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Two modes of enteric gliotransmission differentially affect gut physiology

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Abstract

Enteric glia (EG) in the enteric nervous system (ENS) can modulate neuronally regulated gut functions. Using molecular genetics, we assessed the effects that molecular entities expressed in EG and otherwise mediating two distinct mechanisms of gliotransmitter release, connexin 43 (Cx43) hemichannel vs. Ca^{2+} -dependent exocytosis, have on gut function. The expression of mutated Cx43^{G138R} (which favors hemichannel, as opposed to gap-junctional activity) in EG increased gut motility *in vivo*, while a knock-down of Cx43 in EG resulted in the reduction of gut motility. However, inhibition of Ca^{2+} -dependent exocytosis in EG did not affect gut motility *in vivo*; rather, it increased the fecal pellet fluid content. Hampering either Cx43 expression or Ca^{2+} -dependent exocytosis in EG had an effect on colonic migrating motor complexes, mainly decreasing frequency and velocity of contractions *ex vivo*. Thus, EG can differentially modulate gut reflexes using the above two distinct mechanisms of gliotransmission.

Keywords

enteric glial cells; Cx43; dnSNARE; VIPP; IP₃; calcium; exocytosis

INTRODUCTION

Local gut reflexes are controlled by the enteric nervous system (ENS), a network of neurons and glia residing in the gut wall. While neuronal innervation and control of the ENS are well established [reviewed in (Furness et al. 2014)], enteric glia (EG) were traditionally recognized as supportive cells due to their inability to generate action potentials (Hanani et al. 2000). However, cell specific ablation or metabolic poisoning of EG demonstrated their indispensable function in maintenance of gut homeostasis (Bush et al. 1998; Nasser et al. 2006), and only recent studies have begun to uncover truly active roles of EG in gut motility and physiology.

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EG express receptors to all major classes of enteric neurotransmitters [reviewed in (Grubisic and Gulbransen 2016)], allowing them to respond to neuronal signaling. For instance, neuronal purinergic signaling activates EG *in situ* via P2Y4 receptor causing a downstream inositol 1,4,5-trisphosphate (IP₃)-dependent increase in intracellular calcium (Ca²⁺) concentration ([Ca²⁺]_i) (Gulbransen and Sharkey 2009). This Ca²⁺ increase in EG was also observed during colonic migrating motor complexes (CMMCs) (Broadhead et al. 2012), indicating that EG get activated during these physiological, neuronally regulated, propulsive contractions of the large intestine. Furthermore, recent studies showed that inhibition or activation of [Ca²⁺]_i dynamics specifically in EG resulted in reduced or enhanced gut motility/contractility (McClain et al. 2015; McClain et al. 2014), respectively, demonstrating the active role of EG in gut motility.

Ca²⁺ excitability of EG suggests that these glial cells may release neuroactive chemical substances known as gliotransmitters. Although gliotransmission is a fairly well studied phenomenon, particularly in astrocytes of the central nervous system (CNS) [(reviewed in (Parpura and Zorec 2010))], it has been understudied in the ENS. Nonetheless, adenosine triphosphate (ATP) release through functional unopposed/unpaired connexons, i.e. hemichannels, was found required for propagation of Ca²⁺ responses between EG *in vitro* (Zhang et al. 2003). These hemichannels are composed of connexin 43 (Cx43) and are required for EG propagated Ca²⁺ responses *in situ*, consequently modulating gut motility/contraction (McClain et al. 2014). However, the extent to which Cx43 mutations, like Cx43^{G138R}, in EG affect gut motility is unknown; Cx43^{G138R} causes human pathology of oculo-dento-digital dysplasia (ODDD) and hampers gap-junctional coupling yielding an augmented ATP release via unopposed connexons (Dobrowolski et al. 2007). Furthermore, it is unknown whether EG may use Ca²⁺-dependent exocytosis, the best known pathway for gliotransmitter release from astrocytes of the CNS (Parpura et al. 1994), otherwise involved in many processes, from memory formation (Pascual et al. 2005) and sleep homeostasis (Halassa et al. 2009) to breathing (Gourine et al. 2010) and nociception (Foley et al. 2011). We set to address these issues.

Since EG *in situ* do not share the same characteristics with the cultured cells [reviewed in (Grubisic and Gulbransen 2016)], in the present study, we performed *in vivo* and *ex vivo* experiments using various inducible, glial specific, mouse models to: i) enhance (using a mutated Cx43 form) or reduce availability of Cx43 hemichannel-mediated gliotransmission; and ii) inhibit exocytotic release machinery or the upstream IP₃-dependent Ca²⁺ signaling, which is necessary for Ca²⁺-/SNARE-dependent exocytosis (Supplementary Figure 1). Our results suggest that increased activity or decreased expression of Cx43 hemichannels in EG correspondingly affects both gut transit *in vivo* and CMMCs *ex vivo*. Inhibition of Ca²⁺-dependent exocytosis in EG specifically modifies CMMCs and fecal pellet composition. This study demonstrates differential roles of two distinct mechanisms of gliotransmission, with some apparent cross-talk between them, on the functional output of the gut.

MATERIALS AND METHODS

Animals

Thirteen- to sixteen-week-old hemizygous and wild-type (C57BL/6) mice of both sexes were used. All mice were *Helicobacter spp.* free and maintained in a temperature (24–25 °C)-controlled environment on a 12-hour light (7 am on):12-hour dark (7 pm off) cycle, with ad libitum access to food and water. Genotyping was performed by a commercial vendor (Transnetix, Cordova, TN). All procedures were performed in strict accordance with the National Institutes of Health Guide for the Care and were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

To investigate specific roles in gut function exhibited by EG Cx43 in gliotransmission, we used two mouse models that utilize inducible Cre-lox system and concurrently report on the Cre activity at the very locus involved in the recombination. The inducible and conditional knockdown Cx43 mice (Cx43-icKD) were generated by crossing a knock-in line with floxed *wild-type* Cx43 followed by the enhanced cyan fluorescent protein (eCFP) (Cx43^{fl::eCFP}) (Degen et al. 2012) and *hGFAP::CreERT²* transgenic line (The Jackson Laboratory, Bar Harbor, ME; stock number 012849) (Ganat et al. 2006) that utilizes the *Gfa2* fragment of the human glial fibrillary acidic protein (GFAP) promoter (Brenner et al. 1994) to drive the expression of a fusion protein between Cre recombinase and the mutated estrogen receptor (ERT²), responsive to the active 4-hydroxy form/metabolite of tamoxifen. The other genetically modified line (Cx43^{G138R}) is a cross between the above-mentioned *hGFAP::Cre:ERT²* transgenic parental line and a knock-in line with floxed *wild-type* Cx43 gene followed by a bicistronic cassette consisting of Cx43 gene with a missense mutation in the codon 138, an internal ribosome entry site (IRES) sequence and enhanced green fluorescent protein (eGFP) gene (Cx43^{fl::Cx43G138R::IRES::eGFP}) (Dobrowolski et al. 2008). Animals were gavaged with tamoxifen free base (2 mg per 10 g of body weight; Sigma, St Louis, MO; cat. no. T5648) once a day for 5 consecutive days to induce Cre-lox recombination in GFAP positive cells, resulting in deletion of the floxed *wild-type* Cx43 gene and consequent expression of either eCFP in Cx43-icKD or both Cx43^{G138R} and eGFP in Cx43^{G138R} mice. Experiments started one week after the final gavage to allow animals to recover from the tamoxifen treatment. The control group of tamoxifen treated animals consisted of pooled wild-type mice and littermates carrying single parental lines genes.

To test possible roles in gut function exerted by exocytosis and IP₃-dependent Ca²⁺ signaling in EG, we used two double transgenic mouse models that utilize tetracycline (tet)-off system. Dominant negative SNAP (Soluble NSF Attachment Protein; NSF stands for N-ethylmaleimide-sensitive fusion protein) Receptor mice (dnSNARE) were obtained by crossing *hGFAP::tTA* and *tetO::dnSNARE/tetO::eGFP* parental lines, all described in detail elsewhere (Pascual et al. 2005). The *hGFAP::tTA* line utilizes the *hGFAP* promoter fragment to drive the expression of its tet transactivator (tTA), which in turn activates tet operon (*tetO*) provided from the other *tetO::dnSNARE/tetO::eGFP* parental line. Briefly, in the absence of doxycycline (Dox), a tet analog, the dnSNARE mice express the entire cytosolic tail (amino acids 1-96) of synaptobrevin 2 (Sb2₁₋₉₆), which prevents the formation of ternary SNARE complexes and thus blocks exocytotic gliotransmitter release from GFAP positive cells

(Zhang et al. 2004); eGFP fluorescence validates the transgene expression. Another mouse model referred to as VIPP (Marpegan et al. 2011) was generated by crossing the above-mentioned *hGFAP::tTA* parental line with the *tetO::VIPP* transgenic line that encodes a fusion protein between the N-terminal Venus, an improved version of a yellow fluorescent protein, and the type I IP₃ 5-phosphatase (IPP), an enzyme that terminates IP₃ signaling (De Smedt et al. 1997); VIPP expression impedes IP₃-dependent calcium signaling pathways, otherwise necessary and sufficient to drive Ca²⁺-dependent vesicular secretion. To prevent expression of the *tetO* driven transgenes during the ENS development, both breeders and weaned animals were fed with a diet supplemented with 200 mg/kg of doxycycline hyclate (Harlan Laboratories Inc, Indianapolis, IN; cat. no. TD.140783). Regular diet (lacking Dox) was introduced six to seven weeks before the experiments to induce expression of the transgenes in GFAP positive cells, as indicated directly or indirectly by either Venus or eGFP fluorescence, respectively. Control group consisted of *hGFAP::tTA* mice that were subjected to the above Dox treatment.

In vivo assays for fecal pellet output and gut motility

Pellet output and composition, as well as whole gastrointestinal (GI), upper GI and distal colon transit velocities were determined as previously described (Grubisic et al. 2015).

Evaluation of colonic migrating motor complexes using spatiotemporal maps

Tissue preparation, video recordings and spatiotemporal map generation/analysis were previously described elsewhere (Roberts et al. 2007). Briefly, the colon was removed, flushed cleaned, cannulated at the both ends (oral and anal) and placed in an organ bath containing physiological saline (20 ml) composed of (in mM): NaCl (118), KCl (4.6), NaH₂PO₄ (1), NaHCO₃ (25), MgSO₄ (1.2), D-glucose (11) and CaCl₂ (2.5). The saline was gassed with 95% O₂ and 5% CO₂, heated (27–29 °C), and continuously superfused through the organ bath at the flow rate of 7 ml/min. Intraluminal pressure of 2 cm H₂O was hydrostatically maintained using saline at room temperature (24–26 °C). After a 30-minute equilibration period, tissue movements were recorded for 30–45 min using an 8-bit digital scope (Celestron, Torrance, CA; Model 44302A) at a rate of 15 frames/s, sampled at the pixel density of 640 × 480, and saved in the AVI format. The recordings were subsequently processed offline to generate and analyze spatiotemporal maps with tandem software (Scribble2 and Analysis, respectively) developed by Dr. Joel C. Bornstein's group (The University of Melbourne, Australia) (Roberts et al. 2007), using MATLAB 8.2.0.701 (MathWorks®, Natick, MA).

Myenteric whole-mount immunohistochemistry

Colon was removed, flushed with phosphate buffered saline (PBS), longitudinally cut along the mesenteric border, flattened out, pinned down on a silicone elastomer (Sylgard® 182; Dow Corning, Auburn, MI) -coated dish, and fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS for 2 hours at room temperature (RT, 20–25 °C). The PBS solution was composed of (in mM): NaCl (137), KCl (2.7), Na₂HPO₄ (10) and KH₂PO₄ (1.8) (pH 7.2). After washing three times with PBS, mucosa and submucosa were removed under a Leica GZ6 stereomicroscope (Bannockburn, IL) and immunohistochemistry was performed on the remaining tissue that contained the myenteric plexus. Cells within the

tissue were permeabilized with 0.25% (v/v) Triton X-100 in PBS for 1 hour, followed by sequential tissue incubations with 10% (v/v) goat serum in PBS (GS-PBS) for 1 hour at RT and then overnight at 4°C with mouse anti-GFAP antibody [clone GA-5, ICN Biomedicals Irvine, CA, cat. no. 691102 (now MP Biomedicals, LLC, Santa Ana, CA, cat. no. 0869110)] diluted at 1:100 in GS-PBS. Following incubation with primary antibody, tissue was washed three times with PBS and incubated for 2 hours at RT with goat anti-mouse antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Southern Biotechnology Associates, Inc., Birmingham, AL; cat. no. 1030-03) and diluted 1:200 in GS-PBS. Tissue was then washed four times with PBS, mounted in ProLong® Gold Antifade Mountant (Thermo Fischer Scientific, Waltham, MA; cat. no. P36930) and left overnight to harden. Images were acquired through 40× and 60× oil-immersion objectives (UPLFLN and PLAPON, 1.30 and 1.42 numerical apertures, respectively) of a FluoView FV1000 (Olympus, Tokyo, Japan) confocal laser scanning microscope.

Statistics

The number of mice required for an individual set of experiments was pre-assessed using power analysis (set at 80% and $\alpha = 0.05$). Kruskal-Wallis one-way analysis of variance followed by either Newman-Keuls or Dunn's Multiple Comparisons tests were used to compare groups with equal or different sizes, respectively, using GB-Stat 6.5 (Dynamic Microsystems Inc., Silver Spring, MD) and GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA) software. Additionally, Microsoft Excel 2013 Data Analysis ToolPack (Microsoft Corporation, Redmond, WA) was used for *F*-test two-sample for variances applying the Bonferroni correction for experiment-wide significance level. For all statistical comparisons significance was established at $P < .05$.

RESULTS

The extent of availability of Cx43 hemichannels in EG affects gut motility *in vivo*

Cx43 hemichannels play a role in physiological colonic motility *in vivo* (McClain et al. 2014). However, it is unknown whether the gut function can be affected by an increased availability of Cx43 hemichannels, as seen in Cx43^{G138R} point mutation which hampers gap-junctional coupling and leads to an ~ 2-fold augmented ATP release via unopposed connexons (Dobrowolski et al. 2008). To address this issue, we used tamoxifen-inducible Cx43^{G138R} mice to express this Cx43 point mutation in GFAP positive EG. We also used tamoxifen-inducible Cx43-icKD mice to reduce the expression of Cx43 and consequently the availability of hemichannels in EG (Figure 1A and Supplementary Figure 1A). Tamoxifen administration induced the deletion of the floxed *wild-type* Cx43 gene in both Cx43^{G138R} and Cx43-icKD mice and concurrent expression of either both enhanced green fluorescent protein (eGFP) and Cx43^{G138R} (Dobrowolski et al. 2008), or only the fluorescent marker enhanced cyan fluorescent protein (eCFP) (Degen et al. 2012), respectively (Figure 1A). Specificity of DNA recombination was confirmed by the expression of fluorescent proteins in the GFAP positive cells of the ENS, i.e. EG (Figure 1B). Only hemizygous animals were used to avoid compensatory mechanisms due to complete Cx43 knockout and/or inhibition of gap junction coupling. It should be noted that homotypic Cx43^{G138R} connexons do not functionally couple, but can form gap junctions

when wild-type Cx43 is expressed. Consequently, combination of these two models allows us to obtain EG with a dynamic range for Cx43 hemichannel activity/expression for a single allele, i.e. reduction or enhancement in Cx43-icKD or Cx43^{G138R} mice, respectively.

In vivo experiments were performed one week after tamoxifen administration. Body weight and total fecal pellet count were similar among the mice in all three groups: control, Cx43^{G138R}, and Cx43-icKD (Supplemental Figure 2A–B). Furthermore, there was no significant difference in pellet weight and composition (Figure 2A–B). Due to reduced intestine length in Cx43^{G138R} and Cx43-icKD mice when compared to controls (Supplemental Figure 2C–E), the measurements of *in vivo* transit assays were expressed as transit velocities (Figure 2C), as opposed to plain transit times. While the whole GI transit velocity was similar between the groups, the upper GI transit and colon transit velocities were significantly higher in Cx43^{G138R} mice in comparison to the Cx43-icKD group (Figure 2C). Our results indicate that the molecular genetic manipulations of a single allele resulting in modulation of the expression level/availability of Cx43 hemichannels in EG led to changes *in vivo* gut motility; a decreased availability hampers motility, while an increase availability of hemichannels increases gut motility. As the latter manipulation is based on a Cx43 point mutation seen in ODDD, these are clinically relevant findings.

Inhibition of Ca²⁺-dependent exocytosis in EG affects fecal pellet composition

Regulated exocytosis is triggered by an increase in [Ca²⁺]_i and requires the activity of a set of proteins, collectively known as SNAREs. These proteins are necessary for the fusion of secretory vesicles with the plasma membrane, which warrants release of the intravesicular content, i.e. transmitters and modulators, into the extracellular space. One of the SNAREs, synaptobrevin 2 (Sb2), is widely expressed in neurons and non-neuronal tissue (Rossetto et al. 1996). This vesicular protein is necessary for Ca²⁺-dependent exocytosis from astrocytes of the CNS (Jeftinija et al. 1997) and hampering its operation, using the dominant negative SNARE (dnSNARE) approach, leads to the reduction of ATP release from astrocytes *in situ* (Pascual et al. 2005). We tested whether EG utilize the exocytotic molecular machinery to regulate gut physiology. Since activation of EG mainly leads to Ca²⁺ release from intracellular stores via IP₃R (Gulbrandsen and Sharkey 2009; Kimball and Mulholland 1996), we also tested if IP₃ signaling is involved in this process.

We used dnSNARE (Pascual et al. 2005) and VIPP (Marpegan et al. 2011) mice containing tet operon (*tetO*) driven cassettes that encode either the cytoplasmic domain of Sb2 (dnSNARE) and eGFP (expression marker) or the fusion protein between Venus fluorescent protein and IP₃ 5-phosphatase (VIPP), respectively (Figure 3A). Transcription of each *tetO* driven cassette is controlled by tetracycline transactivator, expression of which is driven by the *hGFAP* promoter fragment. Consequently, after the removal of tetracycline analog doxycycline from animals diet, the cistron products in the gut are only expressed in the GFAP positive cells, i.e. EG of the ENS (Figure 3B), which leads to tampering directly with the exocytotic secretory machinery (dnSNARE mice) or with the upstream Ca²⁺/IP₃ signaling (VIPP mice). The experiments below were conducted on animals that were withdrawn from doxycycline containing food for 6–7 weeks.

Body weight, total fecal pellet count and intestine length were not significantly different between the mice in all three groups: control, dnSNARE and VIPP (Supplementary Figure 3). Additionally, pellet weight (Figure 4A) and *in vivo* gut transit velocities did not significantly differ among the groups (Figure 4C). However, there was a significant increase in the fluid content of pellets originating from dnSNARE mice when compared to that of the control group (Figure 4C); the VIPP group showed only a trend in an increase of median fluid content.

Overall, these results indicate that exocytotic gliotransmitter release from EG affects fluid secretion/reabsorption, while is not involved in regulation of gut motility *in vivo*. However, there could still be subtle effects on gut motility exerted solely via the ENS, otherwise masked in our *in vivo* approach. We next addressed this issue using an *ex vivo* approach.

Gut contractions are modifiable by both forms of local gliotransmission in an *ex vivo* colon preparation

Our *in vivo* data reported of the gut function governed by the complex interplay between the local ENS activity along with that of reflex loops at the level of sympathetic ganglia and the CNS [reviewed in (Furness et al. 2014)]. To assess gut motility solely driven by local ENS activity in the complete absence of the extrinsic innervation, we utilized an *ex vivo* preparation of a perfused isolated colon segment combined with video recording, generation of CMMC spatiotemporal maps (Figures 5A and 6A) and analysis of CMMC characteristics (Roberts et al. 2007) (for details see Supplementary Figure 4). The characteristics of CMMCs were differentially affected by molecular genetics manipulations of two forms of local gliotransmission.

Baseline colon width and that during CMMCs, along with relative amplitude and duration of CMMCs were unaffected in Cx43^{G138R} and Cx43-icKD colon samples when compared to their controls (Figure 5B, B' and C, respectively). However, colons from Cx43-icKD mice showed a decrease in CMMC dwell time, velocity and frequency when compared to colons isolated from control animals (Figure 5C', D and E, respectively). Additionally, CMMC dwell time in Cx43-icKD colons was significantly smaller than that recorded from Cx43^{G138R} colons (Figure 5C'). These findings are in a good agreement with the *in vivo* gut transit assays (Figure 2C).

CMMCs were also affected by EG specific inhibition of exocytotic molecular machinery and IP₃-dependent Ca²⁺ signaling (Figure 6). When compared to control, colons from dnSNARE mice showed a significant reduction in baseline colon width and CMMC velocity (Figure 6B and D, respectively), while colons from VIPP mice had significantly decreased relative CMMC amplitude (Figure 6B') and CMMC frequency (Figure 6E). All the other CMMC characteristics were statistically insignificant in colons isolated from dnSNARE, VIPP and control mice. Thus, gut motility *ex vivo*, which is solely driven by the ENS, is modulated by EG exocytosis and IP₃-dependent Ca²⁺ signaling. In experiments *in vivo*, this effect seems to be masked due to extrinsic innervation of the gut and/or tampering with GFAP-positive cells residing out of the gut (see discussion).

Taken together, our results suggest that EG diversely modulate CMMCs using two distinct mechanism of gliotransmitter release through Cx43 hemichannels and Ca^{2+} -dependent exocytosis.

DISCUSSION

This study provides evidence for the role of two modes of local gliotransmission in gut physiology *in vivo* and colonic contractions *ex vivo*. We used genetically modified mice to assess the role of EG transmission. The cell specificity of molecular genetics was achieved using the fragment of GFAP promotor. This was combined with inducible gene expression/excision to guard against otherwise unwanted developmental effects. For instance, vesicular SNAREs are required for proper migration of neural crest cells, which differentiate into enteric neurons and glia (Vohra et al. 2006), thus, tampering with SNAREs in early development would obscure the interpretation of data. Albeit we selectively targeted EG in the ENS, other GFAP expressing cells regardless of their locations would be affected in our mouse models. Thus, the observed *in vivo* effects could be in part due to modified gliotransmission from astrocytes. These glial cells play a role in the operation of the gut; e.g., astrocytes in the hindbrain play a role in gastric vagal reflex circuits and gastric motility (Hermann et al. 2014). Consequently, we implemented an *ex vivo* approach using isolated colons, which are devoid of any extrinsic input and solely operate under the control of local ENS circuits. This approach unmasked the role of glial exocytosis and $\text{Ca}^{2+}/\text{IP}_3$ signaling in gut contractions otherwise not seen in the *in vivo* approach.

This “masking concept” begs for some speculation on its possible mechanism; we briefly explore one possible hypothetical scenario. EG residing in proximity to enteric neurons receive direct nerve fiber inputs through synapse-like, synaptoid contacts (Gabella 1972). Through these contacts and/or volume transmission, EG can respond to the activity of extrinsic sympathetic fibers by an increase in cytosolic Ca^{2+} levels (Gulbransen et al. 2010). EG can also display Ca^{2+} dynamic in the absence of extrinsic fibers input (McClain et al. 2014). In either case, EG Ca^{2+} excitability can cause gliotransmission. As in the case of astrocytes (Araque et al. 1999; Zorec et al. 2012), gliotransmission could modulate synaptic transmission and plasticity. However, in the absence of extrinsic input, modulatory effect by EG on the local circuitry could differ, quantitatively and/or qualitatively (being even opposing), perhaps due to difference in spatio-temporal nature of heterocellular signaling. For instance, the local circuit may be susceptible to modulation by gliotransmission that is time-dependent on the activity of the specific extrinsic input. Similarly, the external input may recruit a subset of EG release sites, the spatio-temporal organization of which may differ from that of sites recruited without this input and rather based on intrinsic ENS or spontaneous EG activity. Certainly, the effects that EG may have on gut reflexes in presence/absence of the extrinsic nerve input(s) represent a research area that needs future experimental work.

Pre-existing literature highly favors ATP as a gliotransmitter being released via Cx43 hemichannel (McClain et al. 2014) or exocytosis (Pascual et al. 2005) in animal models used and to carry out the effects we observed. ATP can be measured in isolated gastrointestinal tissue using enzyme-linked microelectrodes with excellent sensitivity (Patel 2014; Patel et

al. 2011); however, spatial resolution allowing for resolving the cellular source (e.g., neuron vs glia) of ATP using this approach has not been attained, yet. While imaging of ATP at the cellular level is possible in isolated tissue (e.g., retina) (Newman 2004), it will likely be technically challenging task in the gut *in vivo* and we have not directly measured it. By no means have we claimed that the reported effects were mediated by ATP. Rather, a variety of gliotransmitters released by either, or both mechanisms (Malarkey and Parpura 2009) could mediate them. For instance, glutamate can modulate GI motility (Filpa et al. 2016). The identity of gliotransmitter(s) utilized in EG-mediated modulation of gut physiology remains to be studied.

Release through Cx43 hemichannels on astrocytes in the CNS likely occurs only in pathological conditions. Namely, Cx43 hemichannel activity in astrocytes appears at odds with their voltage sensitivity opening as membrane potentials become positive (Trexler et al. 1996); astrocytes normally do not depolarize to such extent unless under pathophysiological conditions such as stroke and ischemia. This raises an issue of whether the results we observed might reflect upon the importance of gap junctions growing the diffusion capacity of molecules throughout the EG syncytium as opposed to Cx43-mediated gliotransmission. While diffusion capacity could play a role, glial Cx43 hemichannels have been reported fully operational in the ENS and to play a role in gut physiology (McClain et al. 2014).

Cx43 knock-down hemizygous approach we used here differs from our prior work using Cx43 knock-out homozygous animals (McClain et al. 2014). The Cx43 floxed animals not only differed in zygosity but were also made and sourced in a different fashion. At present work the excision of Cx43 gene drives the *in locus* expression of a fluorescent marker, while in the previous work the Cre-recombination was indirectly reported by the excisions of a stop codon downstream of the *Rosa26* promoter driving the expression of a fluorescent marker. Of note, the *hGFAP::CreER^{T2}* parental line was obtained from a different source. Nonetheless, the present data using the Cx43 knockdown approach is qualitatively similar to that previously obtained using the knock out approach, although the effects seem less robust. For example, the statistically significant reduction in gut motility when compared to controls was obtained in Cx43 knockout animals [Fig. 4A of (McClain et al. 2014)], while the present knock-down approach showed only a trend, while the statistical significance occurred when comparison was made to Cx43 mutant with enhanced hemichannel activity but not to control (Fig. 2C). This seemingly incongruent findings could be perhaps simply explained by a dynamic range; the comparison of excision of both alleles to wild-type control in previous work would have an equivalent effect on gut function as that seen by comparison between a single allele excision to a single mutated Cx43 allele knock-in. Additionally, the effect of decreased fecal pellet weight and increased fluid content seen in previous work [Fig. 4B of (McClain et al. 2014)], was not obtained in Cx43 knockdown here (Fig 2B). However, such an effect on mucosal secretion/reabsorption is seen here in hemizygous dnSNARE animals.

Be that as it may, our data might be providing for a novel hypothesis that these two gliotransmission mechanisms are, in fact, linked: connexin43 (Cx43), the major constituent of hemi-channels in EG, and exocytotic release sites interact, so that perturbing one would affect the other; *ex vivo*, this is perhaps evident from the common effects on CMMC

frequency and velocity (Fig. 5D, E and Fig. 6D, E). Cx43 hemichannels are not only permeable to ATP being released but also to Ca^{2+} , which enters from the extracellular space into the cytosol of EG *in situ* (McClain et al. 2014). This Cx43-mediated increase in cytosolic Ca^{2+} could in turn trigger regulated exocytosis. Thus, a manipulation that would affect availability/activity of Cx43 hemichannels could also affect vesicular release. Vice versa, dnSNARE manipulation hampering gliotransmitter release could also affect the delivery of vesicular membrane cargo including that of Cx43 to the plasma membrane, as well as the retrieval/recycling of Cx43 from the plasma membrane. In addition, Cx43 hemichannels are regulated by cytosolic Ca^{2+} in a bimodal temporal fashion; after initial enhancement of their permeability by increasing $[\text{Ca}^{2+}]_i$, the sustained presence of this ion leads to inhibition of Cx43 hemichannel permeability (De Bock et al. 2012). While this raises an interesting possibility for self-regulation of Cx43 hemichannels, it also means that IP_3 -dependent Ca^{2+} response in VIPP mice, besides hampering Ca^{2+} -dependent exocytosis, could also affect the activity of Cx43 hemichannels. Indeed, we expect the proposed cross-talk to be multifaceted as spatiotemporal Ca^{2+} dynamics represent a complex and adaptive process with various effector proteins having different sensitivity to concentration and duration of Ca^{2+} signals (Berridge et al. 2003). Of course, as gliotransmitter gets released into the extracellular space by either mechanism, the additional complexity presents itself in paracrine and autocrine signaling as EG express variety of receptors for transmitters. Furthermore, in the case of ATP, as per its extracellular degradation generating additional signaling species in the extracellular space, such as adenosine and ADP, there could be diversification of purinergic signaling events.

Cx43^{G138R} mutation, used here in one of the model animals, is found in the ODDD syndrome. This disorder is caused by various mutations of the Cx43 coding gene that either enhance (e.g., Cx43^{G138R}) or reduce Cx43 hemichannel activity (Dobrowolski et al. 2008). Our results indicate that variable bowel disturbances in ODDD patients (Loddenkemper et al. 2002) could stem, at least in part, from the direct modification of EG Cx43 hemichannel availability, activity, permeability and/or coupling.

EG can respond to ADP/ATP by P2Y_{1/4} receptor activation, which leads to IP_3 -signaling and Ca^{2+} release from the endoplasmic reticulum (McClain et al. 2014). Thus, we investigated VIPP mice. However, there could be additional sources of Ca^{2+} for cytosolic Ca^{2+} increase necessary and sufficient to trigger Ca^{2+} -dependent exocytotic gliotransmitter release. If astrocytes be our guide [reviewed in (Parpura et al. 2011; Parpura and Verkhratsky 2012)], besides IP_3 receptors, ryanodine receptors/channels could serve as conduits for Ca^{2+} delivery from the ER to the cytosol of EG. The accumulation of Ca^{2+} in the cytosol could also be caused by the entry of Ca^{2+} from the extracellular space via voltage-gated Ca^{2+} channels, store-operated Ca^{2+} entry and the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Furthermore, cytosolic Ca^{2+} levels could be modulated by mitochondria that can take-up Ca^{2+} via the Ca^{2+} uniporter during the cytosolic Ca^{2+} increase and as cytosolic Ca^{2+} declines due to extruding mechanisms, most notably the Ca^{2+} ATPase/pump of the ER store, Ca^{2+} would be slowly released by mitochondria into the cytosol via mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger and by the formation of the mitochondrial permeability transition pore. The role of these and perhaps other molecular entities in sourcing Ca^{2+} for EG transmission is yet to be investigated.

The observed physiological effects of gliotransmission on the gut motility depend on the activity of enteric neurons (McClain et al. 2015). Cx43 hemichannel might also be a conduit for release of metabolites and neurotransmitter precursors, such as lactate and glutamine, which can affect normal neuronal functioning (Giaume et al. 2004). Furthermore, it is possible that here observed effects of gliotransmission are also regulated, at least in part, on the afferent side of gut local reflex loops – perhaps by communication between EG with enteroendocrine cells (Bohorquez et al. 2014) or mechanosensitive enteric neurons (Mazzuoli-Weber and Schemann 2015). These are all open issues that need to be experimentally addressed in future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

Enteric glial cells modulate neuronally regulated reflexes that govern gut functions. Two distinct mechanisms of enteric gliotransmission, connexin 43 (Cx43) hemichannel vs. Ca^{2+} -dependent exocytosis, differentially affect gut physiology.

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Figure 1.

Cx43^{G138R} and Cx43-icKD animal models. **A.** Cx43^{G138R} and Cx43-icKD mice utilize inducible Cre-lox system and concurrently report on the Cre recombinase activity at the particular locus; dashed horizontal line separates illustration of these two models. Both models utilize a fragment of human glial fibrillary acidic protein promoter (*hGFAP*) to drive the expression of a fusion protein between Cre recombinase and the mutated estrogen receptor (ER^{T2}) responsive to the active 4-hydroxy form/metabolite of tamoxifen. The other parental lines had floxed *wild-type* Cx43 gene followed by either: (top) bicistronic cassette consisting of mutated Cx43 (Cx43^{G138R}), an internal ribosome entry site (IRES) sequence and enhanced green fluorescent protein (eGFP) gene; or (bottom) enhanced cyan fluorescent protein (eCFP). Tamoxifen administration (+Tam) induces Cre-lox dependent deletion of the floxed *wild-type* Cx43 gene in both models and consequently results in expression of either both Cx43^{G138R} and eGFP (top) or eCFP (bottom). **B.** Reporter expression in the enteric nervous system (ENS). Tamoxifen treated Cx43^{G138R} (top) or Cx43-icKD (bottom) mice express eGFP or eCFP (respectively, left panel) specifically in GFAP expressing cells (middle panel) as seen by the colocalization of the fluorescent protein expression and GFAP immunoreactivity (-ir) (merge). Scale bar, 10 μm.

Figure 2.

Fecal pellet composition and *in vivo* gut motility obtained from Cx43^{G138R} and Cx43-icKD mice. **A–B.** Total pellet weight, wet or dry (A), and liquid content (B). **C.** Gut motility *in vivo*, shown as velocities of the whole gastrointestinal (GI) tract (left), upper GI tract (middle) and distal colon (right). Data are shown as median $\pm$ interquartile range (IQR). *, $P < .05$, Kruskal-Wallis one-way analysis of variance (KWA) followed by Dunn's Multiple Comparisons (DMC). Number of animals per group is given parenthetically in A (for A and B) and C.

Figure 3.

DnSNARE and VIPP animal models. **A.** DnSNARE (top) and VIPP (bottom) mice utilize tetracycline-off system. Both models have *hGFAP::tTA* transgene utilizing *hGFAP* promoter fragment to drive the expression of tetracycline transactivator (tTA). This transactivator cannot bind tetracycline operon (*tetO*) in the presence of doxycycline (Dox), a tetracycline analogue. Dox removal (-Dox) permits expression of transgenes driven by *tetO*, i.e. *dnSNARE* and *eGFP* (top), encoding a dominant negative SNARE (the cytoplasmic tail of synaptobrevin 2 that blocks exocytosis) and an expression marker, respectively; or *VIPP* (bottom), encoding a fusion protein between Venus fluorescent protein (expression marker) and the cytosolic type I IP₃ 5-phosphatase (IPP). **B.** Reporter expression in the ENS. In the absence of Dox, dnSNARE (top) or VIPP (bottom) animals express eGFP or Venus (left column), respectively. This expression occurs specifically in GFAP expressing EG (middle column), as indicated by the colocalization of the fluorescent protein expression and GFAP-ir (merge). Scale bar, 10 μ m.

Figure 4.

Fecal pellet composition and *in vivo* gut motility obtained from dnSNARE and VIPP mice.

A–B. Total pellet weight, wet or dry (A), and liquid content (B). **C.** Gut motility *in vivo*, shown as velocities of the whole GI tract (left), upper GI tract (middle) and distal colon (right). Data are shown as median $\pm$ IQR. *, $P < .05$ versus control (KWA followed by DMC). Number of animals per group is given parenthetically in A (for A and B) and C.

Figure 5.

Cx43-dependent gliotransmission affects a subset of colonic migrating motor complexes (CMMCs) characteristics. **A**. Representative spatiotemporal (ST) maps obtained from isolated colons originating from control, Cx43^{G138R} and Cx43-icKD mice (left to right). ST maps depict the colon width (pseudocolor coded bar) along the colon length (x axis) over time (y axis). **B–E**. CMMC properties: (B) baseline colon width (blue), i.e. colon width between the CMMCs, and the colon width during CMMC (red), i.e. the colon width during the maximal contraction; (B') relative CMMC amplitude; (C) CMMC duration; (C') CMMC dwell time; (D) CMMC velocity; and (E) CMMC frequency. For details about ST map generation and analysis see Supplementary Figure 4. *, $P < .05$; **, $P < .01$; versus control with the exception of the comparison indicated with the bracket, which points to the

specific difference between the groups [KWA followed by Newman-Keuls Multiple Comparisons (NKMC)]. All groups had equal number of colons/animals (n=6).

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Figure 6.

Enteric glial exocytosis and IP₃-dependent calcium signaling have modulatory roles on CMMCs. **A.** Representative ST maps obtained from isolated colons originating from control, dnSNARE and VIPP mice. As in Figure 5, changes of the colon gut width are pseudocolor coded. **B–E.** CMMC properties: (B) baseline colon width (blue) and that during CMMCs (red); (B') relative CMMC amplitude; (C) CMMC duration; (C') CMMC dwell time; (D) CMMC velocity; and (E) CMMC frequency. *, $P < .05$ versus control (KWA followed by NKMC). #, $P < .05$ versus control (F -test two-sample for variances with the Bonferroni correction for experiment-wide significance level). All groups had equal number of colons/animals ($n=4$).