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RESEARCH ARTICLE

Cell and Animal Models of Gastrointestinal Disease

Characterization of Fabry disease-associated lyso-Gb_3 on mouse colonic ion transport and motility

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Abstract

Fabry disease (FD) is a rare X-linked lysosomal storage disorder caused by a deficiency in α-galactosidase A leading to the accumulation of globotriaosylceramide (Gb_3) and subsequent increase in globotriaosylsphingosine (lyso-Gb_3) in different cells and organs, including the gastrointestinal (GI) tract. GI symptoms represent some of the earliest manifestations of FD and significantly impact quality of life. The origin of these symptoms is complex, and the exact mechanisms remain poorly understood. Here, we sought to determine whether lyso-Gb_3 contributes to the pathophysiology of GI symptoms associated with FD by examining its effects on mouse colonic ion transport and motility ex vivo using Ussing chambers and organ baths, respectively. Lyso-Gb_3 significantly increased colonic baseline short-circuit current (I_{sc}). This increase in I_{sc} was insensitive to inhibition of the cystic fibrosis transmembrane conductance regulator and Na-K-Cl cotransporter 1, suggesting that the increase in I_{sc} is Cl^- ion independent. This response was also insensitive to inhibition by the neurotoxin, tetrodotoxin. In addition, pretreatment with lyso-Gb_3 did not significantly influence subsequent responses to either veratridine or capsaicin implying that the response to lyso-Gb_3 does not involve the enteric nervous system. In terms of colonic motility, lyso-Gb_3 did not significantly influence colonic tone, spontaneous contractility, or cholinergic-induced contractions. These data suggest that lyso-Gb_3 significantly influences ion transport in mouse colon, but that accumulation of Gb_3 may be a prerequisite for the more pronounced disturbances in GI physiology characteristic of FD.

NEW & NOTEWORTHY Fabry disease-associated lyso-Gb_3 significantly influences mouse colonic ion transport in a Cl^- ion-independent manner.

biomarker; Fabry disease; gastrointestinal; lyso-Gb3; Ussing chamber

INTRODUCTION

Fabry disease (FD) is a progressive X-linked lysosomal storage disorder characterized by dysfunction of multiple organs including the gastrointestinal (GI) tract. Although the exact mechanisms that lead to multiorgan dysfunction are not yet completely understood, the characteristic accumulation of globotriaosylceramide (Gb_3), due to a genetic deficiency or absence of its metabolizing enzyme α-galactosidase A (α-GalA), is likely involved (1, 2). The first symptoms of classic FD usually appear in childhood and early adulthood, and ultimately lead to organ failure and premature death (3, 4). Moreover, excess Gb_3 is metabolized by ceramidase, which gives rise to its deacetylated derivative, globotriaosylsphingosine known as lyso-Gb_3 (5). Plasma lyso-Gb_3 has been identified as a sensitive diagnostic marker of FD (6, 7), but its role in the pathophysiology of the disease is less clear.

GI symptoms are among the earliest reported by patients with FD, are the most difficult to interpret, and occur in ~70% of FD males (8, 9). Of the GI symptoms, abdominal pain and diarrhea are the most common (10–12). Significantly, these symptoms have a substantial impact on quality of life (13, 14). In a recent molecular and morphological characterization of the GI tract of the α-GalA knockout mouse model of FD (α-$GalA^{-/0}$ mice), Masotti and coworkers demonstrated the presence of Gb_3 deposits in the GI tract (15). In particular, the structure of the enteric nervous system (ENS) was found to be altered, characterized by fragmented nerve patterns and enlarged myenteric ganglia (15). This is consistent with those observed in patients with FD (16). We also recently determined that α-$GalA^{-/0}$ mice display a diarrhea-like phenotype characterized by increased fecal water content and decreased transit time (17), suggesting a link between Gb_3 deposits within GI tissue and GI dysfunction.

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Despite the observation that intestinal lyso-Gb_3 concentrations exceed those in plasma of a mouse model of FD (18), a causal relationship between lyso-Gb_3 and GI symptomology has not yet been explored. Other studies have demonstrated that lyso-Gb_3 can promote Notch1-mediated inflammation and fibrogenesis associated with kidney dysfunction (19) and that lyso-Gb_3 can sensitize peripheral nociceptive neurons related to the development of pain (20). Supporting our hypothesis that lyso-Gb_3 may play a role in the pathophysiology of GI symptoms associated with FD, enzymatic replacement therapy has been shown to reduce not only the load of lyso-Gb_3 but also the severity of GI symptoms (12, 21, 22). Therefore, we sought to determine whether lyso-Gb_3 may play a contributory role in the pathophysiology of GI symptoms associated with FD by examining its effects on colonic ion transport and motility ex vivo.

MATERIALS AND METHODS

Materials

Lyso-Gb_3 was purchased from Avanti Polar Lipids (Alabama, USA), PPQ-102 from Medchemexpress (New Jersey, USA), and tetrodotoxin (TTx) from Tocris (Bristol, UK). All other drugs were obtained from Merck Life Science Limited (Wicklow, Ireland). Lyso-Gb_3, forskolin, furosemide, PPQ-102, and veratridine stocks were prepared in DMSO. Carbachol (CCh) was diluted in water. Capsaicin stock was prepared in 95% ethanol. The concentrations of CCh, forskolin, TTx, and veratridine were determined based on those we have previously used in mouse colon (23, 24). Capsaicin (3 μM) was chosen as this concentration has previously been shown to activate nociceptive enteric neurons in mouse colon (25). Concentrations of furosemide in the range 10 μM–1 mM have been used previously to investigate the contribution of the Na-K-Cl cotransporter 1 (NKCC1) to Cl^- ion transport in mouse intestine and colon (26–28) and concentrations of 20–50 μM PPQ-102 have been shown to inhibit the forskolin-induced increase in I_{sc} in bronchial and pancreatic ductal epithelial cells (29, 30).

Animals

Adult male C57BL/6J mice (18–25 g; aged 10–18 wk) were purchased from Envigo (Bicester, UK) and were group-housed and maintained on a 12-h:12-h dark/light cycle (0700–1900 h) with a room temperature of 22 ± 1°C. Standard rodent chow and water were available ad libitum. All experimental procedures were conducted in accordance with European Community Council Directive (86/609/EEC) and approved by the local University College Cork Animal Experimentation Ethics Committee (No. 21-003).

Ussing Chamber Experiments

Mice were euthanized by decapitation. A laparotomy was performed, and the entire intestine was carefully removed and placed into carbogenated Krebs solution, consisting of 1.2 mM NaH_2PO_4, 117 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 11 mM $CaCl_2$, and 10 mM D-glucose. The colon was dissected and flushed of fecal matter. Seromuscular stripping was carried out by blunt dissection under a stereomicroscope, and both the longitudinal and circular muscle layers were removed. The resulting mucosa-submucosa preparations were mounted in Ussing chambers with an area of 0.12 cm^2 (Harvard Apparatus, Kent, UK). Both chambers were filled with 4 mL of Krebs solution, maintained at 37°C, and oxygenated with carbogen gas (95% O_2-5% CO_2). Up to six colonic preparations were obtained from each mouse. Tissues were then voltage-clamped at 0 mV using an automatic voltage clamp (DVC-1000; World Precision Instruments, Hertfordshire, UK). Short-circuit current (I_{sc}) was continuously recorded on a computer using LabTrax data acquisition hardware and LabScribe software (World Precision Instruments). Once a stable baseline was achieved, baseline I_{sc} and transepithelial electrical resistance (TEER; Ω·cm^2) were recorded. TEER was calculated using Ohm's law by recording the change in I_{sc} evoked by a 2-mV pulse. All additions were made to the basolateral side of the tissue except for PPQ-102, which was added apically.

Plasma lyso-Gb_3 may reach values of 400–600 nM, and in mice, intestinal values can be even higher (18). Therefore, we examined the effects of clinically relevant concentrations of lyso-Gb_3 (1 μM and 3 μM; 30 min) on baseline I_{sc}. Given that 1 μM lyso-Gb_3 did not significantly influence I_{sc} relative to vehicle (vehicle, 0.9 ± 1.8 μA·cm^{-2}, $n = 10$ vs. lyso-Gb_3, 4.0 ± 0.6 μA·cm^{-2}, $n = 8$, $P > 0.05$), all further experiments were conducted with 3 μM lyso-Gb_3. Subsequent responses to CCh (100 μM) and forskolin (10 μM) in the presence of vehicle or lyso-Gb_3 were also determined. To investigate whether the change in I_{sc} induced by lyso-Gb_3 was Cl^- ion dependent, tissues were pretreated for 15 min with either PPQ-102 (50 μM) or furosemide (100 μM). To investigate the involvement of enteric nerves, tissues were either pretreated with the neurotoxin, TTx (300 nM) for 15 min and then with lyso-Gb_3 or were preincubated with lyso-Gb_3 for 30 min followed by either veratridine (30 μM) or capsaicin (3 μM), and resultant changes in I_{sc} were recorded (see Supplemental Fig. S1 for experimental protocol; all Supplemental material is available at https://doi.org/10.6084/m9.figshare.27170994).

Organ Bath Experiments

Mice were euthanized as described under *Ussing Chamber Experiments*. The proximal colon was carefully removed, and the tissue was suspended from a tension transducer under 1 g of tension in an organ bath filled with 10 mL of Krebs solution maintained at 37°C and bubbled with carbogen gas. The tissue was then allowed to equilibrate (1 min) before reagents were added to the bath. Changes in tension were recorded and analyzed using Powerlab and LabChart 8 (ADInstruments, Oxford, UK). The effects of lyso-Gb_3 (3 μM) on colonic tone, spontaneous and CCh (100 μM)-induced contractility were measured. The effect of lyso-Gb_3 on tone was determined relative to basal tone [Δforce (g) of basal tone]. Spontaneous contractility is quoted as area under the curve per second (AUC/s).

Statistical Analysis

Data were analyzed using GraphPad Prism for Windows (v.10, GraphPad Software, Massachusetts, USA). Data are presented as means ± SE. Student's unpaired or paired t tests were used throughout. $P < 0.05$ was considered significant.

RESULTS

Lyso-Gb_3 (3 μM) significantly increased I_{sc} after basolateral application relative to vehicle control (Fig. 1, *A–C*) and had no significant effect on TEER (Fig. 1*D*). To determine whether the lyso-Gb_3-induced increase in I_{sc} was Cl^- ion dependent, we examined the effects of the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor, PPQ-102 (50 μM) and the NKCC1 inhibitor, furosemide (100 μM) on this response. Neither inhibition of the CFTR nor NKCC1 significantly influenced the subsequent response to lyso-Gb_3 (Fig. 1, *E* and *F*). In terms of intracellular signaling, responses to forskolin (10 μM), which induces an increase in I_{sc} through activation of adenylate cyclase, and to the cholinomimetic, CCh (100 μM), which increases intracellular Ca^{2+}, were also unaffected by lyso-Gb_3 pretreatment (Fig. 2, *A* and *B*).

Inhibiting enteric neural Na^+ channels with the neurotoxin, TTx (300 nM) also had no significant effect on the subsequent response to lyso-Gb_3 (3 μM; Fig. 3*A*). Furthermore, pretreatment with lyso-Gb_3 did not affect the tissue response to veratridine (30 μM), an activator of voltage-dependent Na^+ channels (Fig. 3*B*). Given that lyso-Gb_3 has been shown to increase intracellular calcium in capsaicin-sensitive neurons in vitro (20), we also sought to determine whether lyso-Gb_3 could influence capsaicin (3 μM)-evoked changes in I_{sc} in mouse colon. With respect to the nature of the capsaicin response, tissues responded with either an increase in I_{sc} (Fig. 3*Ci*), a biphasic change in I_{sc} (Fig. 3*Cii*), or a decrease in I_{sc} (Fig. 3*Ciii*), similar to that recently described by Evans and colleagues in mouse colon (31). The proportion of tissues responding with either an increase [primary (1°) phase only], biphasic change [1° and secondary (2°) phase], or a decrease (2° phase only) in I_{sc} did not differ between vehicle- or lyso-Gb_3-treated tissues (Supplemental Fig. S2*A*). Given that the 1° phase of the response to capsaicin was sensitive to neurokinin 1 receptor antagonism in mouse colon (31), suggesting that this response is sensory, we pooled the data from tissues that exhibited an increase in I_{sc} only with the 1° phase data from tissues which responded with a biphasic change in I_{sc}. Pretreatment with lyso-Gb_3 did not have a significant effect on this response. When data were disaggregated into 1° phase only (Supplemental Fig. S2*B*), biphasic (1° and 2° phases; Supplemental Fig. S2*C*), and 2° phase only (Supplemental Fig. S2*D*), no significant differences between vehicle and lyso-Gb3-treated tissues were detected, although there was a tendency for the 2° response to be greater in the lyso-Gb_3-treated group.

Given that we recently described altered GI transit time as a characteristic feature of the α-$GalA^{-/0}$ mouse model of FD (17), we sought to investigate whether lyso-Gb_3 can directly influence colonic motility. We examined the effects of lyso-Gb_3 (3 μM) on colonic tone, spontaneous contractility, and CCh (100 μM)-induced contractility in organ baths (Fig. 4*A*). Despite the overt changes in GI function observed in the α-$GalA^{-/0}$ mouse (17), lyso-Gb_3 did not significantly influence colonic tone (Fig. 4*B*), spontaneous contractility (Fig. 4*C*), or cholinergic-induced contractility (Fig. 4*D*).

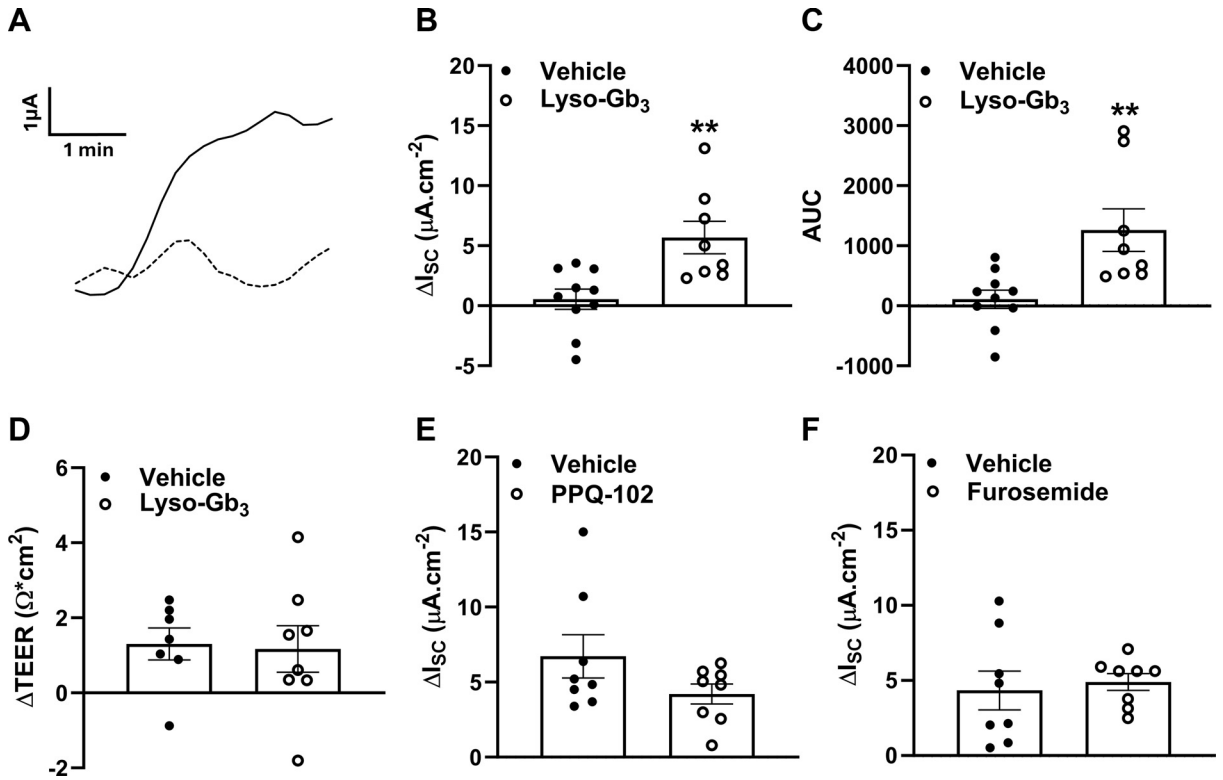

Figure 1. Representative response to vehicle (dashed lined; 0.1% DMSO) and lyso-Gb_3 (solid line; 3 μM; *A*) from mouse colonic mucosa-submucosa tissue preparations. Lyso-Gb_3 significantly increased short-circuit current (I_{sc}; *B*) and area under the curve (AUC; *C*) but had no effect on transepithelial electrical resistance (TEER; *D*). Neither inhibition of the cystic fibrosis transmembrane regulator (CFTR) with PPQ-102 (50 μM; *E*) nor of the Na-K-Cl cotransporter 1 (NKCC1) with furosemide (100 μM; *F*) influenced the lyso-Gb_3-induced increase in I_{sc}. Data are presented as means ± SE; $n = 7–10$ mice. $^{**}P < 0.01$, Student's *t* test.

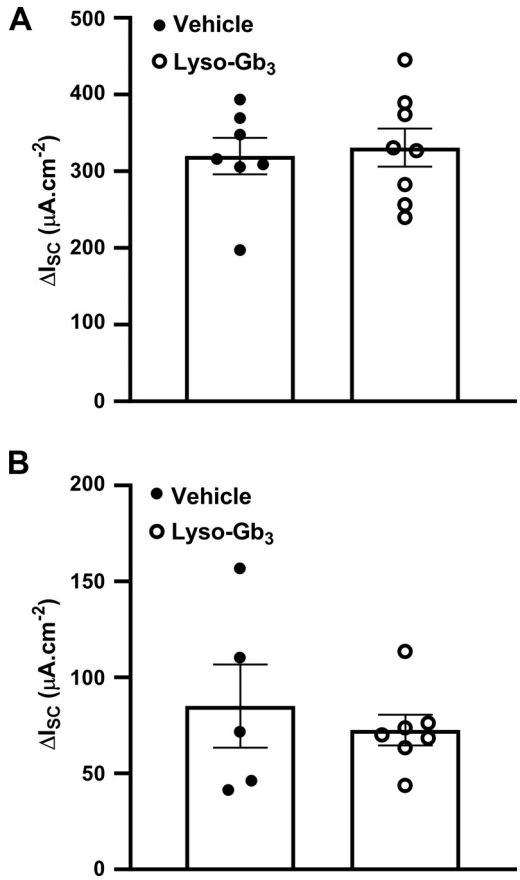

Figure 2. Neither cAMP (*A*)- nor calcium (*B*)-mediated ion transport, stimulated by forskolin (10 μM) or carbachol (100 μM), respectively, were significantly influenced by lyso-Gb_3 (3 μM). Data are presented as means ± SE; $n = 5–8$ mice.

DISCUSSION

Little is known regarding the effects of lyso-Gb_3 on peripheral tissues, particularly the GI tract. The primary goal of this work was to investigate whether lyso-Gb_3 contributes to the pathophysiology of GI symptoms associated with the disease. Our findings demonstrate that lyso-Gb_3 can significantly influence colonic ion transport, suggesting that local changes in the concentration of lyso-Gb_3 have the potential to contribute to GI symptoms observed in patients with FD (32) as well as in mouse models of the disease (17). The increase in I_{sc} we recorded reflects the electrogenic transport of ions across the colonic epithelium, which can, in turn, affect paracellular water transport. Significant changes in ion transport can influence the amount of water in the GI lumen, potentially contributing to the development of diarrhea or constipation under pathophysiological conditions. It has been well established that Cl^- ion secretion significantly contributes to the water loss associated with secretory diarrhea (33). Moreover, α-$GalA^{-/0}$ mice exhibit a diarrheal-like phenotype (17). Despite this, however, neither the inhibition of Cl^- uptake by NKCC1 nor the inhibition of Cl^- secretion through the CFTR significantly affected the change in I_{sc} elicited by lyso-Gb_3, inferring that the response we observed is Cl^- ion independent. Nonetheless, given that both diarrhea and constipation are common GI symptoms in FD (10–12), any substantial change in I_{sc} could have clinical significance.

Previous work has suggested that lyso-Gb_3 can influence the activity of other ion channels, including the intermediate-conductance calcium-activated potassium channel [$K_{Ca}3.1$; (34)]. Although this channel has been implicated in the regulation of mouse colonic ion transport (35), $K_{Ca}3.1$ knockout mice displayed a decreased response to CCh-induced Cl^- ion transport (35). Given that we observed an increase in I_{sc} and no significant effect of lyso-Gb_3 on CCh-induced responses, $K_{Ca}3.1$ is unlikely to be the molecular target involved in our study. Another family of ion channels, acid-sensing ion channels (ASICs), also display sensitivity to lyso-Gb_3 in vitro (36). ASICs are also expressed by mouse intestinal epithelium and by the human colonic epithelial cell line, HT29 where they appear to contribute to acid-induced HCO_3^- secretion (37). Although HCO_3^- secretion can cause an increase in I_{sc} (38), this is largely facilitated by electroneutral Cl^-/HCO_3^- exchangers (39), and therefore would not result in a change in I_{sc}.

Both intracellular cAMP and Ca^{2+} play an important role in the modulation of intestinal ion transport (40). In addition, lyso-Gb_3 has been shown to increase intracellular cAMP (34) and is associated with elevated plasma cAMP levels in FD (41). If this were to occur in intestinal epithelial cells, this could influence the activity of epithelial ion channels and ion transport. Nonetheless, in our study, preincubation of tissues with lyso-Gb_3 did not alter the subsequent I_{sc} response to forskolin, a direct activator of adenylyl cyclase used to stimulate intracellular cAMP formation. Similarly, lyso-Gb_3 had no effect on CCh-stimulated, Ca^{2+}-mediated ion transport. Although we did not observe altered sensitivity to cholinergic stimulation, previous work has demonstrated that inhibition of α-GalA, the enzyme deficient in FD, significantly decreased acetylcholine release from neuronal-like cells, which may involve the accumulation of Gb_3 and decreased synthesis of acetylcholine (42). This perhaps supports the idea that lyso-Gb_3 alone may not be sufficient to induce overt GI dysfunction in the context of relatively short-term exposure ex vivo. Nonetheless, an inhibition of acetylcholine synthesis or release from enteric nerves or extrinsic parasympathetic nerve fibers would undoubtedly have a significant impact on GI physiology, including effects on ion transport.

To determine whether enteric neurons were involved in mediating the lyso-Gb_3-induced increase in I_{sc}, we examined the effects of lyso-Gb_3 in the presence of the voltage-gated Na^+ channel inhibitor, TTx which had no significant effect on the lyso-Gb_3 response. In support of this finding, the response to veratridine, which activates predominantly voltage-gated Na^+ channels, was not significantly influenced by pretreatment of tissues with lyso-Gb_3. Taken together, these data suggest that the effects of lyso-Gb_3 on I_{sc} may occur directly at the level of epithelium or may be mediated by TTx-insensitive neural pathways. Previous work by Choi et al. (20) demonstrated that lyso-Gb_3 induced calcium transients in capsaicin-sensitive primary dorsal root ganglia (DRG) neurons. In the GI tract, transient receptor potential cation channel subfamily-V member-1 (TRPV1) receptor expression is not only associated with extrinsic sensory nerve endings but also with intrinsic enteric neurons and glia (43–45). Therefore, we sought to investigate the effects of lyso-Gb_3 on capsaicin-

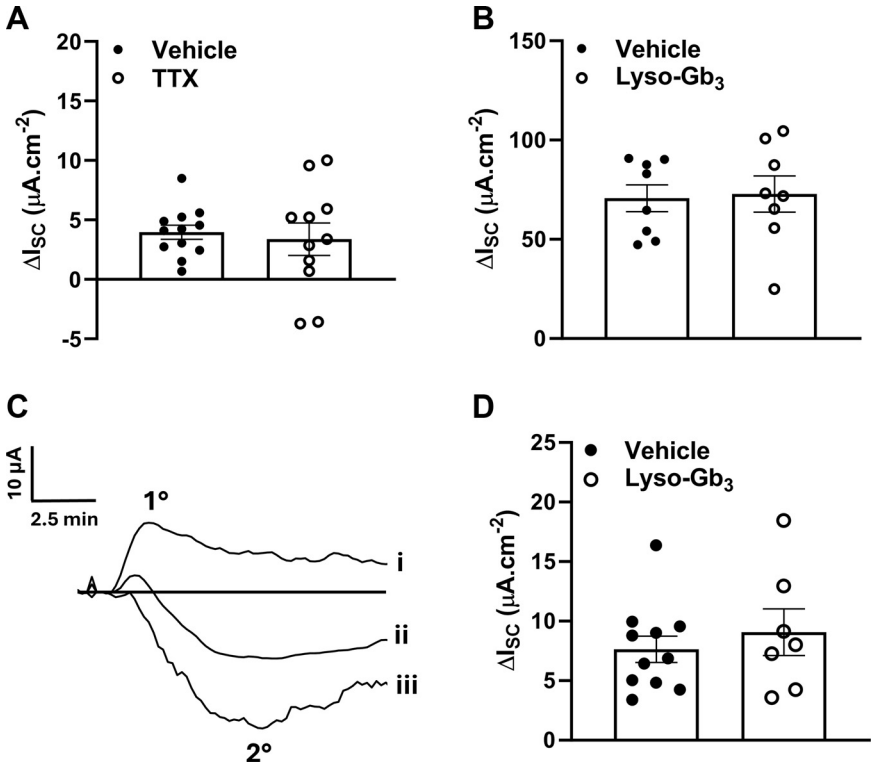

Figure 3. Inhibition of enteric neurones with tetrodotoxin (TTx; 300 nM) did not affect the lyso-Gb_3 (3 μM)-induced change in short-circuit current (I_{sc}; *A*) nor did lyso-Gb_3 (3 μM) pretreatment influence the subsequent response to the Na^+ channel activator veratridine (30 μM; *B*). Capsaicin (3 μM) evoked characteristically different changes in I_{sc}, resulting in either an increase I_{sc} (*Ci*), a biphasic response (*Cii*), or a decrease in I_{sc} (*Ciii*). Pooled data from those tissues exhibiting a primary (1°) response, as part of the biphasic response, or 1° response only, were not significantly different between vehicle- and lyso-Gb_3-treated tissues (*D*). Data are presented as means ± SE; $n = 8$–12 mice (*A* and *B*), and $n = 7$–11 tissues (*C*), which responded with a 1° response or biphasic response.

induced responses in mouse colon. Although there was a tendency for an enhanced "antisecretory" or secondary phase of the capsaicin response in the presence of lyso-Gb_3, this did not reach statistical significance. Moreover, we did not observe any significant effect on the primary phase of the capsaicin response which has been attributed to the activation of neurokinin receptors (31). It is possible, however, that only a proportion of capsaicin-sensitive neurons in the colon may be sensitive to lyso-Gb_3, as not all capsaicin-sensitive DRG neurons were sensitive to the effects of lyso-Gb_3 in vitro (20). This coupled with the heterogeneous nature of the capsaicin response we observed could account for the absence of a significant effect of lyso-Gb_3 on capsaicin-induced changes in I_{sc}. It may also be the case that intracellular accumulation of Gb_3

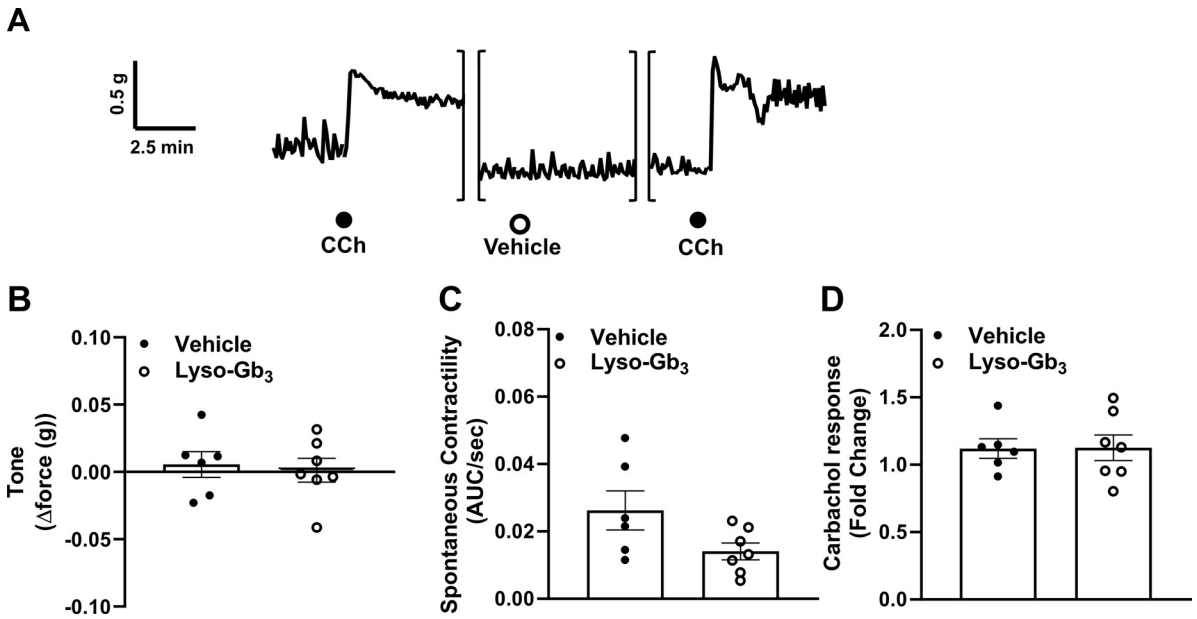

Figure 4. Representative contractile response to carbachol (CCh; 100 μM; ●) before washout, spontaneous contractile activity before and after DMSO (0.1%; ○) and subsequent contractile response to CCh in the presence of DMSO (*A*). Bath application of lyso-Gb_3 (3 μM) did not significantly affect colonic tone (*B*), spontaneous contractility (*C*), or the subsequent response to CCh relative to vehicle (*D*). Data are presented as means ± SE; $n = 6$ or 7 mice.

may be a prerequisite for subsequent changes in TRPV1 receptor expression and sensitization (46).

As we previously observed altered GI transit in α-$GalA^{-/0}$ mice (17), implying a possible role for lyso-Gb_3 in the regulation of GI motility, we examined the direct effects of lyso-Gb_3 on colonic motility ex vivo. Despite our previous observations in vivo (17), we did not observe any significant effects of lyso-Gb_3 on colonic tone, spontaneous contractile activity, or on carbachol-induced contractility in our organ bath experiments. This may again support the notion that the full onset of GI dysfunction associated with FD is not only associated with increased production of lyso-Gb_3 but also occurs alongside the gross ultrastructural changes to the myenteric and submucosal ganglia as well as smooth muscle cells in the GI tract, which are characteristic features in FD and in α-$GalA^{-/0}$ mice (15, 47). Recent clinical data also suggest that dysmotility alone in patients with FD may only partly account for the GI symptoms associated with the disease (48).

Another intriguing possibility is that lyso-Gb_3 could exert indirect effects on GI physiology through modification of the gut microbiome, including biofilm formation, growth of particular taxa, and effects on short-chain fatty acid production (49). The most pronounced effects of lyso-Gb_3 were on *Bacteroides fragilis* (49), components of which have been shown to engage with enteric sensory neurotransmission (50) and can stimulate Cl^- secretion in colonic epithelial cells (51). Nonetheless, this putative mechanism is unlikely to contribute to the response observed to lyso-Gb_3 in our Ussing chamber or organ bath experiments given the nature of the assays and relatively short incubation with lyso-Gb3 but could account for the absence of a more robust effect of lyso-Gb_3 on GI physiology which warrants further investigation.

We previously determined that alterations in gut motility and fecal water content (17) as well as Gb_3 deposits are a characteristic feature of the α-$GalA^{-/0}$ mouse model of FD at 8–10 wk of age (15), a time at which they appear otherwise clinically normal (52). Notwithstanding some of the limitations of FD animal models (53), this might suggest that the presence of a GI phenotype supports earlier interventional therapy in FD before other manifestations of the disease develop. The developmental trajectory of GI dysfunction from birth, however, has yet to be fully characterized in the α-$GalA^{-/0}$ mouse, which would provide further insight into the pathophysiological role of Gb_3 in the development of GI dysfunction. Nonetheless, here, we report that a clinically relevant concentration of lyso-Gb_3 also has a significant effect on intestinal ion transport independent of Gb_3 accumulation. Taken together, these data suggest that Gb_3 and lyso-Gb_3 collectively contribute to morphological and functional changes in the GI tract, which manifest as the more profound GI symptoms associated with FD.

DATA AVAILABILITY

The source data are available to verified researchers upon request by contacting the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1 and S2: https://doi.org/10.6084/m9.figshare.27170994.

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GRANTS

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DISCLOSURES

The authors are not aware of any conflicts of interest that might be perceived as affecting the findings of this study.

AUTHOR CONTRIBUTIONS

F.U. and N.P.H. conceived and designed research; C.D. and F.U. performed experiments; C.D., F.U., and N.P.H. analyzed data; C.D., F.U., and N.P.H. interpreted results of experiments; C.D., F.U., and N.P.H. prepared figures; C.D., F.U., M.C., and N.P.H. drafted manuscript; C.D., F.U., M.C., and N.P.H. edited and revised manuscript; C.D., F.U., M.C., and N.P.H. approved final version of manuscript.

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