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TOPICAL REVIEW

Enteric glia: the most alimentary of all glia

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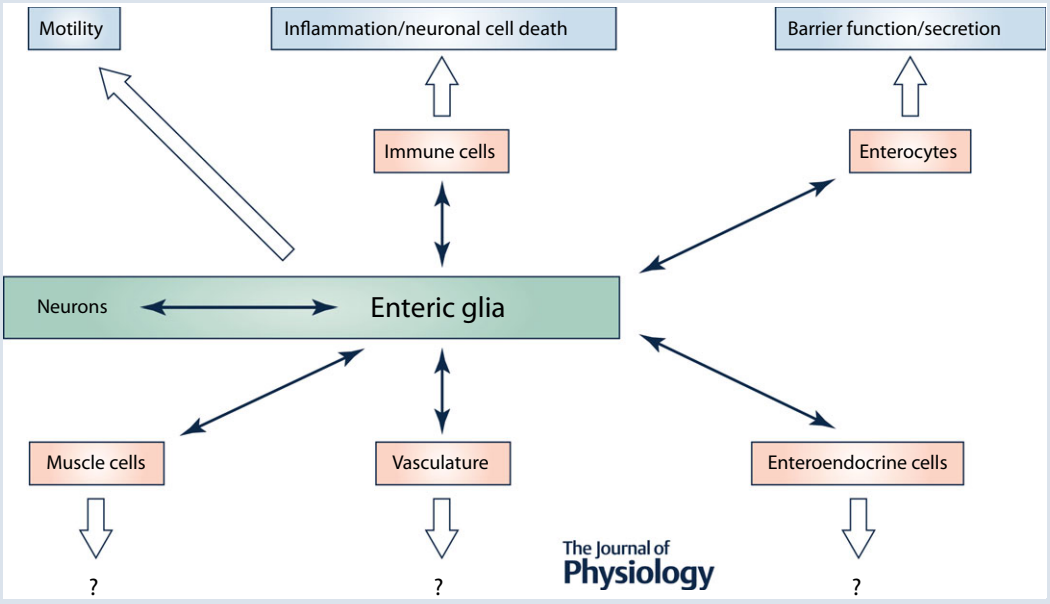

Abstract Glia (from Greek *γλοία* meaning 'glue') pertains to non-neuronal cells in the central (CNS) and peripheral nervous system (PNS) that nourish neurons and maintain homeostasis. In addition, glia are now increasingly appreciated as active regulators of numerous physiological processes initially considered exclusively under neuronal regulation. For instance, enteric glia, a collection of glial cells residing within the walls of the intestinal tract, regulate intestinal motility, a well-characterized reflex controlled by enteric neurons. Enteric glia also interact with various non-neuronal cell types in the gut wall such as enterocytes, enteroendocrine and immune cells and are therefore emerging as important local regulators of diverse gut functions. The intricate molecular mechanisms that govern glia-mediated regulation are beginning to be discovered, but much remains unknown about the functions of enteric glia in health and disease. Here we present a current view of the enteric glia and their regulatory roles in gastrointestinal (GI) (patho)physiology; from GI motility and epithelial barrier function to enteric neuroinflammation.

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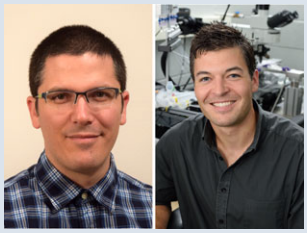

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Abstract figure legend Enteric glia are local regulators of gut (patho)physiological processes. Enteric glial cells interact with neurons within the enteric nervous system (green box) and with many other cells within the wall of the alimentary canal. Recent studies demonstrated regulatory roles of enteric glia in gastrointestinal motility, inflammation/neuronal cell death and epithelial secretion/barrier function via active interplay with neurons, immune cells and enterocytes, respectively. Physiological effects of direct interplay between the enteric glia and muscle, vasculature and enteroendocrine cells are still unknown (?).

Introduction

At its heart, the discipline of physiology aims to understand processes that govern homeostasis (Michael *et al.* 2009). In this regard, glia can be considered the seat of nervous system physiology. Indeed, we are now well aware of many of the essential roles glia play in the maintenance of homeostasis within the central nervous system (CNS) and some of the potentially catastrophic effects if these functions are perturbed. Astroglia, in particular, are essential for the regulation of neuronal microenvironments and neural network functions (reviewed in Parpura *et al.* 2012). Similar types of glial cells are associated with neurons in peripheral neural networks, but the roles of these glial cells in the regulation of homeostasis outside the brain are much less defined.

The largest collection of glia outside the brain and spinal cord is housed within the walls of the intestines in the enteric nervous system (ENS). The ENS is the largest division of the autonomic nervous system (ANS) and consists of approximately 100 million enteric neurons that are surrounded by 1–7 times as many glial cells depending on the species (reviewed in Gulbransen, 2014). Enteric neurons and glia are housed in two major ganglionated plexuses in the gut wall (Fig. 1) and the integrative circuitry within these networks is sufficient to control reflex behaviours of the intestine such as peristalsis (Bayliss & Starling, 1900), fluid exchange across the mucosal surface and the regulation of local blood flow.

The basic neural circuitry underlying intestinal reflexes is now relatively well understood and many of the key neurotransmitters are known (Furness, 2012). Yet, new observations suggest that this level of understanding of ENS function is grossly inadequate to understand the control of intestinal reflexes. Indeed, a growing body of work is currently modifying old neurocentric models to include an unexpected layer of complexity provided by glial cells. The picture that is emerging is one where glial cells have a strong influence on the physiological control of gut functions at multiple levels (see recent reviews by Neunlist *et al.* 2014; Coelho-Aguiar Jde *et al.* 2015; Sharkey, 2015). This is an intriguing concept but many important questions remain unanswered about basic glial functions in the gut. In this review, we focus on our current understanding of how enteric glia participate in the regulation of intestinal homeostasis and discuss some of the important unanswered questions in the field. We examine recent studies that probe enteric glial cell identity and discuss novel findings that demonstrate active roles of enteric glia in the regulation of gut motility reflexes, barrier function and inflammation.

What are enteric glia?

Enteric glia are a large population of non-myelinating peripheral glial cells that derive from neural crest precursors that colonize the intestinal tract between embryonic (E) days 9 and 13.5 in mice (Rothman *et al.* 1986; Kapur *et al.* 1992). Precursor cells begin to commit to a glial fate near E11.5 and cells expressing markers of terminally differentiated glia such as the calcium-binding protein S100β and the intermediate filament glial fibrillary acidic protein (GFAP) are present by E14.5–16 (Fig. 2). Mature enteric glial cells display a strong morphological resemblance to astrocytes (Reichenbach *et al.* 1992) and express similar molecular markers such as the astrocyte-associated determinant of GFAP (Jessen *et al.* 1983), vimentin (Jessen & Mirsky, 1983), connexin-43 (McClain *et al.* 2014) and S100β (Ferri *et al.* 1982). No unique enteric glial marker has been identified, but the unique compilation of characteristics displayed by enteric glia specifically set this class of glia apart from other classes of glia (Fig. 3). Indeed, not all astrocytic properties can be generalized to enteric glia because the two cell types are fundamentally different. For example, enteric glia require neuregulin signalling through the ErbB3 receptor for development while astrocytes do not (Riethmacher *et al.* 1997). Likewise, enteric glia lack expression of some key astrocytic proteins such as aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (Boesmans *et al.* 2014) and express non-astrocytic molecules like Sox10 (Young *et al.* 2002), a transcription factor more common in oligodendrocytes. Indeed, the transcriptional profile of enteric glia shows significant overlap with oligodendrocytes, astrocytes and even neurons of the CNS (Fig. 3*A*; Rao *et al.* 2015). This finding may partly illuminate the remarkable plasticity of enteric glial cells. For example, enteric glia are capable of forming enteric

neurons *in vitro* (Joseph *et al.* 2011) or performing the functions of oligodendrocytes and astrocytes when transplanted into the CNS (Jiang *et al.* 2003, 2005). However, enteric glia are mainly restricted to a glial fate in their native intestinal environment (Joseph *et al.* 2011) and only form neurons under very rare circumstances (Laranjeira *et al.* 2011). These studies clearly show that the fate of the enteric glia is heavily influenced by external signals. Yet the specific conditions and factors that drive the heterogeneity of enteric glia are still poorly understood. A deeper understanding of these transformative factors holds great promise for the development of novel therapies for many diseases by harnessing the plastic capabilities of enteric glia.

Beyond inter-glial expression differences, new data indicate significant intra-glial variability in the expression levels of key markers such as GFAP, S100β and Sox10 (Fig. 3*B*; Boesmans *et al.* 2015). The significance of this variability within enteric glia is not currently understood and at this point, there is no consensus on what the 'best' enteric glial cell marker might be. In light of these discoveries, future studies should consider the following points. (i) Current markers may not be pan-enteric glial at any given time. Sox10 and proteolipid protein 1 (PLP1) are the closest pan-enteric glia markers and even these are not entirely reliable (Boesmans *et al.* 2015; Rao *et al.* 2015). (ii) Current 'enteric glial-specific' markers are not confined to enteric glia. Sox10 and GFAP are widely expressed by other glia, as mentioned above, and other non-glial cells such as melanocytes (Potterf *et al.* 2001) and hepatic stellate cells (Gard *et al.* 1985), respectively. Likewise, S100β is expressed in subpopulations of neurons in the CNS (Vives *et al.* 2003). This is a significant problem because it confounds the interpretation of experiments that aim to understand the integrative functions of enteric glia by selectively modulating their functions *in vivo*. This problem is made even more challenging by the recent discovery of intramucosal neuroglial cells that express both neuronal and glial markers (Badizadegan *et al.* 2014). (iii) The expression of various markers by enteric glia is a dynamic process that reflects many changes in glial maturity and phenotype. For example, expression of GFAP appears to reflect both enteric glial cell maturity and glial cell reactivity as a response to inflammatory stimuli (von Boyen *et al.* 2004). Clearly, understanding these processes will be an important aspect when both planning and interpreting future experiments.

Enteric glial cell numbers and their morpho-functional characteristics also vary widely depending on their location in the GI tract, age, sex and species (Table 1). This is an important consideration while comparing findings from multiple studies and interpolating results from animal models to human physiology. For instance, the glia index (glia-to-neuron ratio) in the human intestine is approximately sevenfold greater than in the mouse intestine (Gabella & Trigg, 1984; Hoff *et al.* 2008). This potentially indicates a more prominent role of glia in the human intestine than in rodents, but this concept

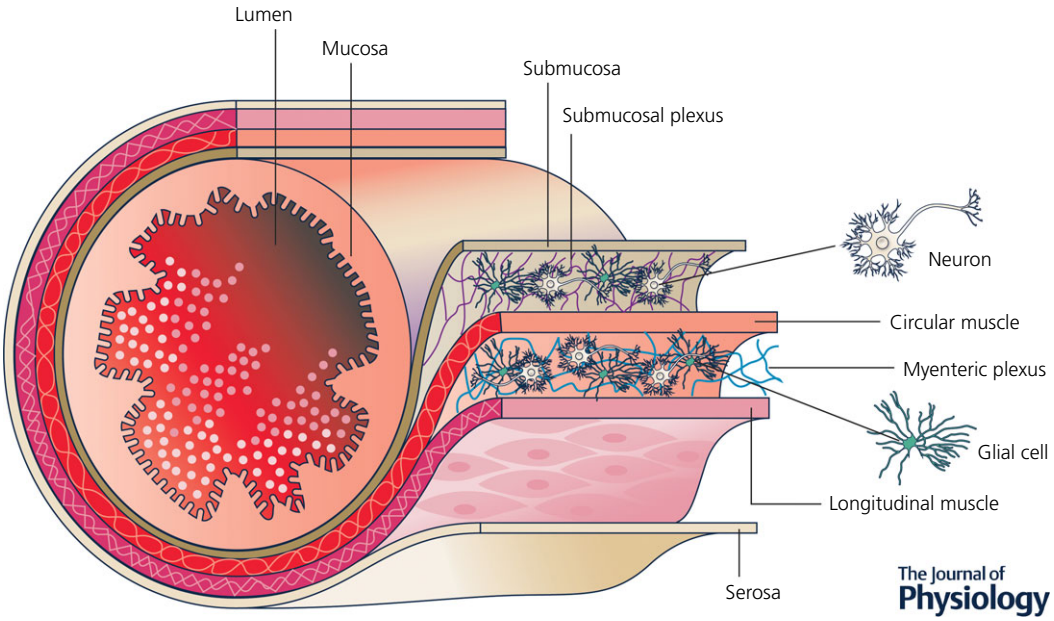


Figure 1. Schematic depiction of the intestine showing the general arrangement of the enteric nervous system in the gut wall
Enteric neurons and glia are housed within the submucosal and myenteric plexuses. Neural programmes in the submucosal plexus regulate fluid exchange across the intestinal mucosa and neural programmes in the myenteric plexus coordinate the contractile activity of the intestine. Image courtesy of David E. Fried.

is still theoretical and direct experimental confirmation is still lacking. Additionally, enteric glia may exhibit differences in their expression of particular receptor subtypes (Table 2) and signal transduction cascades in different species and regions of the gut. Some signal pathways seem well conserved, but it is unknown how similar human and murine enteric glia actually are. Future efforts, therefore, should be directed towards closing the gap in knowledge between human enteric glia and those in experimental animals.

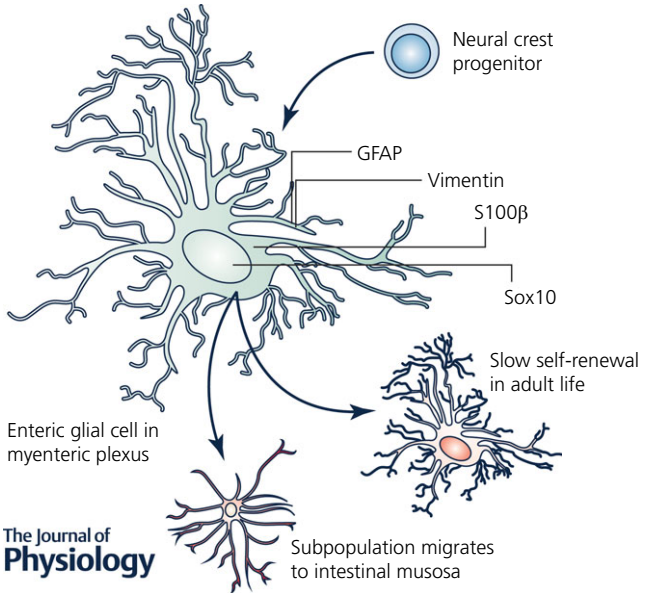


Figure 2. Enteric glial cells derive from neural crest precursors and mature into neuroglia in the enteric nervous system
Enteric glia within the myenteric plexus are slowly replaced under physiological conditions (Joseph *et al.* 2011; Laranjeira *et al.* 2011) and are responsible for generating glia that migrate to the intestinal mucosa (Kabouridis *et al.* 2015).

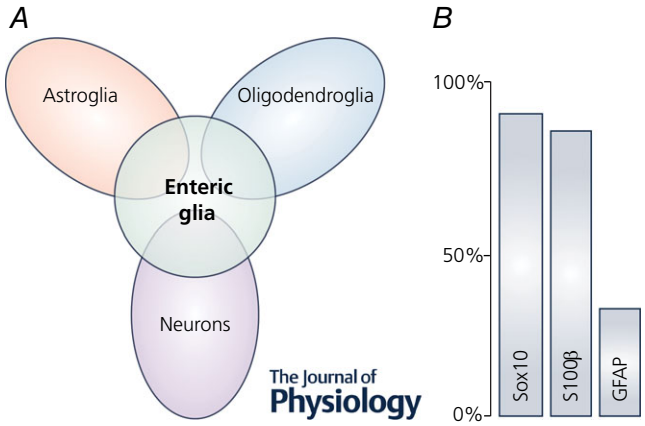


Figure 3. Gene expression in enteric glia
A, transcriptional profile of enteric glia compared with the profiles of neurons and glia from the CNS (Rao *et al.* 2015). Not drawn to scale. *B*, expression of common markers for enteric glia estimated from Boesmans *et al.* (2015); co-localization among the glial markers omitted for clarity.

Neuron–glia crosstalk and the regulation of gut motility

Until recently, neurons were considered the only active cells in the ENS. Consequently, the major part of our understanding of the enteric reflexes that underlie gut motor activity is extremely neurocentric. However, mounting evidence over the past decade shows that enteric glia play an active role in enteric neural circuits that control motility (Gulbransen & Sharkey, 2009; Broadhead *et al.* 2012) and that manipulating enteric glia can have profound effects on gut functions (Aube *et al.* 2006; Nasser *et al.* 2006*a*). We are now well aware of the fact that glia are also excitable cells. Like astrocytes, enteric glial excitability is mainly encoded by transient elevations of intracellular calcium concentration ($[Ca^{2+}]_i$) and a number of studies have shown that glial activity is recruited by neurotransmitters/neuromodulators released during synaptic communication (Table 2). Importantly, Broadhead *et al.* (2012) demonstrated that these glial $[Ca^{2+}]_i$ transients are entrained with endogenous neuronal reflexes that underlie peristalsis (Broadhead *et al.* 2012). Yet the significance of neural recruitment of enteric glial activity has remained enigmatic. Enteric glia are clearly capable of 'listening' to enteric neurons (Table 2), but if and how they 'talk back' is only beginning to come to light. Two studies from our own laboratory provide the first hints that the activation of enteric glia is an important modulator of enteric reflexes. First, we found that agonist-evoked $[Ca^{2+}]_i$ responses in enteric glia lead to the opening of glial connexin-43 (Cx43) hemichannels (Fig. 4) and that the selective ablation of Cx43 in GFAP-expressing enteric glia limits the propagation of $[Ca^{2+}]_i$ responses through the glial network (McClain *et al.* 2014). Importantly, we found that impairing the activity of glial cells *in vivo* disrupts the neural control of gut motility and produces constipation in mice (Fig. 5*A*–*C*). Based on these data, it is tempting to hypothesize that the mechanisms enacted by $[Ca^{2+}]_i$ responses in enteric glia function to regulate the activity of enteric neural networks. We recently tested this hypothesis using *GFAP::hM3Dq* transgenic 'DREADD' (designer receptors exclusively activated by designer drugs) mice to selectively trigger Gq-G protein-coupled receptor (GPCR)-dependent $[Ca^{2+}]_i$ responses in GFAP-expressing enteric glia (McClain *et al.* 2015). Our results show that enteric glia exert a surprisingly robust and selective influence on neuronal circuits in the gut. Perhaps the most surprising finding in this study was that the activation of glial $[Ca^{2+}]_i$ responses alone was sufficient to drive intestinal contractility (Fig 5*D*–*F*). Glial-driven contractions were entirely tetrodotoxin-sensitive so presumably the effects of

Table 1. Variability in numbers and morpho-functional characteristics of enteric glial cells

Variables	Description			References
Species	↑ Glia index in larger species (1 in mouse and 7 in human MP)			(Gabella & Trigg, 1984; Hoff *et al.* 2008)
Sex	↑ Glia index in males (human ileum SMP)			(Hoff *et al.* 2008)
	↑ GFAP expression in females (mouse MP)			Unpublished*
Age	↓ Number of Sox10 expressing cells with age (mouse MP)			(Stenkamp-Strahm *et al.* 2013)
	↑ Glial density with age (human ileum/ sigmoid colon MP)			(Hoff *et al.* 2008)
	↑ transcription of Cx43			(McClain *et al.* 2014)
Location along the gut length	↑ Glial density in ileum (guinea pig MP, interganglionc area)			(Hoff *et al.* 2008)
Location within the gut wall	↑ Glia index in MP than in SMP (mouse, guinea pig, rabbit, sheep, human)			(Gabella & Trigg, 1984; Hoff *et al.* 2008)
	Four types	Within SMP/MP	I – protoplasmic (intraganglionic)	(Gulbransen & Sharkey, 2012; Boesmans *et al.* 2015)
			II – fibrous (interganglionic)	
		Extraganglionic	III_{MUCOSA} – mucosal	
			$III_{SMP/MP}$ – at the level of ganglia	
			IV – intramuscular	

Glia index, number of glial cells per neuron. MP, myenteric plexus. SMP, submucosal plexus. *Courtesy of Ninotchska Del Valle Dorta (Gulbransen lab).

glial activation were mediated through direct actions on neurons. Importantly, glial cell activation had no effect on neurogenic relaxations in the intestine. This is important because it suggests that gliotransmission in the intestine is highly specific.

The studies described above provide strong support for the notion that enteric glia actively participate in regulation of gut motility (Fig. 5). However, a great deal of work is still needed to dissect the exact mechanisms involved. One obvious question at this point is how glia excite neurons. Is this via gliotransmitter release? If so, what is the identity of the gliotransmitter(s), what are the release mechanisms and how does the transmitter exert a selective effect on excitatory circuits? Some data from our lab (Brown *et al.* 2015) and the work of others (Zhang *et al.* 2003) suggest that ATP fits the criteria for a candidate gliotransmitter in the ENS. Enteric glial cells release ATP through Cx43 hemichannels when stimulated (McClain *et al.* 2014; Brown *et al.* 2015), but whether glial ATP release is responsible for the observed excitatory effects *in vivo* is unclear. Likewise, it is not clear how Cx43-dependent ATP release from glia could exert such specific effects on enteric circuits. Furthermore, it is still unknown whether enteric glia exhibit other modes of gliotransmission, such as Ca^{2+}-dependent exocytosis, a well-studied process in astoglia of the CNS (Zorec *et al.* 2012). If they do, it will be important to determine how certain conditions favour and/or modulate any certain mode of gliotransmission.

Beyond understanding transmitters and release mechanisms, it is also important to understand how enteric glia process information. Glial information processing could occur at multiple levels including within single cells or within networks of glia. At the single cell level, the soma appears to be a centre of integration for $[Ca^{2+}]_i$ transients generated in fine processes (Broadhead *et al.* 2012). However, significant integration also seems to occur directly in the processes prior to summation and propagation to the cell soma (Broadhead *et al.* 2012). $[Ca^{2+}]_i$ responses in the soma recruit activity in the surrounding glia in the form of Ca^{2+} waves. This network level integration could be an extremely important aspect of GI physiology, but its significance remains relatively unclear. In our experiments, we find that reducing the propagation of Ca^{2+} waves through the glial network by ablating *Cx43* blunts GI motility (McClain *et al.* 2014). However, it is unknown if this outcome reflects abnormalities in glia-to-neuron communication mediated by Cx43 hemichannels or altered glial network integration mediated by Cx43 hemichannels and gap junctions. In any case, changes that affect glial integration such as changes in glia numbers or their cellular Ca^{2+} handling could play major roles in GI dismotility disorders such as chronic constipation (Bassotti *et al.* 2013) and functional dyspepsia (Cirillo *et al.* 2015), and this will be an important point to address in future work.

One major deficiency in our current understanding of glial activity is in regard to the activation of intracellular signal transduction that does not involve Ca^{2+}. The change in $[Ca^{2+}]_i$ is currently the most studied mode of the glial activation simply due to the availability of Ca^{2+}-sensitive dyes and genetically encoded proteins. Glial Ca^{2+} dynamics are clearly important for normal gut physiology (Broadhead *et al.* 2012; McClain *et al.* 2014) and also play an important role in pathological processes

Table 2. Neurotransmitters that (could) activate enteric glia

Neurotransmitter	Receptor (subunit or substrate)	Method	Source	References
Acetylcholine	Muscarinic*	fluo4	CRL-2690 cell line and human duodenal SMP *in vitro*	(Boesmans *et al.* 2013)
	Nicotinic*	fluo4	Mouse colon MP *in situ*	(Broadhead *et al.* 2012)
	α3-Nicotinic	IHC	Mouse colon MP *in situ*	(MacEachern *et al.* 2011)
Catecholamines	α2a-Adrenergic receptor	IHC	Rat ileum/colon MP *in situ*	(Nasser *et al.* 2006*b*)
Glutamate	mGluR5	IHC	Guinea pig ileum/colon SMP/MP *in situ* Mouse ileum/colon SMP/MP *in situ* Rat ileum/colon SMP/MP *in situ*	(Nasser *et al.* 2007)
	AMPA (GluR1 and GluR3) KA (GluR5) NMDA (NR2A/B)	ICC	Rat small intestine MP *in vitro*	(von Boyen *et al.* 2006)
	NMDA (NR1)	IHC	Human colon MP *in situ*	(Giaroni *et al.* 2003)
Purinergic	A2B (adenosine)	IHC	Rat ileum MP *in situ* Human jejunum/colon *in situ*	(Vieira *et al.* 2011) (Christofi *et al.* 2001)
	P2Y1 (ADP)	fluo4	Mouse colon MP *in situ*	(McClain *et al.* 2014)
		fluo4	Guinea pig colon MP *in situ*	(Gulbransen & Sharkey, 2009)
		fluo4	Mouse embryonic cultures *in vitro*	(Gulbransen *et al.* 2012)
	P2Y4 (ATP, UTP)	IHC/fluo4	Mouse/guinea pig colon MP *in situ*	(Gulbransen & Sharkey, 2009)
		IHC	Guinea pig ileum/colon SMP *in situ*	(Van Nassauw *et al.* 2006)
		ICC/fura2	Guinea pig colon MP *in vitro*	(Kimball & Mulholland, 1996)
Serotonin	Not tested	fura2	Guinea pig MP *in vitro*	(Kimball & Mulholland, 1996)
	5-HT_2*	fluo4	CRL-2690 cell line and human duodenal SMP *in vitro*	(Boesmans *et al.* 2013)

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; ATP, adenosine triphosphate; ICC, immunocytochemistry; IHC, immunohistochemistry; KA, kainate; metabotropic glutamate receptor; MP, myenteric plexus; NMDA, *N*-methyl-D-aspartate; SMP, submucosal plexus. *Receptor determined only by pharmacological inhibition and not confirmed by ICC/IHC.

(see below). Yet Ca^{2+} is only one second-messenger and there are many signalling pathways in glial cells. For example, Christofi *et al.* (1993) showed that glia are the major contributors to cAMP formation in the myenteric plexus, but the significance of cAMP elevations in glia is not understood at all. Could glial cAMP signalling be of equal or greater importance than glial Ca^{2+} signalling? Questions such as this clearly need more attention and addressing other second messengers such as cGMP, an effector molecule of NO signalling (Denninger & Marletta, 1999), or cAMP is becoming more feasible with the use of readily available sensors (Nikolaev *et al.* 2006; Borner *et al.* 2011). As a final note, the glial influence on gut motility may extend well beyond their interactions with neurons. Indeed, enteric glia interact with many non-neuronal cells that are important for peristalsis. For instance, glia-derived ATP could also signal directly to interstitial cells of Cajal or to smooth muscle (Sanders, 2000). Furthermore, Bohorquez *et al.* (2014) recently described a novel relationship between enteric glia and enteroendocrine cells (Bohorquez *et al.* 2014). Such interactions with non-neuronal cells have the potential for major effects on gut function. But to what extent these interactions influence intestinal reflexes is still unknown (Fig. 6).

Enteric glia at the mucosal interface

A growing number of studies strongly support the notion that enteric glia are important regulators of physiological processes in the gut mucosa. For example, mice with a targeted ablation of enteric glia exhibit a dramatic loss of epithelial barrier function (Bush *et al.* 1998; Cornet *et al.* 2001; Aube *et al.* 2006). Subsequent *in vitro* work has identified several enteric glial-derived molecules that impact gut barrier function through direct actions on epithelial cells (Table 3). Based on these findings, Neunlist *et al.* (2013) recently coined the term 'neuronal–glial–epithelial unit' to describe the anatomical proximity and functional interaction between enteric glia and the intestinal epithelium. However, emerging data

draw some aspects of the neuronal–glial–epithelial unit into question. For example, inhibition of glial functions with fluoroacetate, a glial cell metabolic toxin, had no effect on electrogenic ion transport under physiological conditions (MacEachern *et al.* 2015). A regulatory role of glial cells did emerge under pathological conditions during inflammation, but these findings suggest that glia do not play a major role in the regulation of secretomotor functions under normal circumstances. Likewise, mucosal glial cells are absent in both germ-free mice and mice treated with antibiotics (Kabouridis *et al.* 2015). Yet secretomotor function and transepithelial resistance are preserved in germ-free mice (Lomasney *et al.* 2014). In light of these new findings, it would seem that mucosal glia do not play an essential role in the regulation of epithelial barrier function in the short term.

Many of these discrepancies may be reconciled by considering the mechanisms and kinetics of the release of the proposed glial-derived factors. For example, the tonic release of glial factors may play an important role in the maturation of the epithelium but not in the neurogenic regulation of secretomotor functions. In support, germ-free mice do have less mucosal thickness in the absence of mucosal glia (Lomasney *et al.* 2014). Many of the studies that have identified glial mediators have used *in vitro* systems that study the interaction between Caco-2 cells and cultured enteric glia or the supernatants from glial cultures. These conditions lack neuronal input and thus, any factors released by glia under these conditions do not require glial excitation or activity-dependent processes. Some glia-derived substances such as nitric oxide (NO) can freely diffuse across membranes but many others require regulated transport. For example, prostaglandin E_2 (PGE2) and small peptides ($\leq$10mer) could be released through Cx43 hemichannels (Jiang & Cherian, 2003; Neijssen *et al.* 2005) while larger proteins like fibroblast growth factor (FGF) and transforming growth factor β1 (TGF-β1) may require Ca^{2+}-dependent exocytosis. One possibility is that glial mediators that act as trophic factors to support the growth and differentiation of enterocytes are constitutively released while the release of those that affect secretomotor functions are more tightly regulated and activity dependent. In support, cholinergic signalling in the ENS induces NO production in enteric glia that modulates the epithelial secretion (MacEachern *et al.* 2011). However, this is an indirect effect that is mediated by neuron–glia interactions within myenteric ganglia (MacEachern *et al.* 2011). Furthermore, recent evidence indicates that enteric glial-derived NO contributes to epithelial barrier dysfunction in animal models of colitis (MacEachern *et al.* 2015). These studies highlight the need for a more comprehensive understating of glial inter- and intracellular signalling mechanisms to understand their roles in health and disease.

One very exciting aspect of glia in the intestinal mucosa is their potential for bi-directional interactions with the gut microbiome (Liu *et al.* 2013). Whether glial cells directly influence the microbiome is not currently clear but new data suggest that the presence of gut bacteria has a major effect on the development of mucosal glia. These studies, performed by Kabouridis *et al.* (2015), show that mucosal glia are continuously replenished by precursor cells in the enteric plexuses and that the replenishment did not occur in antibiotic-treated animals or germ-free mice. These results indicate that cues from the gut microbiota are essential to promote the migration of glia from the plexuses into mucosa. Interactions between the microbiota and the immune system are implicated in this process (reviewed in Kabouridis & Pachnis, 2015) but the exact mechanisms are currently unknown. Theoretically, it is possible that bacterial and viral components directly influence enteric glia through actions on glial Toll-like receptors (TLR-3, -4 and -7; Barajon *et al.* 2009; see below). However, this would imply that glia in the myenteric plexus are exposed to bacteria or bacterial components on a regular basis, and to what extent this occurs under physiological conditions is unclear. Perhaps a more likely explanation is that the microbiota indirectly influence glia through interactions

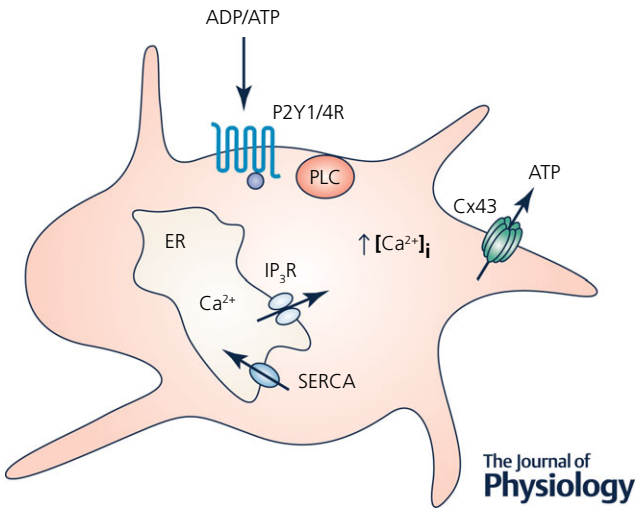
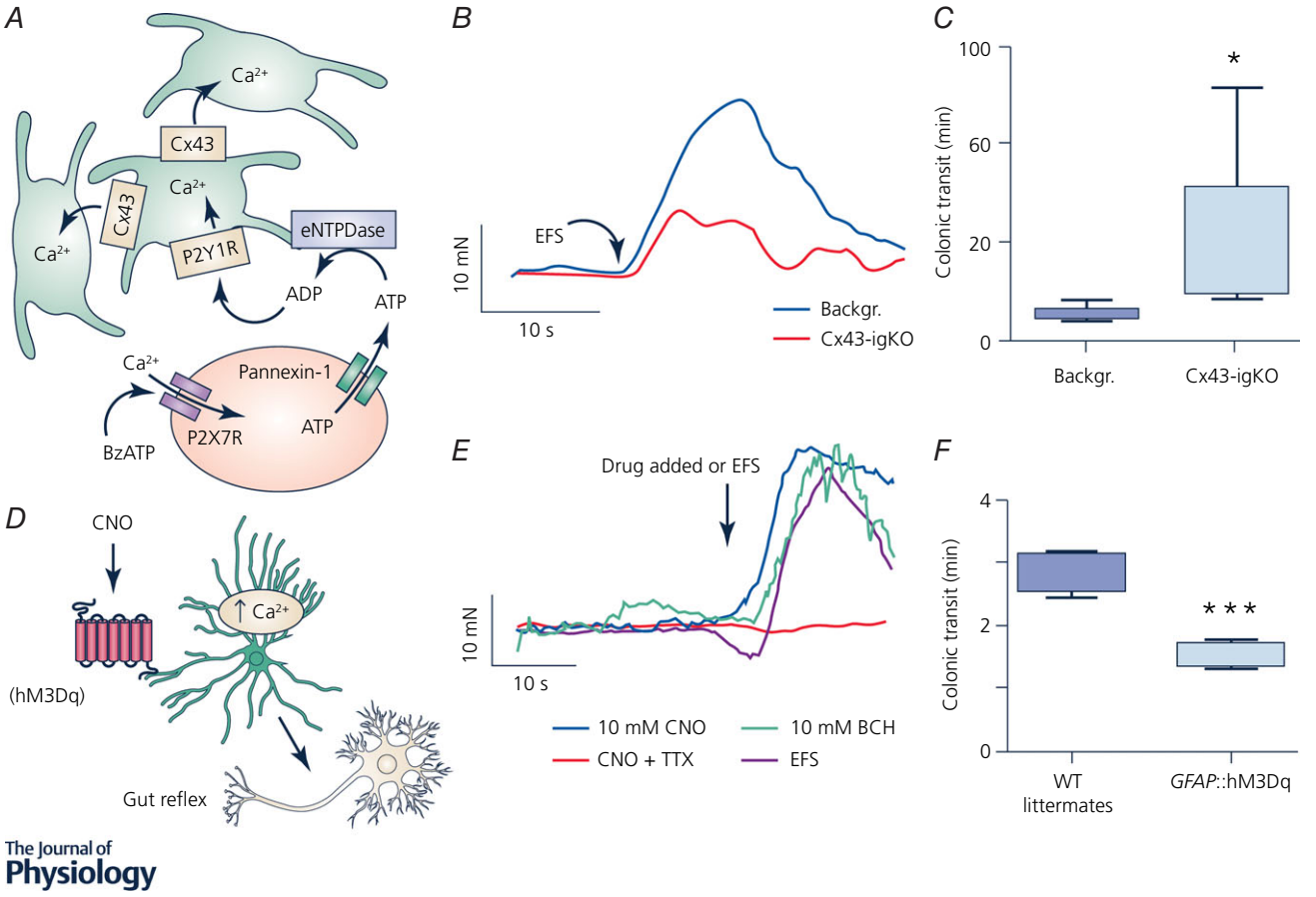


Figure 4. Enteric glia actively participate in purinergic neuron–glia signalling
ADP and ATP bind to G-protein coupled purinergic receptors P2Y1R and P2Y4R, respectively, and activate phospholipase C (PLC) and subsequent production of inositol 1,4,5-trisphosphate (IP_3). This consequently activates IP_3 receptors (IP_3R) inducing the release of Ca^{2+} from endoplasmic reticulum (ER). Increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ induces ATP release through Cx43 hemichannels. Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} ions back into the ER. Based from original research on enteric glial cells (Kimball & Mulholland 1996; Zhang *et al.* 2003; McClain *et al.* 2014). Not drawn to scale.

with the gut epithelium (reviewed in Abreu, 2010) and mucosal immune cells (Round & Mazmanian, 2009). Indeed, microbiota-driven neuroimmune interactions have already been documented (Muller *et al.* 2014). In any case, these are very exciting findings that raise many questions about the role of microbiota–glial interactions in gut physiology and pathophysiology.

Enteric glia and intestinal inflammation

Enteric glia actively participate in immune responses in the intestine. It is now clear that enteric glia have the potential to modulate immune response by both responding to and secreting inflammatory mediators that include interleukin (IL)-1 and IL-6 (Ruhl *et al.* 2001) and purines (Gulbransen *et al.* 2012; Brown *et al.* 2015). However, the mechanisms



The Journal of Physiology

Figure 5. Enteric glia actively regulate gut motility
Inhibition (*A–C*) or activation (*D–F*) of glial calcium (Ca^{2+}) signalling (*A* and *D*) results in reduction or stimulation of the gut motor reflex assessed by smooth muscle tension recordings (*B* and *E*), respectively, and corresponds to changes in the distal colon motility tested *in vivo* (*C* and *F*). *A–C*, experiments from tamoxifen-induced glia-specific knock out (igKO) of connexin 43 (Cx43) mice (Cx43-igKO) and the tamoxifen-treated background strains (Backgr.); figures obtained from McClain *et al.* (2014). *A*, neuron-specific stimulation activates Ca^{2+} responses in enteric glia and Cx43 is required for the propagation of the glial Ca^{2+} response (see original work for details). *B*, electrical field stimulation (EFS) elicits muscle contractions and the contraction force is reduced in the Cx43-igKO mice. *C*, selective reduction of the Ca^{2+} response in the enteric glia reduces distal colon motility *in vivo*. *D–F*, experiments from *GFAP*::hM3Dq transgenic mice, where glial fibrillary acidic protein (*GFAP*) promoter drives expression of an engineered G_q-coupled human M3 muscarinic receptor (hM3Dq) and their wild-type (WT) littermates; figures obtained from McClain *et al.* (2015). *D*, enteric glia expressing hM3Dq respond to clozapine N-oxide (CNO) with an increase in cytosolic Ca^{2+} and subsequently affect neurally controlled gut reflexes. *E*, glia-specific stimulation with CNO evoked response in *GFAP*::hM3Dq mice similar to stimuli with bethanechol (BCH) and EFS that directly activate smooth muscle and enteric neurons, respectively. Note that CNO effect was blocked by tetrodotoxin (TTX) indicating that glia-specific effects are mediated via enteric neurons. Also, CNO stimulation evoked no response in WT littermates (see original work). *F*, selective activation of glial Ca^{2+} signalling enhances *in vivo* motility of the distal colon.

underlying the glial responses to gut inflammation and their contribution to the development of functional gastrointestinal disorders are still poorly understood. One emerging theme is that pro-inflammatory stimuli enact glial signalling pathways that involve nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and NO. This pathway has emerged as the main effector pathway for pro-inflammatory stimuli in enteric glia and it seems to be a critical mediator of the detrimental effects of reactive glia. For example, NF-κB signalling is involved with the elevation of S100β content and release by glia and elevated glial NO production during GI inflammation induced by pathogenic bacteria (Turco *et al.* 2014), DSS-colitis in mice (Esposito *et al.* 2014; MacEachern *et al.* 2015) and ulcerative colitis in humans (Cirillo *et al.* 2011; Esposito *et al.* 2014). Likewise, our group has discovered that the activation of glia by purinergic danger cues released by neurons in the context of neuroinflammation drives glial NO production (Brown *et al.* 2015). Importantly, glial activation and NO production is a critical mediator of neurodegeneration during intestinal inflammation and neurodegeneration is a major contributor to functional bowel disorders (Gulbransen & Sharkey, 2012; Brown *et al.* 2015). In this case, the mechanisms involve the pathological opening of glial Cx43 hemichannels, glial ATP release and the activation of neuronal P2X7 receptors (Gulbransen *et al.* 2012; Brown *et al.* 2015). Precisely how purines drive an up-regulation of inducible nitric oxide synthase (iNOS) activity in glia is currently unclear but it may also involve NF-κB signalling downstream of the $\beta\gamma$ subunits of GPCRs or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Jones *et al.* 2007).

Whether similar signalling pathways are involved in other aspects of reactive gliosis in inflamed gut such as the upregulation of major histocompatibility complex class II (MHC-II) (Koretz *et al.* 1987; Turco *et al.* 2014) is unknown. This molecule is typically expressed by antigen presenting cells and the expression by enteric glia suggests that glia may instruct immune cells in unique ways (Geboes *et al.* 1992). Thus, understanding if shared signalling pathways drive diverse glial contributions to immune responses that include the secretion of inflammatory mediators and a gained antigen presenting capability would be important in the quest for new therapeutics. Turco *et al.* (2014) observed some support

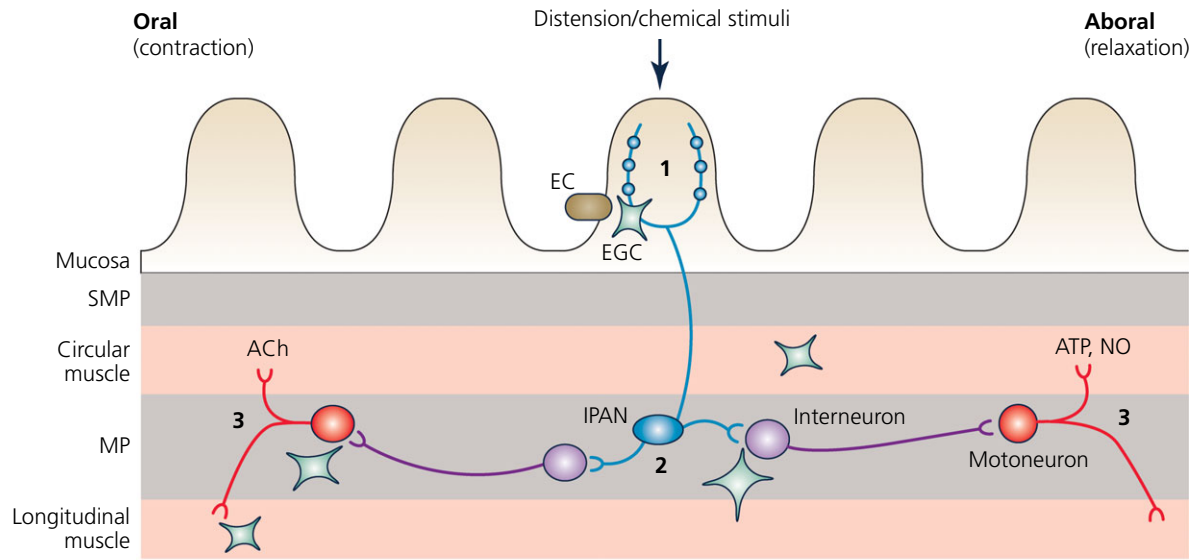


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Figure 6. Enteric glia as active players in the peristaltic reflex
The accepted circuitry of the peristaltic reflex involves the following chain of events: (1) mechanical or chemical stimuli in the gut lumen activate intrinsic primary afferent neurons (IPANs) residing in both plexi; (2) IPANs activate interneurons that project in both oral (ascending) and aboral (descending) directions; (3) ascending interneurons activate excitatory motorneurons that cause smooth muscle contraction by releasing acetylcholine (ACh) and neuropeptides while descending interneurons produce relaxation below the point of stimulation by activating inhibitory motorneurons that release nitric oxide (NO), purines and other inhibitory molecules (Kunze & Furness, 1999). Enteric glia cells (EGC) could interact with the circuit at multiple levels (see text for details), from the release of serotonin from enterochromafine cells (EC) to the direct interaction with the smooth muscle cells. Other abbreviations: MP, myenteric plexus; SMP, submucosal plexus. This schematic representation is not drawn to scale.

Table 3. Enteric glia cell (EGC)-derived molecules that regulate intestinal epithelial function

Molecule	Effect on GI epithelium	Source	References
TGF-β1	↓ Proliferation	*In vitro* (Caco-2 and primary EGCs, MP derived)	(Neunlist *et al.* 2007)
GSNO	↓ Permeability	*In vitro*, *in vivo*, human tissue	(Savidge *et al.* 2007)
15dPGJ2	↓ Proliferation	*In vitro* (Caco-2 and primary EGCs, MP derived)	(Bach-Ngohou *et al.* 2010)
GDNF*	↓ Permeability	*In vivo*	(Zhang *et al.* 2010)
proEGF	↑ Wound healing	*In vitro* (Caco-2 and EGC lines)	(Van Landeghem *et al.* 2011)

Abbreviations: 15dPGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; Caco-2, human epithelial colorectal adenocarcinoma cell line; GDNF, glial-derived neurotrophic factor; GSNO, *S*-nitrosoglutathione; proEGF, pro-epidermal growth factor precursor; TGF-β1, transforming growth factor β1. *Release of GDNF was not directly associated to EGCs.

for a common signalling pathway because an upregulation of MHC-II by glia accompanied the glial response to either S100β or TLR signalling (Turco *et al.* 2014).

Of course, an important task for future research will be to determine how to mitigate the detrimental effects of reactive glia in the intestine without interfering with their physiological functions. This may be challenging given that glial mediators such as ATP and NO play important roles in both GI physiology (MacEachern *et al.* 2011; McClain *et al.* 2014) and pathophysiology (Fig. 7; Brown *et al.* 2015; MacEachern *et al.* 2015). However, the distinct signal transduction mechanisms involved with either physiological or pathophysiological glial functions may allow for dampening of pathophysiological functions without interfering with GI physiology. In support, palmitoylethanolammide (PEA) was found to improve colonic inflammation by inhibiting NF-κB and NO release (Esposito *et al.* 2014).

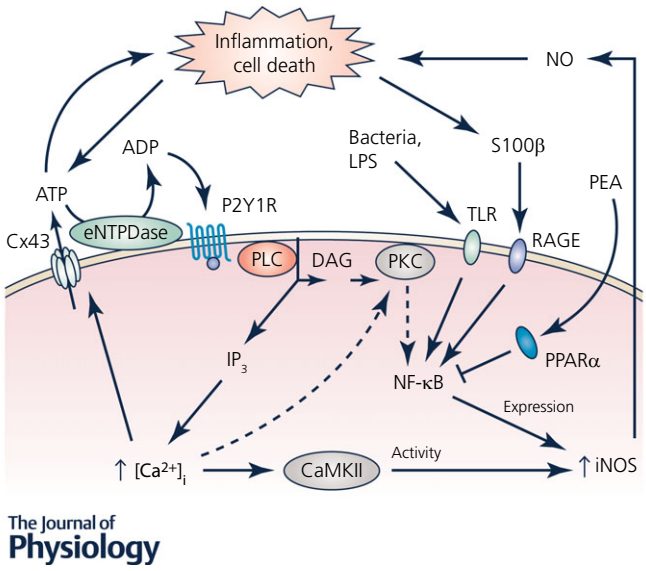

Figure 7. The role of enteric glia in inflammation – feed-forward loop leading to increased cell death
Both ATP and nitric oxide (NO) released from enteric glia regulate normal gut physiology (see text for details). Infection-induced TLR signalling increases iNOS expression via NF-κB and results in increased release of NO, a molecule with an antimicrobial effect. Excessive NO release, either by the infection or by other inflammatory signals (omitted for clarity) can also damage the cells leading to a surge of purines and S100β. While S100β enhances NO release via the increased iNOS expression, purine signalling increases intracellular calcium concentration ($[Ca^{2+}]_i$) and increased ATP release via Cx43 hemichannels. Increased $[Ca^{2+}]$ can also lead to increased iNOS activity and expression via CaMKII and PKC, respectively. Both PKC and CaMKII were not directly investigated in enteric glia (light grey); our findings indirectly show that PKC does not play a role in enteric glia (dashed arrows). The main findings are summarized from Esposito *et al.* (2014), Turco *et al.* (2014) and Brown *et al.* (2015); see text for details. Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; eNTPDase, ecto-nucleoside triphosphate diphosphohydrolase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKC, protein kinase C; PLC, phospholipase C; PPARα, peroxisome-proliferator-activated receptor-α; S100β, S100 calcium-binding protein β; TLR, toll-like receptor.

Conclusions

Enteric glia are clearly necessary for the maintenance of gastrointestinal functions and have the potential to profoundly influence gut physiology (Fig. 6) and pathophysiology (Fig. 7). The field is now poised to begin asking more pointed questions about why enteric glial cells are so important and what mechanisms they contribute to. Specifically, understanding the intricacies of the glial cell interface with multiple cell types is a relatively poorly understood area that holds great promise to further our understanding of the pathogenesis of many gastrointestinal diseases. New experimental tools such as glial-specific mutant mice (McClain *et al.* 2014, 2015) and glial-specific viral vectors (Benskey *et al.* 2015; Gombash *et al.* 2015) are now readily available and provide ample opportunities to selectively alter defined mechanisms in enteric glial cells at will. The incorporation of these types of technologies into future work will be extremely important to gain a more in-depth understanding of these fascinating glial cells and, in turn, the gastrointestinal tract itself.

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Additional information

Competing interests

The authors have no financial, professional, or personal conflicts that are relevant to the manuscript.

Author contributions

Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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