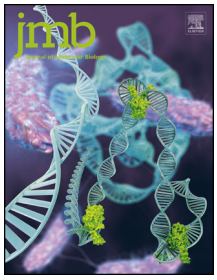

The Role of Connexins in Gastrointestinal Diseases

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Abstract

Gap junctions are hexagonal arrays of protein molecules in the plasma membrane and were first described in Mauthner cell synapses of goldfish. They form pathways for coupling between cells, allowing passive, electrotonic spread of ions and also passage of larger molecules such as amino acids and nucleotides. They are expressed in both excitable and non-excitable tissues. Each gap junction is made of two connexons, which are hexameric proteins of the connexin subunit. In this review, the roles that connexins play in gastrointestinal motility, the mechanisms of altered connexin expression leading to inflammatory bowel disease, gastrointestinal infections, and gastrointestinal symptoms in autistic spectrum disorder are discussed in detail.

Introduction

Gap junction proteins are high-resistance pathways that mediate intercellular coupling, allowing passive spread of ions and of larger molecules such as amino acids and nucleotides [1–3]. Currently, more than 20 connexin isoforms [4,5] and 21 human genes have been identified [6]. Gap junctions consist of two connexon hemichannels, each of which is a hexamer of connexin molecules [7]. It is also known that connexons can be in homomeric or heteromeric forms, with the latter formed from co-expression of multiple connexin isoforms. Studies have shown that homomeric connexons of different isoforms can also couple and communicate with each other to form heterotypic gap junctions [8]. It is speculated that this formation may be the key to attenuating or facilitating impulse relay at certain sites [9].

Gap junction intercellular communication is crucial in tissue electrical excitability such as synchronizing smooth muscle contraction [10], equalizing and buffering cytoplasmic metabolic substrate concentrations [11], and mediating inflammation [12] and immunity by cross-presentation [13]. It is also known to interact with other proteins such as enzymes, cytoskeletal molecules, adhesion complexes, and signaling elements [14]. Unpaired connexon hemichannels relay signals between cytosolic and extracellular environments [15,16]. Altered function of these channels have been implicated in many pathologies, ranging from cardiac arrhythmias [17] to abnormal wound healing [18], inflammatory, and neoplastic disorders [19].

Distribution and physiological roles of connexins in gastrointestinal tract

The gastrointestinal (GI) tract consists of various cell types along its length, including smooth muscle cells, fibroblasts, lymphoid tissue, exocrine and endocrine glands, and specialized absorptive epithelial cells.

Gap junction proteins are abundantly expressed throughout the GI tract, and its distribution has been described in several studies: Cx26, Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, and Cx57 have been reported in the small intestine, while Cx26, Cx31, Cx31.1, Cx32, Cx36, Cx40, Cx43, and Cx45 have been found in the colon [7]. In the liver, Cx32 is predominantly expressed by hepatocytes [20], Cx43 by non-parenchymal cells [21], and Cx26 mainly by periportal hepatocytes [22,23]. Apart from organ to organ variation, layer-specific connexins have been described [24]. For example, Cx43 is only found in circular but not longitudinal muscle layers of the stomach [25–28]. Also, Cx43 is the predominant connexin subtype at interstitial cells of Cajal (ICC) [7].

Previous studies found pieces of evidence supporting the roles of connexins in (1) gastric cell differentiation and homeostasis [20,29], (2) gastric acid secretion [30,31], (3) gastric mucosal barrier protection [32–34], and (4) involvement of Cx32 and Cx43 in neoplastic progression of gastric cancers [35]. Nevertheless, gap junction is key to intestinal signaling, of which one of the most prominent examples would be gap junctions mediating electrical communication between ICC in the myenteric plexus (ICC-MP) and smooth muscle cells through maintaining interstitial excitability [36–38], which is often referred to as the "pacemaker region" of the GI tract [39].

The Role of ICCs in Enteric Neurotransmission

Evidence based on morphological experiments suggests that ICCs mediate neurotransmission of enteric motor neurons via the formation of synaptic-like structures between the nerve terminals of enteric cells and ICCs [40]. A study was conducted to determine if the conservation of these structures in the gastric antrum of canines would translate into similar functional roles in mice and larger mammals [41]. The results showed synaptic-like close contacts between ICCs and excitatory and inhibitory nerve fibers, which contains electron-dense regions at the pre- and post-junctional spaces. However, no synaptic contacts were observed between smooth muscle cells and enteric neurons, suggesting that innervated ICCs conduct neurotransmission by forming gap junctions with adjacent smooth muscle cells.

A genetic loss-of-function study was conducted to demonstrate how ICCs transmit both excitatory and inhibitory signals to smooth muscle cells from enteric neurons [42]. An inducible *c-Kit*^{CreERT2} allele was knocked in and this allowed ICCs to be manipulated during development and adulthood at various time points in vivo. These findings demonstrate that ICCs are able to integrate enteric neurotransmission with pacemaker activity in the intestine to control and maintain the activity of the digestive tract in adult animals.

Connexins 43 and 36 and GI Motility

In the gut, gap junctions are found abundantly among ICC and smooth muscle cells [28,43,44]. While Cx43 is extensively found in a plethora of gut tissues (e.g., deep muscular and myenteric plexus cells, muscle and epithelial cells, etc.), Cx36 is reported to be localized to only myenteric ganglia cells [45]. As mentioned before, ICC are populations of noncontractile interstitial cells that serve as enteric neuromodulators [39], and various populations have been identified: ICC of myenteric plexus (ICC-MP), ICC of deep muscular plexus (ICC-DMP) and intramuscular ICCs (ICC-IMs). ICC-MP lies between circular and longitudinal smooth muscle layers throughout the gut and is between circular smooth muscle and submucosa in the colon [39]. In contrast, ICC-IMs are located between smooth muscle cells in the stomach and colon but are clustered as the ICC-DMP in the deep muscular plexus of the intestine [46–48].

ICC-MP spontaneously generates and coordinates rhythmic electrical slow waves that propagate to adjacent smooth muscles via gap junctions [31,49–51]. In contrast, ICC-DMP and ICC-IM do not share the autorhythmicity and are speculated to modulate electrical signals from enteric neurons to gut smooth muscle cells. However, it was found that slow wave depolarization alone does not always induce contraction, suggesting that a second stimulus may be required to reach the depolarization threshold, which studies attributed this to phase-amplitude coupling interactions between low-frequency rhythmic transient depolarization from ICC-DMP and slow waves from ICC-MP [52].

Furthermore, it has been proposed that segmentation contractions are a result of alternative smooth muscle inhibition and excitation by the enteric nervous system (ENS) [53]. However, a study was conducted that showed that segmentation contractions can occur when nerve blockade does not occur as well as through a different mechanism, which consists of two networks of ICCs [54]. Moreover, the modulatory phase-amplitude coupling is responsible for coordinating the checkered segmentation motor pattern, involving two pacemaker activities. Specifically, ICC-MP slow wave activity is modulated by the phase of lower frequency rhythmic transient depolarizations that originate from ICC-DMP.

A study was conducted to investigate the role of Cx43 in mouse intestinal tunica muscularis [55]. Mice transfected with Cx43 knockdown in intestinal smooth muscle cells were studied (Fig. 1). The following findings were observed: (1) smooth muscle

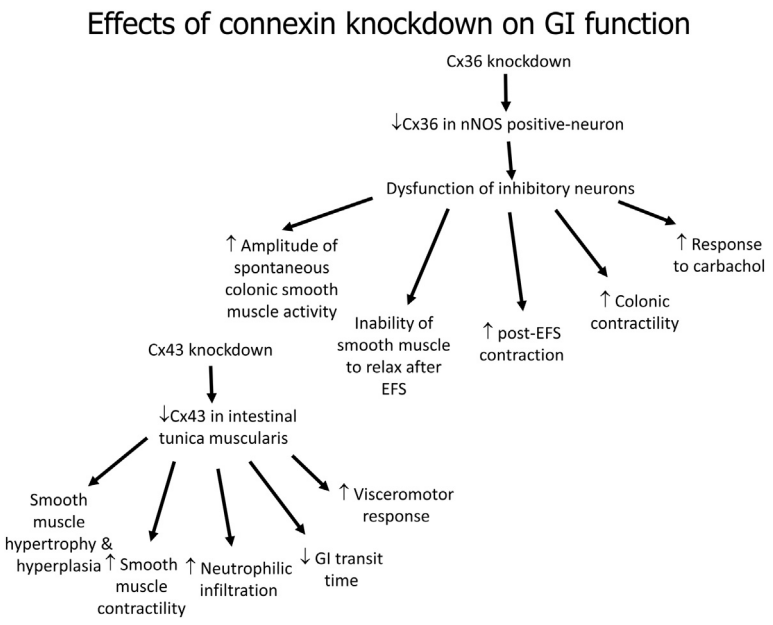

Fig. 1. Effects of connexin knockdown on GI function.

hypertrophy and hyperplasia, (2) 13-fold increase of neutrophilic infiltration, (3) GI transit time slowed down by 29%, (4) hypercontractility, and (5) enhanced visceromotor response. At cellular levels, loss of connexins, namely Cx43, was shown to lead to cytoskeletal changes resulting in altered cell morphologies, adhesion, polarity, and migration [56–58]. The findings were consistent with that in previous studies on chronic inflammatory conditions of the GI tract [59,60]. Also, visceral hypersensitivity and hyperalgesia are often observed in irritable bowel syndrome and functional disorders [61,62]. This led to postulations that Cx43 may contribute to GI motor and visceral sensory dysfunction in certain pathologies [55].

Cx36, a neurotransmitter that is often found in gamma-aminobutyric acid neurons [63–65], was shown to localize at nitric oxide synthase (nNOS)-containing neuronal cell bodies throughout the GI tract [66]. Abnormal patterns of contractility were detected in the gut after Cx36 knockdown: (1) enhanced amplitude of spontaneous smooth muscle activity in the colon, (2) inability to relax during electric-field stimulation, and (3) increased post-EFS contraction [66]. Also, previous experiments have demonstrated that nitric oxide acts as a neurotransmitter in mouse colonic longitudinal muscle [66]. Elevated colonic contractility in Cx36 knockdown mice associated with enhanced responsiveness to carbachol (cholinergic receptor agonist) was noted [66]. These phenomena were attributed to nNOS-positive neurons in MP, which are known to take part in relaying descending pathway of local reflex, inhibition of circular muscles, and, to a smaller extent, longitudinal muscles [67,68]. These data suggest that loss of Cx36 was responsible for the dysfunction of inhibitory nNOs-positive cells that would otherwise exert tonic inhibition on gut muscle contraction with nitric oxide as the neurotransmitter [66]. Future investigative approach will involve methods of higher-resolution microscopy to confirm more precise distribution of gap junction proteins. The complex interplay underlying the roles of altered Cx36 expression on GI motor activity is yet to be determined.

Apart from the intrinsic enteral nervous system, GI motility is also modulated by extrinsic afferent neurons. Cx43-based hemichannels take part in intercellular communication between enteric glial cells [69,70]. It was also demonstrated that application of octanol, a nonspecific gap junction inhibitor, hinders gap junction intercellular communication (GJIC) in the form of inositol-1,4,5-triphosphate molecules transfer between rat colonic myocytes and dorsal root ganglion neurons in a co-culture [71–73]. However, this may be a candidate for an alternative connexin-mediated regulatory pathway in GI motility.

Role of Cx43 in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of unknown etiology and is characterized by an early neutrophilic and macrophage infiltration in the GI mucosa and submucosa [74]. Epithelial cell lining of the intestine mucosa is responsible for maintaining both structural and immunological protection against pathogens [75]. It also regulates homeostatic processes such as proliferation, migration, and differentiation through

modulating GJIC [76]. The role of Cx43 in intestinal epithelial injury has been investigated [77]. Toll-like receptor 2 is a member of a receptor family that recognizes bacterial epitopes [78] and is induced by gut commensals [79,80]. It was shown that TLR2 stimulates GJIC during acute injury [77], and the inhibition of GJIC suppresses epithelial cell migration (Fig. 2) [81]. This suggests that TLR2 may be a "warning" signal from intestinal epithelial disruption [80,82]. Nevertheless, Cx43 was found to be part of the downstream cascade in TLR2-mediated GJIC induction, where Cx43 transcription, post-translational modification, and assembly are upregulated in response to TLR2 activation [77,83]. Application of TLR2 agonist was shown to prevent chronic colitis in mice model and disintegration of Cx43 in intestinal tissue cultures [77,83]. In addition, levels of TLR2 and Cx43 appeared to be directly correlated to each other, and its loss of either of them correlates with worsening prognosis in patients with acute colitis [80]. It was hypothesized that Cx43 mediates TLR2-induced GJIC, which regulates and restores intestinal epithelial function during both acute and chronic inflammation [77]. Further elucidation of TLR2–Cx43–GJIC axis will provide valuable direction in managing inflammatory GI diseases [77].

As for the role of GJIC in functional aspects of intestinal physiology, it is known to regulate intestinal motility and it has been detected in direct interactions between macrophages and intestinal epithelial cell lines, suggesting that gap junction proteins play a role in the pathogenesis of IBD [84,85]. Two connexins (Cx26, Cx43) with different trafficking pathways were investigated, and both were found to be redistributed to basolateral surfaces [85]. Communication between epithelial cells and macrophages is suggested to be enhanced by basement membrane degradation through reducing gap junction expression and collagen type IV deposition, disrupting junctional complexes, as well as upregulating metalloproteinase-9 expression and activity [85]. The effects on the downstream pathway coincides with past study results on intestinal epithelial cells and macrophages in IBD [85]. Collectively, these results suggest the role of gap junction proteins in regulating epithelial cell metabolism, and its dysregulation may contribute to pathogenesis of IBD [85].

Connexins in GI Infections

Gastric ulcers have been shown to have significantly fewer connexins (namely Cx32), gap junctions, and GJIC [31]. Two virulence factors, CagA and VacA, have been associated with various gastric pathologies. CagA delays the rate of gastric mucosal

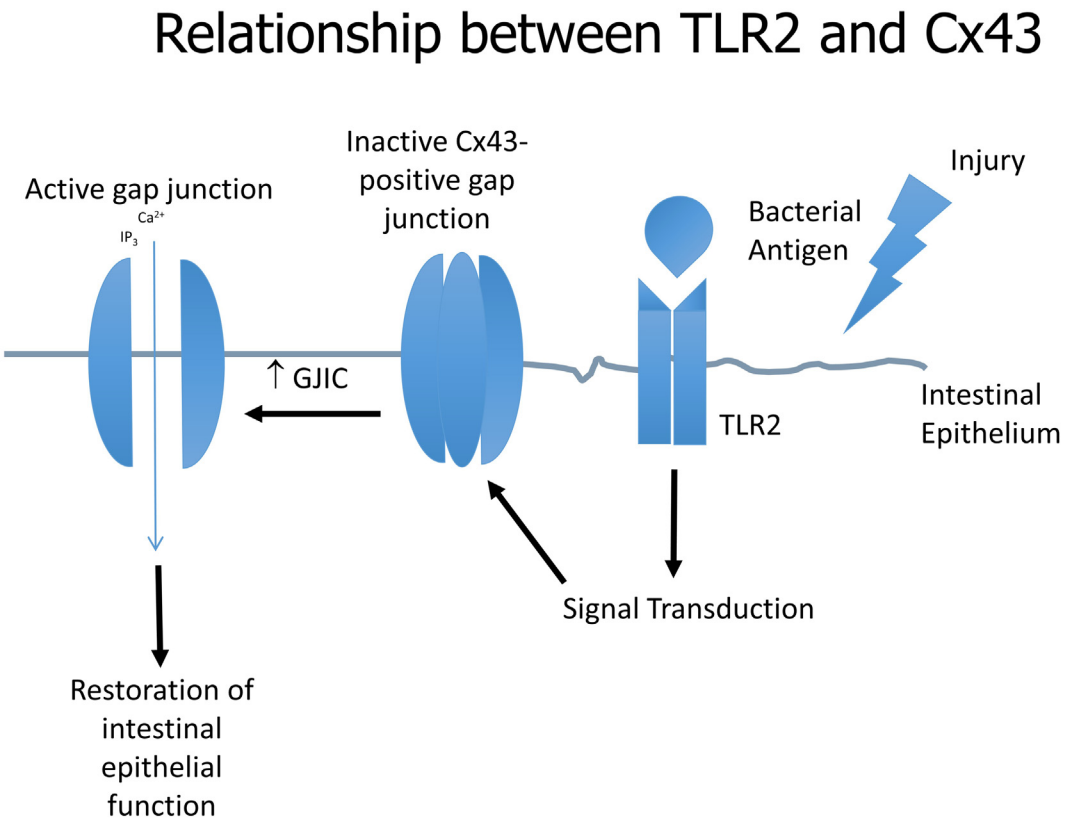

Fig. 2. Relationship between TLR2 and Cx43.

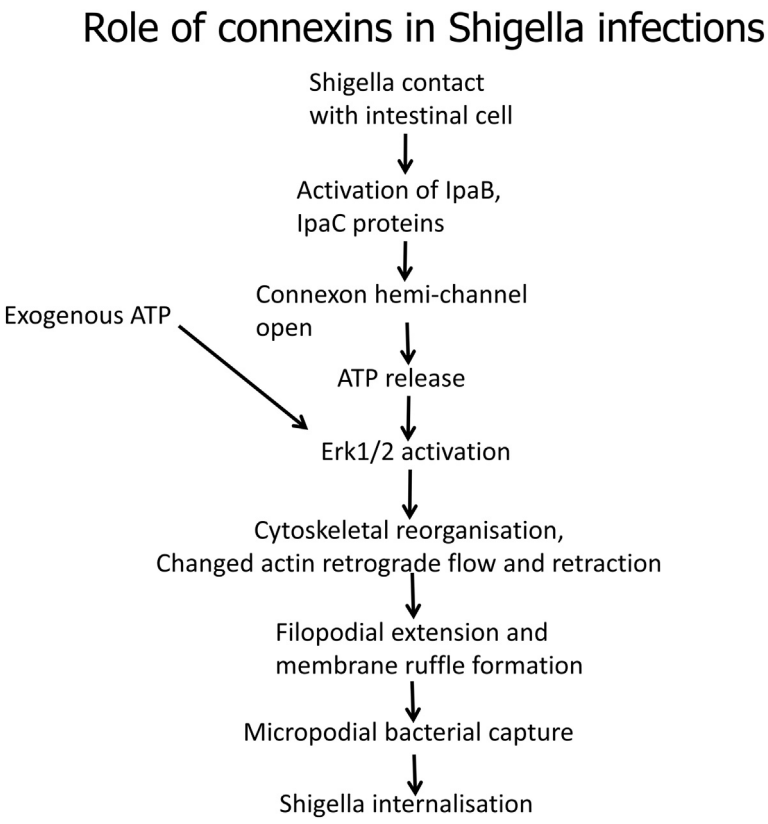

Fig. 3. Role of connexins in *Shigella* infections.

re-epithelialization and suppresses Cx43 in gastric carcinoma cell cultures [86–90]. *Helicobacter pylori* reduces Cx32 and Cx43 levels significantly, which are restored to normal levels upon eradication of *H. pylori* [86,88,90].

Diarrhea is commonly caused by invasion of three main different classes of pathogens: bacteria, viruses, and parasites [91]. The main pathophysiological mechanism has been widely accepted as pathogen interfering with normal ionic and osmotic movements through direct and indirect modulation of transporters [91]. Some notable transporters include sodium-dependent glucose transporters (SGLT1), apical $Cl^-/HCO3^-$ exchanger, and cystic fibrosis transmembrane conductance regulator (CFTR) [91]. Recent study evaluated the correlation between Cx43 and diarrhea-inducing enteric pathogens including enterohemorrhagic *Escherichia coli*, enteropathogenic *E. coli*, and *Citrobacter rodentium* [92]. It was concluded that Cx43 might play a role in diarrheal diseases [64]. The results showed that (1) Cx43 level was elevated during infection, (2) Cx43 protein mainly localized at the apical and lateral cell boundaries, (3) unpaired functional Cx43 hemi-channels were detected during infection, and (4) water in colonic content was significantly reduced [92]. Based on the above data, it was concluded that the altered connexin level at the apical aspects of cells might have caused the efflux of intracellular electrolytes, resulting in increased luminal water content [92]. However, little is known regarding the mechanism of Cx43 upregulation by the pathogens: this will require further experimentation.

Shigella flexneri is a gram-negative rod that causes bacillary dysentery [93,94]. Its invasion is known to trigger a severe inflammatory response of the colonic epithelium [93,94]. This involves activation of *Shigella* IpacB and IpacC proteins upon contact with the host cell, which induces cytoskeletal reorganization and hence internalization of bacteria [95,96]. The functional role of gap junction proteins in *Shigella* dissemination has been examined (Fig. 3). In HeLa cells transfected with human Cx26, Cx32, and Cx43, enlarged foci of *Shigella* dissemination were observed, and the application of the gap junction inhibitor α-glycyrrhetinic acid significantly limited the extent of dissemination [97]. In a different study, connexon hemi-channels was shown to mediate intercellular communication during *Shigella* dissemination [98], suggesting possible involvement of connexins in *Shigella* spreading [97]. Further investigations into the potential mechanism were performed, and ATP was found to be released through connexon hemi-channels upon *Shigella* invasion, which triggers calcium oscillatory waves [97]. Also, filopodial extensions and membrane ruffles were formed in HeLa cells exposed to exogenous ATP [97]. Extension formation is induced by ATP-mediated Erk1/2 activation, leading to actin retrograde flow and retraction, resulting in micropodial bacterial capture

[99]. The consequence is a 50% and a 300% increase in the occurrence and extensiveness of *Shigella* invasion, respectively [97]. Another interesting finding is that ATP purinergic receptors are mainly localized in the apical membranes, while hemi-channels are predominantly at the basal surface [98,100]. These provide insights into the factors and pathways of bacterial penetration, but the exact mechanism is still yet to be fully understood. This model may not apply to other enteropathogenic microorganisms, and the possible interplay with other immunogenic pathways such as release of pro-inflammatory cytokines must be considered [94].

The role of connexins in several other bacterial infections was also investigated. *Pseudomonas aeruginosa*, a gram-negative aerobic rod well known for causing nosocomial infections [101,102], contains an LPS was found to upregulate alveolar Cx43 [103] but depress Cx40 expression in murine nasal epithelial cells [104]. Phospholipase C derived from *Clostridium perfringens* was found to downregulate gap junctions in mouse liver, and gap junction plaque size in murine ventricular cardiomyocytes was also reduced by a chimeric a toxin derived from *C. perfringens* and *Clostridium botulinum* [105]. To sum up, it is evident that connexins may be a mediator in pathophysiology of some GI infections through various signaling pathways [101]. However, the pathophysiology remains largely unknown as it requires further elucidation in several directions: (1) the exact changes in connexin expression associated with infections, (2) biological impact due to the associated GJIC changes, and (3) other cellular changes that may interplay with connexin pathways, for example, pannexins and cytokines [101].

Connexins and GI Symptoms in Autism Spectrum Disorder and Neurodegenerative Diseases

Autism spectrum disorder (ASD) is a collective term for a wide range of heterogeneous neurodevelopmental disorders, which are characterized by defective social communication and limited behavioral patterns without intellectual disability or global developmental delay [106]. There are several proposed mechanisms for ASD, including immunological dysregulation, increased oxidative stress, and environmental factors [107]. However, only in around 20%–25% cases can the genetic causes be identified [108]. Recently, there has been increasing interest in the relationship between the central nervous system (CNS) and ENS. It was found that the incidence rate of general GI symptoms in ASD children is more than five times higher than those without ASD [106]; the incidence of abdominal pain is doubled; and constipation and diarrhea occurred four times more frequently [109]. A possible causative linkage between autism and GI disorders was proposed, attributing it to several factors such as altered composition and metabolic products of gut commensals, maternal infection, and altered gut physiology [110].

Enteric glial cells are astrocyte-like cells that lie underneath intestinal epithelial cells [111]. The functional role of enteric glial cells ranges from a supportive role in the ENS to actively participating in the regulation of GI motility, epithelial integrity, and inflammation [112–115]. EGC dysfunction correlates with gut inflammation and motility impairment [116,117], both of which are commonly observed in ASD patients [106]. Due to the functional and morphological resemblance of EGC to astrocytes, investigations of astrocytic involvement were conducted, revealing alterations in the pattern of astrocytic marker expression, such as elevated Cx43 expression, in brains of ASD patients [118]. Increased Cx43 expression has been observed in brain inflammation [119,120]. First, Cx43 expression is detected in EGCs [106]. Second, selective inhibition and ablation of EGC Cx43 resulted in reduced GI motility and contractility, as well as increased water content in colonic contents [69]. Third, EGCs facilitate blood–brain barrier formation after transplantation, showing high resemblance to the function of astrocytes during normal development of the CNS [121]. Cx43 loss led to a weakened BBB as a result of edema of astrocytic end-foot processes [121]. It was suggested that Cx43 might play a similar role in the development of ENS, which might explain the coincidence of (1) GI inflammatory diseases and (2) brain inflammation that augments CNS symptoms in ASD patients [120]. The proposed connection between EGC, connexins, and ASD is only hypothetical and it is too early to draw any conclusion on the interactive linkage between the two. Further investigations may pinpoint their exact roles, or other concurrent signaling pathways, in GI symptoms in the context of ASD [120]. Loss of Cx43 in the enteric glial cells contributes to brain inflammation in ASD by inducing disturbances in the gut–brain axis [106].

Finally, gap junctions have been proposed to play an important role in the pathogenesis of neurodegenerative diseases [122–125], as well as its GI manifestations [126]. For example, gastric emptying is delayed in Parkinson's disease, which can be related to reduced expression of Cx43 in the stomach [127]. Future studies are needed to elucidate the complex relationship between connexin protein expression and function, enteric glial cells, intestinal epithelial barrier, and intestinal inflammation in the pathogenesis of Parkinson's disease [111,115,128,129].

Conclusion

In summary, connexins play a vital role in the normal function of the GI tract and appear to be involved in

numerous GI diseases. Their expressions are altered in many disease processes, including inflammatory bowel disease, infections of the GI tract, and GI symptoms in autistic spectrum disorder. Animal systems have been extensively used to model human diseases with many opportunities for translational application. Their use will continue to prove useful to elucidate their pathophysiological mechanisms and will be the basis for potential clinical applications in the future. Examples include developing novel therapeutic agents or prophylactic therapy, biomarkers for predicting the likelihood of disease development, and further our understanding of neurochemical homeostasis and the pathophysiology of certain psychiatric disorders.

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Abbreviations used:
GI, gastrointestinal; ICC-MP, interstitial cells of Cajal of myenteric plexus; ICC-DMP, ICC of deep muscular plexus; ICC-IM, intramuscular ICC; nNOS, nitric oxide synthase; IBD, inflammatory bowel disease; GJIC, gap junction intercellular communication; ASD, autism spectrum disorder; CNS, central nervous system; ENS, enteric nervous system.

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