work by Gabella which discovered "neuroglial junctions", a specialized direct contacts between enteric neurons and glial cells [29]. These junctions represent axonal inputs (containing pre-synaptic specialization and vesicles) onto EGCs through synapse-like or synaptoid contacts lacking, however, one essential feature of synaptic contacts, the post-synaptic, i.e. EGC's, membrane specialization. The EGS excitability is mediated by cytosolic Ca²⁺ signals; the first evidence showing ATP-induced increase in intracellular Ca²⁺ concentration of cultured EGCs [53]. Furthermore, myenteric glia *in situ* exhibit Ca²⁺ excitability after electrical field stimulation of both intrinsic, i.e. originating from the ENS, and extrinsic innervation [41, 43], indicating functional neuron-to-glia signaling. It seems that EGCs, at least in the guinea pigs myenteric ganglia, predominantly respond to the sympathetic innervation [41]. Although many studies found EGCs responsive to various neurotransmitters (Table 1, see below), the main component of glial Ca²⁺ excitability *in situ* is sensitive to purinoceptor inhibitors [41, 43]. Purinergic P2Y₁ and P2Y₄ receptors (Table 1 and Figure 1B), both being G_q-protein coupled receptors, are the most studied in EGCs. Their activation leads to an increase in EGCs cytosolic Ca²⁺ concentration mediated by phospholipase C (PLC) and downstream inositol 1,4,5 trisphosphate (InsP₃)-receptor signaling pathway (reviewed in [38]). Activation of InsP₃-receptors located on the endomembrane of the endoplasmic reticulum (ER) leads to release of Ca²⁺ to the cytosol; the ER store (re)filling with Ca²⁺ is accomplished by ER specific Ca²⁺-ATPases of the

SERCA (SarcoEndoplasmicReticulum ATPase) type. The P2Y₁ receptor preferentially binds adenosine 5' diphosphate (ADP), while ATP and uridine 5' triphosphate (UTP) activate P2Y₄ receptor [53]. Following neuronal and/or glial release of ATP to the extracellular space [64], this nucleotide is rapidly degraded by membrane-bound ecto-nucleosides. The triphosphate diphosphohydrolase (eNTPDase/CD39) is expressed throughout the ENS [11, 57]; eNTPDase2 (ATP is the substrate) is expressed on EGCs, while enteric neurons express eNTPDase3 (ATP and ADP are substrates) [54]. The activity of these eNTPDases underlies the extracellular production of adenosine monophosphate, which gets further hydrolyzed to adenosine by ecto-5'-nucleotidase. While adenosine A_{2B} receptor type was detected by immunocytochemistry in the EGCs [16, 90], its functional role remains elusive. Furthermore, it is unclear whether UTP can be generated in the ENS; if so, this could occur via the exchange of Y-phosphates between adenine- and uracil-based nucleotides via nucleoside diphosphokinase (NDPK) (reviewed in [58]). It is possible that this signaling pathway gets activated during the gut infestation with parasites releasing NDPK [36].

Activation of muscarinic and nicotinic receptors *in vitro* [6] and *in situ* [12], respectively, along with the activation of serotonin 5-HT₂ receptors *in vitro* [6] also leads to Ca²⁺ excitability in EGCs (Table 1). Activation of metabotropic glutamate receptor 5 causes a dose-dependent increase in the expression of c-Fos and the phosphorylated form of the mitogen-activated protein kinases 1 and 2 in EGCs [69]. However, expression of α2A-adrenergic and various ionotropic glutamatergic receptors in the gut wall was only detected by immunocytochemistry/immunohistochemistry [34, 68, 69, 93]; functionality and role of these enteric glial receptors is currently unknown. The additional source of Ca²⁺ is provided by Ca²⁺ entry from the extracellular space, for example through functional connexons/hemichannels composed of connexin 43 (Cx43) expressed in EGCs possess. These Cx43 hemichannels not only represent a conduit for ATP release from the EGCs cytosol to the extracellular space, mediating propagation of Ca²⁺ responses between EGCs *in vitro* [97] and *in situ* [64], but also permit Ca²⁺ entry from the extracellular space into the cytosol of EGCs *in situ* [64] (Figure 1B). The pore opening of Cx43 hemichannels is regulated by Ca²⁺ in a biphasic fashion [20]. An initial increase in [Ca²⁺]_i would result in cell depolarization [20] and consequently hemichannel pore opening [18]. As cytosolic Ca²⁺ reaches higher levels, above 0.5 μM, it decreases the pore permeability acting via Ca²⁺/calmodulin complexes, which directly interact with the Cx43 channel [61]. This bidirectional regulation of intracellular Ca²⁺ dynamics through Cx43 hemichannels could reflect the crosstalk between the two distinct modes of enteric gliotransmission, via Cx43 hemichannels and Ca²⁺-dependent exocytosis [40] (Figure 1B, discussed below).

Taken together, EGCs actively respond to neurotransmitters, particularly to ATP and ADP by generating Ca²⁺ signals. Myenteric EGCs exhibit Ca²⁺ responses during physiological intestinal movements, [12]. Accordingly, we next discuss bidirectional EGC to enteric neuron signaling, a heterocellular signaling pathway in the myenteric plexus, along with effects that EGCs, by their Ca²⁺ excitability and releasing ATP, have on the gut motility.

Enteric glia modulate motility of the gut

Enteric neuron-EGC purinergic signaling can be evoked with the neuronal P2X₇ receptor (expressed on enteric neurons but not on EGCs) agonist 2', 3'-O-(4-benzoylbenzoyl)-ATP (BzATP). This agonist elicits robust Ca²⁺ responses in enteric neurons leading to cytosolic ATP release via pannexin-1 hemichannels into the extracellular space [42] (Figure 2A). Subsequently, extracellular ATP gets converted to ADP by eNTPDases. As discussed earlier, ADP activates EGCs P2Y₁ receptors triggering Ca²⁺ signals and release of ATP via glial Cx43 hemichannels which can in turn enhance EGC Ca²⁺ excitability in autocrine/paracrine fashion [64, 97]. ADP-evoked Ca²⁺ responses from EGCs are attenuated by the mimetic peptide 43Gap26, an inhibitor of the Cx43 hemichannels (Figure 2B). The activity of Cx43 connexons in EGCs is not only important for Ca²⁺ excitability but is also required for physiological gut motility [64] (Figure 2C, D). Of note, key components of a reflexive gut motility patterns involve coordinated contraction and relaxation of smooth muscle proximally and distally from the reflex stimuli (for orientation see Figure 5). Interfering with glial Ca²⁺ signaling by inducible EGC-specific ablation of Cx43 gene reduces gut smooth muscle contractions in the *ex vivo* preparations of isolated colons (Figure 2C) and gut motility *in vivo* (Figure 2D). Electrical field stimulation of isolated mice colons that resulted in neuron-evoked muscle responses characterized by colon contractions were greatly reduced in mice with diminished expression of EGCs' Cx43 (Figure 2C). These mice also showed an increase in colonic transit time *in vivo* (Figure 2D). Thus, attenuating glial Ca²⁺ responses by interfering with Cx43 hemichannels availability/activity weakens colonic contractions and slows colonic transit, indicating that EGCs and their Ca²⁺ excitability are necessary for modulation of gut motility.

The next step was to determine whether selective stimulation of EGCs and their Ca²⁺ signaling are sufficient to affect gut motility (Figure 3A). This matter was addressed in experiments using Designer Receptor Exclusively Activated by Designer Drugs (DREDD) technology. Thus, *GFAP*:hM3Dq transgenic mice, where a fragment of human glial fibrillary acidic protein (*GFAP*) promoter drives expression of an engineered Gq-coupled human M3 muscarinic receptor (hM3Dq), were used [63]. Application of clozapine N-oxide (CNO), a synthetic hM3Dq agonist, increased cytosolic Ca²⁺ in EGCs expressing hM3Dq, but not in control wildtype EGCs (Figure 3B). Similarly, CNO caused muscle contractions in isolated mice colons; the duration and magnitude of these contractions were similar to those evoked by electrical field stimulation (Figure 3C). The effect of CNO was blocked by tetrodotoxin indicating that glia-specific effects are mediated by electrical activity of enteric neurons. Additionally *GFAP*:hM3Dq transgenic mice also showed decreased colonic transit time *in vivo*, i.e. increased gut motility, (Figure 3D). Thus, glial Ca²⁺ signals promote colonic contraction and accelerates transit through the gut lumen, indicating that EGCs and their Ca²⁺ dynamics are necessary (as seen in EGCs Cx43 ablation study) and sufficient for modulation of gut motility. The transmitter released through Cx43 hemichannels is ATP, as shown by experiments on EGCs *in vitro* [97] and *in situ* [13]. However, a variety of other transmitters could be additionally released through the Cx43 hemichannel pore (reviewed in [47]).

Purinergic receptor- and/or Cx43 hemichannel- driven EGC Ca^{2+} excitability could lead to exocytotic transmitters release in the ENS, similar to that documented in astrocytes of the CNS (reviewed in [98]). Glia-selective manipulation with Cx43-hemichannel activity (Figure 4A) and Ca^{2+} -dependent exocytosis (Figure 4F) had both distinct (Figure 4B,G; C,H) and similar (Figure 4D,I; E,J) effects on the properties of the *ex vivo* colonic migratory motor complexes [40]. The cross-talk between these two distinct modes of glial signaling is likely to be complex due to variations in spatiotemporal Ca^{2+} dynamics and presence of effector proteins having different sensitivity to Ca^{2+} signals [5]. Consequently, it would be important to assess the composition and characteristics of EGC exocytotic machinery.

The ubiquitous molecular apparatus of exocytosis present in every eukaryotic cell is represented by the ternary soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complexes (reviewed in [49, 74]). These complexes can be composed of synaptobrevin 2, syntaxin and synaptosome-associated protein of 25 kDa or 23 kDa (SNAP25 or SNAP23), the former being present in neurons and the latter in astrocytes of the CNS [75, 88]. The ternary SNARE complex containing neuronal SNAP25B is twice as stable than that containing astrocytic SNAP23A; thus, the composition of the ternary SNARE complex endows the cells with differential ability to control the exocytotic release [65, 88]. Additionally, astrocytic vesicles contain 3–5 times less synaptobrevin 2 than the neuronal ones [82], further affecting the probability of vesicular fusion and gliotransmitter release [99]. Regardless of the exocytosis machinery composition in EGCs, it is imperative to keep in mind that the different physicochemical properties of the cell environment *in situ*, i.e., the gut wall along with the luminal content vs. CNS, can further affect the properties of vesicular release [1].

A perspective on how enteric glia regulate gut motility?

The basic reflex loops that regulate gut motility are formed in the intestinal wall in the neuronal circuits ENS [4, 26]. Recent studies, however, show that EGCs are important components of this reflex: enteric glia are activated during colonic motility [12] while glial activity (Figures 2, 3) [63, 64] and consequent ATP release (by Cx43 hemichannels and Ca^{2+} -dependent exocytosis (Figure 4) [40]) are necessary and sufficient to modulate gut motility. How exactly enteric gliotransmission influences these neuronal circuits is an active area of research and here we present our views.

Conceptually, enteric motility reflex arc is represented by: (i) afferent arm, composed of sensory cells and intrinsic primary afferent neurons (IPANs); (ii) integration center, connected through neuronal synapses in the ENS; and (iii) efferent branch that includes motor neurons and smooth muscle (Figure 5) [55]. Activation of this reflex arc by distension of the gut wall and/or intraluminal chemicals leads to coordinated contraction of mouth muscle proximally and relaxation distally to the stimuli (Figure 5), resulting in gut motility that propagates intraluminal contents. Two populations of IPANs reside in either myenteric or submucosal plexus allowing the coordination between the gut motility and fluid secretion; and allowing sufficient lubrication for fecal matter propulsion. Indeed, colonic motility activates Ca^{2+} transients in submucosal enteric neurons [73] and distension evoked secretory

responses [94]; recently, EGCs activity was found to acutely regulate secretomotor function in distal mouse colon [39].

Enterochromaffin cells, a subtype of enteroendocrine cells residing in the intestinal epithelium, are considered principal sensory cells that, in response to chemical/mechanical stimulation, secrete serotonin and activate mucosal afferents of IPANs [55]. Human mucosa is abundant in EGCs [60] and mucosal glia make connections to enteroendocrine cells [9] in close proximity to synapses between the enteroendocrine cells and IPANs [10]. Since EGCs are responsive to serotonin (Table 1) [6, 53] and release ATP [97] mucosal glial cells could modulate signaling between the principal sensory cell and IPANs. Aside ATP, EGCs could provide for additional signaling molecules, such as bradykinin, endothelin, lysophosphatidic acid, sphingosine-1-phosphate [59, 66, 79, 80, 96]. Furthermore, EGCs could get excited not only by bioactive molecules but by mechanical stimulation [59], which elicits Ca^{2+} responses in EGC. Together, EGCs could serve as sensory cells that contribute to activation of the enteric motility reflex (Figure 5A). Notably, astrocytes of the brainstem chemosensitive areas are primary sensory cells that control breathing reflex [37]. The ability of EGCs to sense their environment could also contribute to various integrative processes (Figure 5B). Within the myenteric plexus EGCs are closely positioned to enteric neurons (Figure 1A), similarly to astrocyte and neurons of the brain forming the multipartite synapse and astroglial cradle [2, 89]. Therefore, EGCs, through releasing various factors, may modulate synaptic connectivity and synaptic transmission in the myenteric plexus. Similar role was found for astrocytes of the hippocampus controlling synaptic networks involved in learning and memory [76].

Finally, EGCs are also positioned between the smooth muscle cells where they could affect effector arm of the motility reflex (Figure 5C). Motoneurons, by releasing neurotransmitters such as acetylcholine or ATP could also stimulate EGCs (Table 1) consequently causing release of glial signaling molecules that could modulate the excitability of motoneurons and smooth muscle cells.

In conclusion, activity of EGCs have stimulatory effect on gut motility (Figures 2, 3, 4) through modulating gut motility reflex at multiple levels (Figure 5). Exactly how and where this modulation occurs are to be clarified in by future studies. Different composition of receptors and receptor subtypes which can be expressed by EGCs may render cell-specific responses to the same stimuli. For instance, selective agonists of P2Y_1 , P2Y_2 , P2Y_4 and P2Y_6 agonists could be used to identify the nucleotide receptors in EGCs. Likewise, EGCs could generate distinct Ca^{2+} signals that can differentially regulate their functional Responses.

Translational potential of enteric glia in GI motility disorders

As already mentioned, the selective knockout of Cx43 in EGCs reduced ATP secretion in mouse myenteric plexus *in situ* and suppressed the velocity of mouse colonic transit *in vivo* [64]. The same study also showed that normal aging causes deregulation of Cx43 expression in the gut [64]. Therefore, the change in EGS signaling could be an underlying cause of impaired intestinal transit due to normal aging as well as in idiopathic constipation.

Functional metabolic restraining of EGCs by gliotoxin fluorocitrate affected GI motility without signs of inflammation or changes in number and morphology of enteric neurons and EGCs [67]. Moreover, selective genetic ablation of EGCs led to fulminant jejunoileitis [14]. Thus, EGCs are essential for maintaining normal bowel function and for safeguarding the homeostasis in the intestines. Conceptually, EGCs are emerging as important regulators in the pathogenesis of inflammatory GI disorders (reviewed in [38, 72, 81]) and some of these mechanisms rely on the active enteric glia signaling. Inflammatory bowel diseases lead to gut dysfunction due to alterations of the ENS and loss of enteric neurons [62]. The neuron death was claimed to be mediated through the pannexin-1 pathway and downstream activation of inflammasome [42]. Inflammation also results in reactive remodeling of EGCs, characterized by an increase in GFAP expression and more “stellate” cell appearance [92], similar to that seen in reactive astrocytes [22]. Astrogliotic response includes actin cytoskeleton reorganization [46] that can enhance Ca^{2+} signaling systems via the cytoskeletal interactions with Cx43 channels [19]. It is possible that similar mechanism could facilitate EGC-mediated inflammation-induced neuronal death, which involves purinergic pathways and Cx43 [13]. Of note, neuronal death could also be the product of EGC interaction with the immune system (reviewed in [15]).

Expression of mGluR5 in EGCs is differentially altered in two models of colitis [69]. In the guinea pig trinitrobenzene sulfonic acid model of colitis, mGluR5 are more diffusely distributed over the colonic myenteric plexus. In the IL-10 gene knock-out mouse model of colitis, mGluR5 expression in EGCs was significantly reduced in the colonic myenteric plexus. Thus, expression of mGluR5 in EGCs and glutamatergic transmission in the ENS may represent targets for therapeutic intervention in various forms of colitis. Dysmotility is also caused by the sterile injury-induced inflammation. Postoperative ileus, a common complication of the abdominal surgery, is mediated by the Il-1 receptor1, which is primarily (but not exclusively) expressed by EGCs [83]. Interfering with the IL-1 signaling is therefore a potential treatment option for the postoperative ileus.

Lastly, EGCs are also involved in pathogenesis of infectious inflammation. Lipopolysaccharide (LPS), a component of the gram-negative bacteria cell wall, stimulates glial toll-like receptors 4 (TLR4) linked to downstream production of nitric oxide (NO) [86]. Glial release of NO release helps to fight the infection [78], but also contributes to the inflammatory process and neuronal cell death [13]. Furthermore, *in vitro* LPS application induces reactive phenotype in human EGCs by changing their biochemistry and affecting purinergic signaling, disrupting Ca^{2+} signaling and increasing ATP release by 5-folds [59]. This change in glial signaling could affect gut motility and needs to be tested in EGC *in situ*. On the other hand, palmitoylethanolamide (PEA) application in animal models of colitis is activates peroxisome proliferator-activated receptor α (PPAR- α)-dependent inhibition of NF- κ B, consequently reducing the TLR4 dependent NO synthesis and inflammation [23]. These mechanism fo action and low toxicity make PEA suitable for potential colitis treatment.

Conclusions

The intent of this mini-review was to provide a summary of the recently discovered modulatory functions of EGCs in the gut motility. The new findings promote EGCs from their supporting cast status into a realm of an active cellular component of the ENS. For now, Cx43 hemichannels along with purinergic signaling system and underlying Ca^{2+} excitability have been identified as components mediating EGCs contribution to gut (patho)physiology. These molecular entities represent possible targets for medical intervention. For instance, boosting Cx43 hemichannel availability/activity in EGCs may be beneficial in bringing a relief to people suffering from age-related constipation. It should be noted, however, that Cx43 hemichannel might also be a conduit for release of metabolites and neurotransmitter precursors, such as lactate and glutamine, which can all affect normal neuronal functioning [35]. Furthermore, it is possible that beside nucleotides, other transmitters and modulators can be involved in enteric transmission and gut functions. Enteric glia sense their environment and receive inputs from neurons and perhaps other non-neuronal cells, such as smooth muscle, epithelial and immune cells, in the gut wall by a wide array of signaling molecules, including nucleotides, nucleosides, lipids, amino acids and/or peptides, opening new venues for exploring neurochemistry of the GI tract in attempt to understand its physiology and curatively intervene in pathophysiology.

Acknowledgments

The authors work has been supported by the Civitan International Emerging Scholar Award at University of Alabama at Birmingham (to VG), the National Institutes of Health (The Eunice Kennedy Shriver National Institute of Child Health and Human Development award HD078678 to VP), and by the Research Agency of Slovenia (grants # P3 0310, J3 6790 and J3 7605 to RZ).

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Highlights

- ▶ The enteric nervous system (ENS) is the largest assembly of neurons and glia outside the central nervous system
- ▶ The ENS resides within the wall of the digestive tract and regulates local gut reflexes involved in gastrointestinal motility, blood flow and fluid transport
- ▶ Enteric glial cells are necessary and sufficient to modulate neuronal reflex circuits controlling gut motility
- ▶ Enteric glia respond to various neurotransmitters via increase in intracellular calcium and release of neuroactive substances, such as ATP through Cx43 hemichannels or Ca²⁺-dependent exocytosis
- ▶ Enteric glial cells are actively involved in gut motility disorders and regulation of inflammation

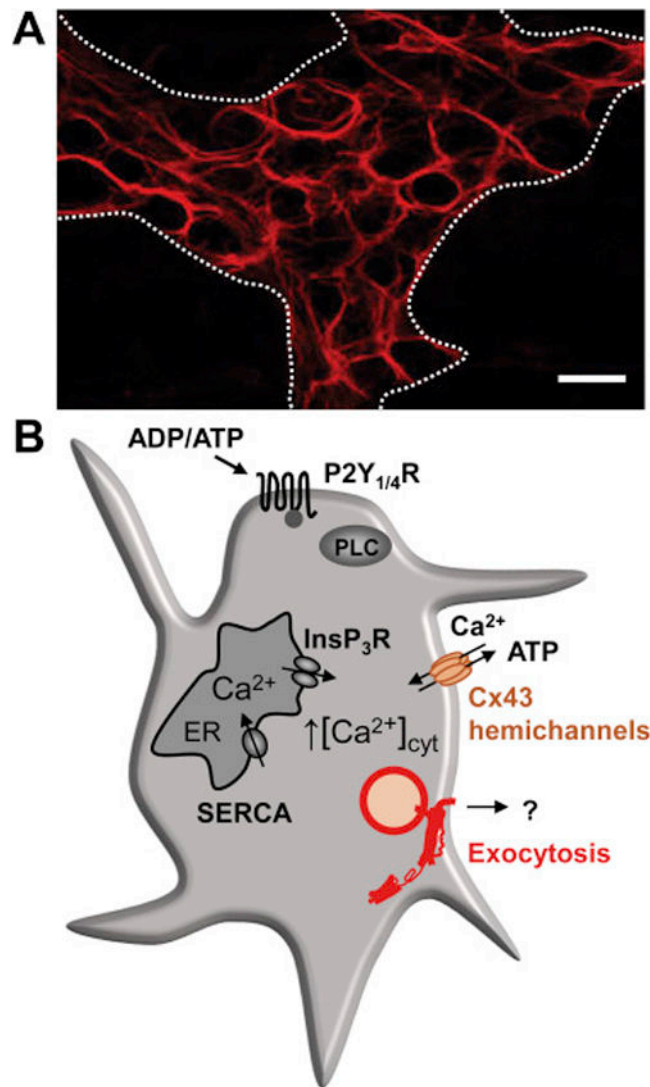


Figure 1. Enteric glial cells (EGCs) in the enteric nervous system (ENS)

A. Expression of the EGC marker, glial fibrillary acidic protein (GFAP), in the myenteric plexus of a mouse colon. Note the close proximity between the EGCs and enteric neurons: GFAP immunoreactivity (-ir) spans across the myenteric ganglion (delineated by the dotted line) while areas within the ganglion that lack the GFAP-ir are occupied by the enteric neurons. Scale bar, 10 μm . Original unpublished image.

B. EGCs exhibit excitability based on the cytosolic Ca²⁺ fluctuations. Preferential binding of natural agonists, ADP or ATP, to G-protein coupled P2Y₁ or P2Y₄ receptors, respectively, activates phospholipase C (PLC)-mediated production of inositol 1,4,5 trisphosphate (InsP₃). This second messenger induces release of Ca²⁺ to the cytosol from the endoplasmic reticulum (ER) upon binding to its InsP₃ receptors (InsP₃R). Increase in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) induces ATP release (and perhaps other gliotransmitters) through Cx43 hemichannels located on the plasma membrane; these channels also represent a conduit for the Ca²⁺ entry from the extracellular space. Increased [Ca²⁺]_i also triggers

gliotransmission via vesicular exocytosis. Store-specific Ca^{2+} -ATPases (SERCA) pump Ca^{2+} to (re)fill the ER. Based on original research on enteric glia [40, 53, 64, 97].

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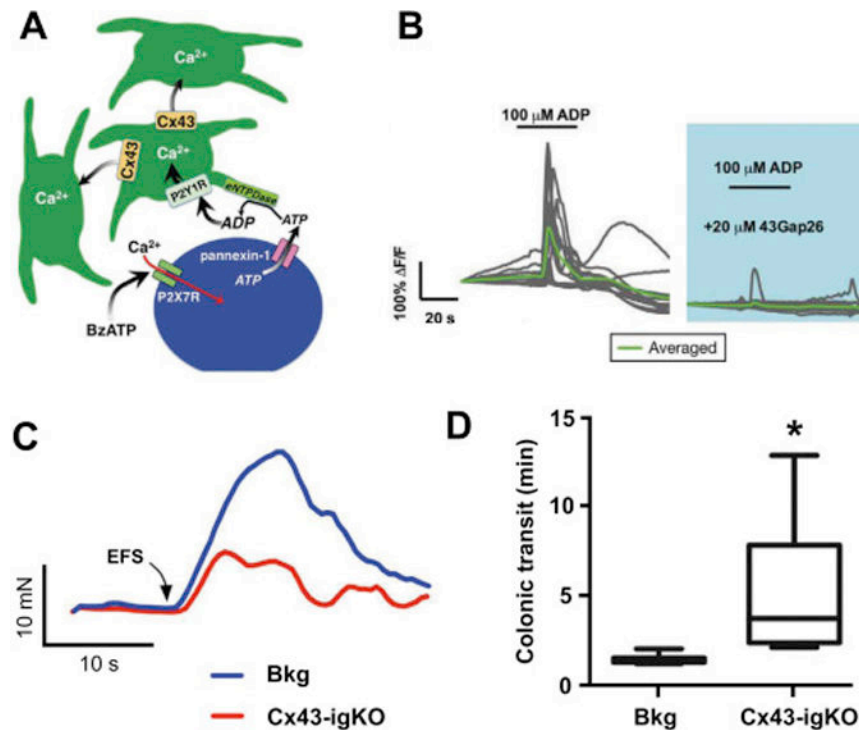


Figure 2. Genetic deletion of Cx43 in EGCs causes inhibition of Ca²⁺ signaling in EGCs and reduction of the gut motor reflexes

A. Model of purine-evoked Ca²⁺ responses through the EGC network; see text for details.

B. ADP-evoked Ca²⁺ responses from wild-type myenteric *in situ* EGCs are blocked by the connexin 43 (Cx43) mimetic peptide 43Gap26, an inhibitor of the Cx43 hemichannels.

C. Electrical field stimulation (EFS)-induced smooth gut muscle contractions are reduced in tamoxifen-induced glia-specific knock out of Cx43 mice (Cx43-igKO) and the tamoxifen treated background (Bkg) strains. Muscle relaxations were also affected (see original publication).

D. Selective genetic deletion of Cx43 in EGCs increases colonic transit time, which reports on the reduction in distal colon motility *in vivo*. Obtained with permission for minor reformatting from [64].

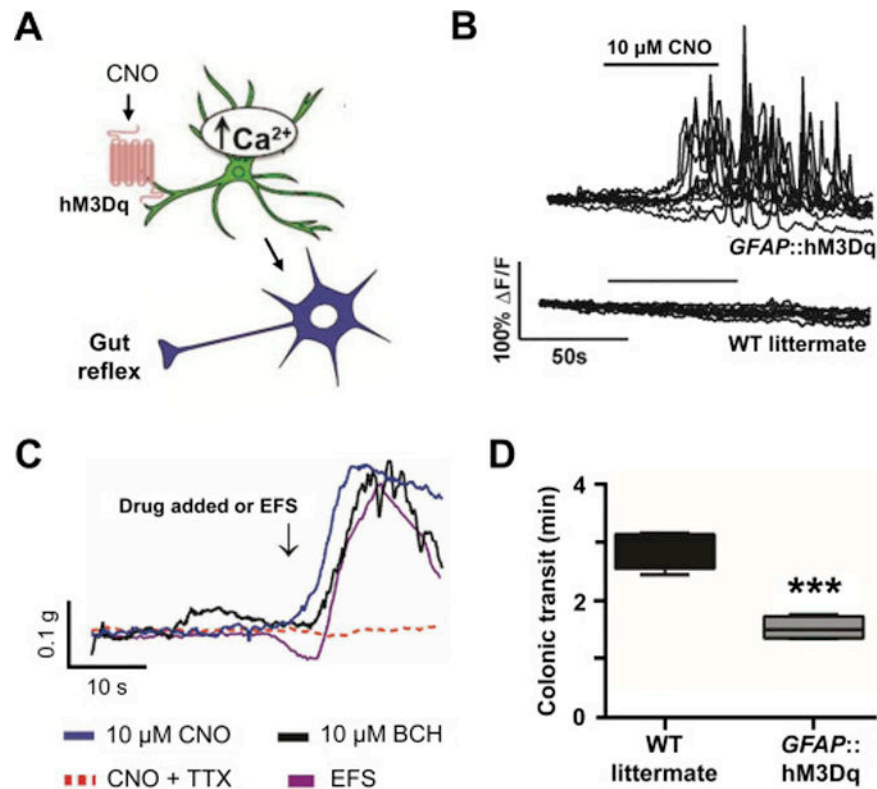


Figure 3. Activation of EGC Ca^{2+} signaling results in stimulation of gut motor reflexes
A. Experimental design using *GFAP::hM3Dq* transgenic mice, where a fragment of human glial fibrillary acidic protein (*GFAP*) promoter drives expression of an engineered Gq-coupled human M3 muscarinic receptor (hM3Dq). Activation of hM3Dq elicited by the synthetic agonist clozapine N-oxide (CNO) leads to cytosolic Ca^{2+} increases in EGCs.
B. CNO-evoked cytosolic Ca^{2+} increases are seen only in myenteric *in situ* EGCs expressing hM3Dq, but not in control cells from wild type (WT) littermate mice.
C. CNO evokes contractions of smooth muscles in colons isolated from *GFAP::hM3Dq* mice. The magnitude and duration of this response is similar to those obtained from direct activation of smooth muscle or enteric neurons using bethanechol (BCH) or EFS, respectively. EGC-specific CNO effect on smooth muscle is mediated via enteric neurons since it can be blocked by tetrodotoxin (TTX). Of note, colonic muscle relaxations and colonic migrating motor complexes (CMMCs) were also affected (see original publication).
D. Selective activation of EGC Ca^{2+} signaling reduces colonic transit time, i.e. enhances *in vivo* motility of the distal colon. Obtained with minor reformatting from [63] (open access article).

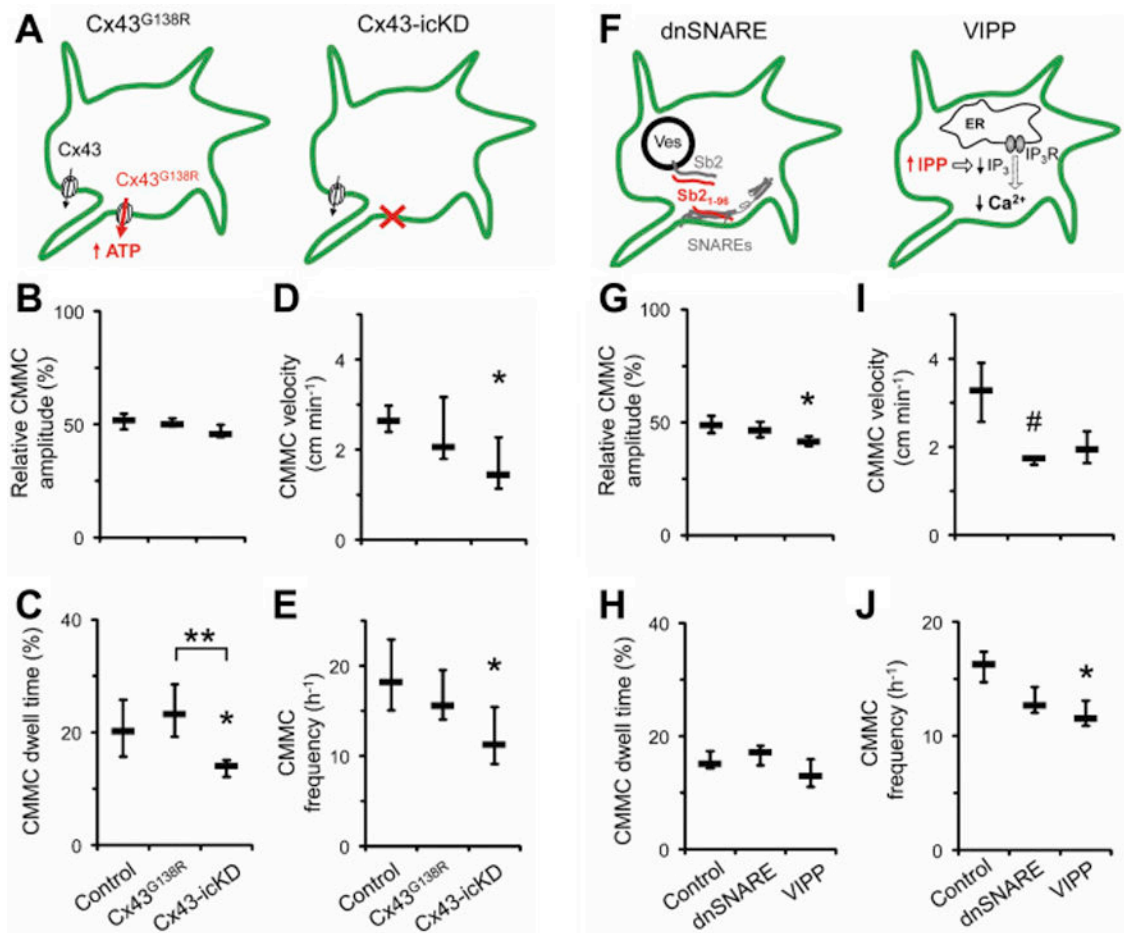


Figure 4. Two modes of enteric gliotransmission affect gut motility. Inducible and glia-selective transgenic mouse strains were used to modify activity of EGCs' Cx43 hemichannels (A) or Ca²⁺-dependent exocytosis (F) and the effect was by video recording of *ex vivo* colonic migratory motor complexes (CMMCs) and offline analysis of their properties (B–E and G–J, respectively). See original work for details. Note the differences and similarities between the modes of gliotransmission. Obtained with permission with minor reformatting from [40].

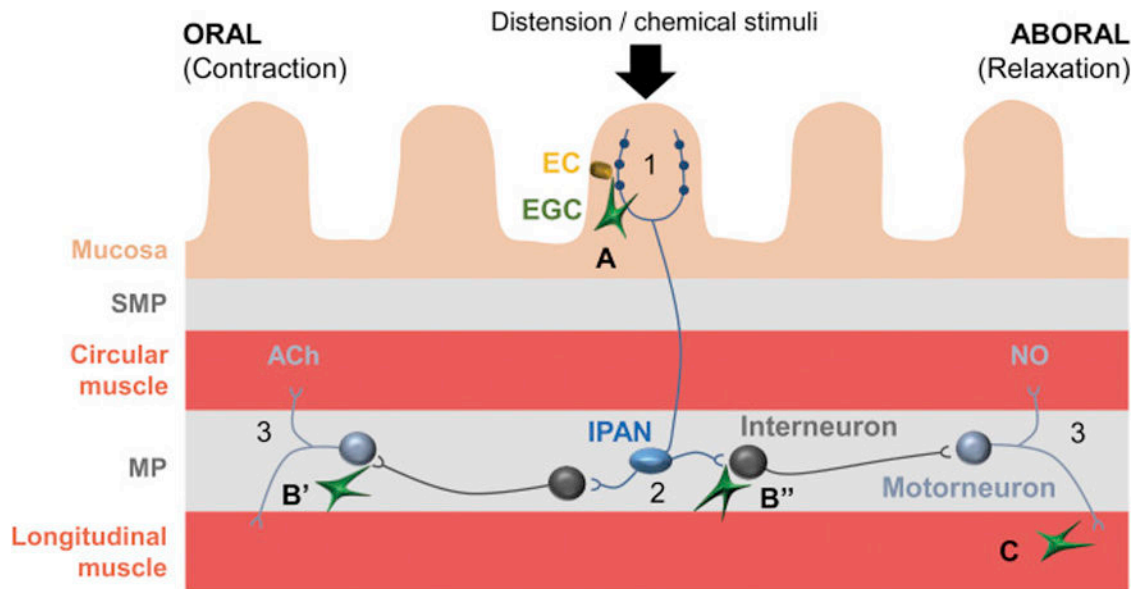


Figure 5.

Potential sites of active EGC modulation of the enteric peristaltic reflex. The peristaltic reflex is described in detail elsewhere (reviewed in [55]) and, briefly, consists of: 1) mechanical or chemical stimulation of the intrinsic primary afferent neurons (IPANs) residing in both plexi [here shown only in myenteric plexus (MP) for clarity]; 2) IPANs activate interneurons that project both proximally and distally from the stimuli; 3) proximal (ascending) interneurons activate excitatory motoneurons that cause smooth muscle contraction by releasing acetylcholine (ACh) and neuropeptides (not shown) while distal (descending) interneurons produce relaxation by activating inhibitory motoneurons that release nitric oxide (NO), purines and other inhibitory molecules (not shown). EGC are present in all layers of the gut wall and perhaps interact with the circuit at several levels: **A**) at the afferent limb by modulation EC-IPAN signaling; **B**) integration of information in the MP by affecting the IPAN-interneuron (**B'**) and interneuron-motoneurons (**B''**) synaptic transmission; **C**) modulation of smooth muscle excitability by the direct interaction with the smooth muscle cells. SMP, submucosal plexus. This schematic was obtained with permission for minor reformatting from [38].

Table 1

Transmitter receptors on enteric glial cells. Receptor types/subunits, if determined, are parenthetically indicated. Alternatively, parentheses disclose agonists used to assess the functionality of receptors. Sensitivity of P2Y_{1/4} receptors to relevant natural agonists is shown parenthetically. Detection methods for the receptor expression (ICC and IHC) and/or functionality (cytosolic Ca²⁺ increase imaged using fluorescent indicator dyes fura-2/fluo-4, or a dose-dependent increase in the expression of specific proteins) are indicated.

Transmitter	Receptor(subunit or agonist)	Method	Refs.
Acetylcholine	Muscarinic	fluo-4	[6]
	Nicotinic ($\alpha 3$)	fluo-4	[12]
Glutamate	mGluR5 (CHPG)	IHC/cFos & pMAPK1/2 expression	[69]
	AMPA (GluA1 and GluA3) *		
	KA (GluK1) *	ICC	[93]
	NMDA (GluN2A, GluN2B) *		
	NMDA (GluN1) *	IHC	[34]
Catecholamines	Adrenergic ($\alpha 2A$) *	IHC	[68]
Nucleoside	A _{2B} *	IHC	[90] [16]
Nucleotides	P2Y ₁ (ADP>ATP)	fluo-4	[64] [43] [42]
	P2Y ₄ (UTP ATP)	IHC/fluo-4 IHC ICC/fura-2	[43] [87] [53]
Serotonin	Not determined	fura-2	[53]
	5-HT ₂	fluo-4	[6]

* Functionality of these receptors has not been demonstrated.

Abbreviations: AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionate; ADP, adenosine 5' diphosphate, ATP, adenosine triphosphate; CHPG, RS-2-chloro-5-hydroxyphenylglycine; ICC, immunocytochemistry; IHC, immunohistochemistry; KA, kainate; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; pMAPK1/2, phosphorylated form of the mitogen-activated protein kinases 1 and 2. Table is abbreviated and updated from a more comprehensive review on enteric glia [38].