Intestinal functions such as peristalsis require the coordinated activity of multiple cell types throughout the gut wall, including enteroendocrine cells, nerves, interstitial cells, and smooth muscle. Yet deciphering the exact contribution of individual cell types is extremely challenging because of the complex nature of tissue and the overlapping expression of many signaling molecules. Each cell type in the chain of command from mucosa to smooth muscle is clearly essential for the transduction of luminal cues into motor responses, and the ultimate integration and execution of motor patterns depends on the neural circuitry within the enteric nervous system (ENS). However, the exact roles of many classes of cells in the gut wall is still highly debated.

One class of cells that have gained particular interest recently are the enteric glia. These astrocyte-like peripheral glial cells surround enteric neurons and play important roles in the maintenance of enteric neurocircuits. Indeed, enteric glia are tuned to detect neuronal activity and express receptors for all major classes of enteric neurotransmitters. New data showing that enteric glia are activated by specific neural pathways and during physiologic patterns of ENS activity raise the possibility that glial activity may be involved in the modulation of ENS circuits in addition to supporting neuronal health. In support, impairing glial functions with a metabolic toxin or the selective genetic ablation of glial channels involved in intercellular communication impairs gut motility. These studies suggest that glial functions are necessary for the maintenance of gut motility, but whether the observed changes reflect poor glial metabolic support of neurons or a change in active glial signaling is not known.

Most enteric glial receptors for neuroactive compounds are G protein-coupled receptors (GPCRs) and many of these
couple to Gq and downstream intracellular signaling cascades that lead to elevations in intracellular Ca\(^{2+}\). These intracellular Ca\(^{2+}\) responses are largely considered central to many glial functions and are currently used as the main readout of glial activity\(^1\) but the significance of glial Ca\(^{2+}\) responses remains a matter of great debate.\(^2\) Thus, despite intense research investigating glial responsiveness to various mediators, major questions remain unresolved concerning the physiological significance glial Ca\(^{2+}\) responses and what information enteric glial Ca\(^{2+}\) responses encode.

New technologies have emerged in recent years that permit the activational control of individual populations of cells in complex tissue.\(^3,4,21\) Importantly, these noninvasive techniques can be employed in vivo or in intact organs to assess the function of specific signaling pathways in defined cell populations such as enteric glia. Here, we used a chemogenetic approach to selectively trigger a glial Gq–GPCR signal cascade leading to intracellular Ca\(^{2+}\) responses to determine the effect of glial Ca\(^{2+}\)-dependent signaling on the neural control of gut motility in vivo and ex vivo. Our results show that Ca\(^{2+}\)-dependent activity within glial cells has a major effect on excitatory neuromuscular transmission in the colon and that the selective activation of Ca\(^{2+}\) signaling in glial cells drives neurogenic contractions. Importantly, our data provide the first conclusive evidence of active glial regulation of enteric neurotransmission. In addition, our findings raise the possibility that the selective modulation of glial cells could be a novel therapeutic approach to improve gut motility in functional gastrointestinal disorders such as slow transit constipation.

**Materials and Methods**

**Animals**

All experimental protocols were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC). The GFAP::hM3Dq transgenic mice were a gift from Dr. Brian Popko\(^24\) (University of Chicago) and were bred with tetO::hM3Dq mice [Tg(tetO-CHRm3*)1Blr/J; Jackson Laboratory, Bay Harbor, MA; RRID: IMSR:JAX:014093] at Michigan State University to obtain double transgenic mice. Mice of both sexes, aged 8–12 weeks, were used for experiments and wild-type (WT) littermates served as experimental controls. Genotyping was performed by the Research Technology Support Facility at Michigan State University. The mice were maintained in a temperature-controlled environment on a 12-hour light/dark cycle, with access to tap water and regular chow ad libitum.

**Whole-Mount Immunohistochemistry**

Whole-mount preparations of the ileal and colonic myenteric plexus were prepared from Zamboni’s-fixed tissue and processed for immunohistochemical analysis with the antibodies shown in Table 1 as previously described elsewhere.\(^22\) Briefly, fixed tissue was pinned flat in a Sylgard (Dow-Corning, Midland, MI)-coated Petri dish, and the mucosa, submucosa, and circular muscle were removed with forceps to expose the myenteric plexus. The resulting longitudinal muscle myenteric plexus (LMMP) tissue preparations underwent three 10-minute washes in 0.1% Triton X-100 in phosphate-buffered saline (PBS-Triton) followed by a 45-minute incubation in blocking solution (containing 4% normal goat or normal donkey serum, 0.4% Triton X-100 and 1% bovine serum albumin). Preparations were incubated in primary antibodies overnight at room temperature and secondary antibodies for 2 hours at room temperature (in blocking solution) before mounting. Dual-labeling with antibodies raised in the same host was performed using a horseradish peroxidase–goat anti-rabbit IgG and Alexa Fluor 568 tyramide signal amplification kit (Life Technologies, Grand Island, NY) following the manufacturer’s instructions. Images were acquired through the 20× (Plan-Fluor, 0.75 numerical aperture (n.a.)) objective of an upright epifluorescence microscope (Nikon Eclipse Ni; Nikon, Melville, NY) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) controlled by QCapture Pro 7.0 (QImaging) or through the 60× (Plan-Apochromat, 1.42 n.a.) oil-immersion objective.

### Table 1. Details of Primary and Secondary Antibodies

| Antibody                  | Source                          | Dilution | RRID      |
|---------------------------|---------------------------------|----------|-----------|
| Primary antibodies        |                                 |          |           |
| Goat anti-c-Kit           | R&D Systems, Minneapolis, MN    | 1:200    | AB 354750 |
| Chicken anti-GFAP         | Abcam, Cambridge, MA            | 1:1000   | AB 304558 |
| Rabbit anti-HA            | Cell Signaling Technology, Danvers, MA | 1:500     | AB 1549958 |
| Anti-human Hu C/D         | Invitrogen, Carlsbad, CA        | 1:250    | AB 87935  |
| Rabbit anti-S100beta      | Molecular Probes, Eugene, OR    | 1:200    | AB 1500232 |
| Secondary antibodies      |                                 |          |           |
| Goat anti-rabbit Alexa Fluor 488 | Invitrogen                  | 1:400    | AB 10562715 |
| Donkey anti-goat Alexa Fluor 568 | Invitrogen                  | 1:400    | AB 10564097 |
| Goat anti-chicken Alexa Fluor 568 | Invitrogen                  | 1:400    | AB 10564483 |
| Goat anti-rabbit Alexa Fluor 568 | Invitrogen                  | 1:400    | AB 10563566 |
| Streptavidin conjugated Alexa Fluor 594 | Jackson ImmunoResearch, West Grove, PA | 1:400 | AB 2337250 |

RRID, Research Resource Identifiers, Antibody Registry (http://antibodyregistry.org).
objective of an inverted Fluoview FV1000 confocal microscope (Olympus, Center Valley, PA).

**Calcium Imaging**

Live whole-mounts of the ileal and colonic myenteric plexus were prepared for Ca$^{2+}$ imaging as described by Fried and Gulbransen. Briefly, distal ileal and colonic segments were collected in ice-cold Dulbecco’s modified Eagle medium (DMEM) and transferred to Sylgard-coated, open diamond shaped bath recording chambers and then opened along the mesenteric border, pinned flat, and microdissected. LMMP preparations were incubated for 15 minutes at room temperature in an enzyme mixture consisting of 150 U/mL Collagenase type II and 1 U/mL Dispase (Life Technologies) dissolved in DMEM before gentle trituration. LMMPs were washed three times with DMEM and incubated with 200 μM water-soluble Probenecid (Life Technologies) in DMEM. LMMPs were washed three times with DMEM and incubated with 200 μM probenecid in DMEM 15 minutes to de-esterify before imaging. Images were acquired every 1–2 seconds (s) through the 40× water-immersion objective (LUMPlan N, 0.8 n.a.) of an upright Olympus BX51WI fixed-stage microscope (Olympus, Tokyo, Japan) using IQ2 software and a Neo sCMOS camera (Andor, South Windsor, CT). Whole mounts were superfused with Krebs buffer (37°C) at 2–3 mL min⁻¹.

**Contractility Studies**

Isometric muscle tension recordings were performed in longitudinally-oriented segments of distal colon and ileum under 1 g passive tension. Muscle strips were affixed to a force transducer (Grass Instruments, Quincy, MA) between two platinum electrodes for electrical field stimulation (EFS) and data was charted with Labscribe (iWorx, Dover, NH) as described previously elsewhere. Responses were normalized to an initial bethanechol (BCH, 10 μM, cholinergic muscarinic agonist)-induced contraction. Neurogenic contractions and relaxations were induced by application of EFS (20V, 0.1 milliseconds, 2–30 Hz). Neurogenic relaxations were studied in tissues precontracted with 5 μM prostaglandin F2-α (PGF₂α). Relaxations were induced when the contractile response to PGF₂α was stable for at least 5 minutes. Tetrodotoxin (TTX, 0.3 μM, voltage-gated sodium channel inhibitor) was applied to block neurogenic responses.

**Colonic Migrating Motor Complexes**

Colonic migrating motor complexes (CMMCs) were recorded from intact colons ex vivo as previously described elsewhere. Colons were collected in warmed media and luminal contents were gently flushed. A stainless-steel rod was inserted into the lumen, and the tissue was secured at both ends with surgical silk. Force transducers (Grass Instruments) were placed 2 cm apart and attached to the oral and aboral ends by surgical silk. Tissue was placed into a bath containing DMEM-F12 media (37°C) and adjusted to an initial tension of 0.5 g. CMMCs were recorded with LabChart 8 (ADInstruments, Colorado Springs, CO) for 20 minutes following an acclimation period and the initial 6-minute interval was used as baseline. Agonists were bath applied and CMMCs recorded for an additional 6-minute interval. CMMCs were defined as a complex in which contraction occurs first at the oral site followed by a contraction at the aboral site. Amplitude, integral, frequency, and propagation velocity were calculated as percent of baseline.

**Colon Bead Assay**

Distal colonic transit was assessed by measuring the latency to expel a small (2 mm diameter) plastic bead inserted 3 cm into the colon.

**Endogenous Pellet Production**

Mice were individually housed, and fecal pellet output was measured on 2 consecutive days. Pellets were collected for 1 hour beginning at 9:00 AM (Zeitgeber +3). The wet weight of fecal matter was measured immediately, and the dry weight was obtained the next day after dehydration. Data from the 2 days was averaged.

**Whole Gut Transit**

Total intestinal transit time was defined as the latency from gavage of 0.2 mL of a 6% carmine red solution in H₂O with 0.5% methylcellulose to the appearance of red dye in fecal pellets.

**Upper Gastrointestinal Transit**

Upper gastrointestinal transit was assessed as described previously. Briefly, mice received a gavage of 0.2 mL of a 6% carmine red solution in H₂O with 0.5% methylcellulose. Mice were euthanized 15 minutes later, and the distance travelled was measured to calculate upper gastrointestinal velocity.

**Solutions**

Calcium imaging experiments were performed in modified Krebs buffer consisting of (in mmol/L): 121 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 10 HEPES, 21.2 NaHCO₃, 1 pyruvic acid, 8 glucose (pH adjusted to 7.4 with NaOH) with 3 μmol/L nicardipine and 1 μmol/L scopolamine to inhibit muscle contractions. Muscle contractility studies were performed in normal Krebs buffer consisting of (in mmol/L): 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃ and 11 glucose. CMMC studies were conducted in DMEM/Nutrient Mixture F-12 (Life Technologies) supplemented with l-glutamine and HEPES.

**Chemicals and Reagents**

Unless otherwise listed, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Clozapine-N-oxide (CNO) was obtained from the National Institute on Drug Abuse Drug Supply Program at the National Institutes of Health and bath applied at 10 μM for isolated preparations or administered via an intraperitoneal injection at 0.25 mg kg⁻¹ for in vivo experiments. Drugs were bath...
Figure 1. Selective expression of hM3Dq receptors by enteric glia in the intestine of GFAP::hM3Dq mice. (A) Model of experimental paradigm used in this study. (B) Schematic of transgene containing hemagglutinin (HA)-tagged hM3Dq driven by the glial fibrillary acidic protein (GFAP) promoter. (C–G) Confocal images of dual-label immunohistochemistry for the HA-tagged hM3Dq protein (green in all panels at left) and enteric glia (C–E center; GFAP immunoreactivity and S100beta immunoreactivity shown in magenta), enteric neurons (F center; HuC/D immunoreactivity shown in magenta) or interstitial cells of Cajal (ICCs) (G center; c-Kit immunoreactivity shown in magenta) in the myenteric plexus of GFAP::hM3Dq mice. Overlays are shown in right-hand panels. The images in C and G are z-stacks and the images in D–F are a single optical slice (1 μm). The dashed line in G denotes the boundary of a myenteric ganglion that lies on top of ICCs and smooth muscle cells. Note that HA-immunoreactivity covers the surface of GFAP-immunoreactive (C, D) and S100beta-immunoreactive (E) enteric glial cells and does not localize with enteric neurons (F) or ICCs (G). Also note that no HA-immunoreactivity is present within the smooth muscle coats (G). Images are representative of labeling in a minimum of 9 animals. Scale bars in C, D: 10 μm; scale bar in C applies to panels C, E, and F; scale bar in G is 20 μm.
applied via a gravity-fed perfusion system in Ca\textsuperscript{2+} imaging experiments and directly added to organ baths for isometric muscle tension recordings and CMMC recordings. Thus, there is a lag between drug application and cellular response in Ca\textsuperscript{2+} imaging experiments that is not present in organ baths because of the time required for the drug to reach the tissue through the perfusion system.

**Statistical Analysis**

Digital images were analyzed offline using ImageJ software (National Institutes of Health, Bethesda, MD). Data were analyzed using Prism 5 (GraphPad Software, La Jolla, CA) and are shown as mean ± standard error of the mean (SEM). Contractility studies were analyzed by two-way analysis of variance (ANOVA) with a Bonferroni post-test. Remaining data were analyzed by Student t test. \( P < .05 \) was considered statistically significant.

**Results**

Enteric glia respond to neurotransmitters\textsuperscript{6,11} and physiologic patterns of neural activity in the ENS\textsuperscript{9} through GPCR pathways that evoke intracellular Ca\textsuperscript{2+} responses.\textsuperscript{6,8} Yet what role, if any, glial signaling downstream of intracellular Ca\textsuperscript{2+} responses plays in the neural control of gut functions is unknown. We addressed this issue directly by using a chemogenetic approach\textsuperscript{22} to selectively trigger glial Ca\textsuperscript{2+} responses and examining the effect on neuromuscular transmission in the gut measured with in vivo and ex vivo motility assays (Figure 1A).

![Figure 2](image)

**Figure 2. Ectopic expression of hM3Dq receptors in the intestines of GFAP::tTA/tetO::hM3Dq transgenic mice.** (A) Breeding scheme to generate double transgenic mice that express the tetracycline-controlled transactivator protein (tTA) driven by the glial fibrillary acidic protein (GFAP) promoter and the tetracycline-responsive cytomegalovirus promoter element (TRE; tetO CMV) upstream of the hM3Dq receptor. Note that glial cell hM3Dq transcription occurs in the absence of the tetracycline analog, doxycycline (dox) in these lines. (B–D) Representative epifluorescence microscopy images of immunoreactivity for GFAP (glia, green, left panels) and the hemagglutinin (HA)-tagged hM3Dq protein (grayscale, middle panels) in whole-mount preparations of myenteric plexus and the adherent longitudinal muscle from the colons of GFAP::tTA/tetO::hM3Dq transgenic mice (overlays of GFAP and HA shown in panels at right). Images are representative of labeling in a minimum of 3 animals from each of the following transgenic lines: (B) GFAP::tTA67/tetO::hM3Dq, (C) GFAP::tTA78/tetO::hM3Dq, and (D) GFAP::tTA110/tetO::hM3Dq. Scale bar in D: 60 \( \mu \)M, and applies to all panels.
Functional Expression of hM3Dq “Designer Receptors Exclusively Activated by Designer Drugs” by Enteric Glia in GFAP::hM3Dq Mice

Our first goal was to establish a suitable model to selectively evoke reliable Ca\(^{2+}\) responses in enteric glial cells. To this end, we obtained several chemogenetic mouse lines (all transgenic) that express designer receptors exclusively activated by designer drugs (DREADDs) under transcriptional control of the glial fibrillary acidic protein (GFAP) promoter and tested their feasibility for use evoking glial Ca\(^{2+}\) responses in the gut. We chose lines expressing the hM3Dq variant of DREADD receptors because this mutant GPCR activates the same canonical Gq signaling pathway as endogenous glial GPCRs.6,8

These mouse lines included one line where hM3Dq is constitutively expressed under the control of the GFAP promoter23 (Figure 1B) and three double transgenic lines that express the tetracycline-controlled transactivator protein (tTA) under regulatory control of the GFAP promoter24 and the tetracycline-responsive promoter element (TRE; tetO) upstream of the hM3Dq receptor29 (Figure 2A). In double mutant offspring of the latter “Tet-Off” system, hM3Dq transcription occurs in the absence of the tetracycline analog doxycycline. Specific details on mouse lines are provided in the Materials and Methods section. We reasoned that a suitable model should meet certain basic criteria including 1) confinement of hM3Dq receptor expression to GFAP-positive enteric glia and 2) reliable Ca\(^{2+}\) responses evoked in glia in response to the physiologically inert hM3Dq agonist CNO.

All transgenic mouse lines tested express hM3Dq with a hemagglutinin (HA) protein tag, and we took HA immunoreactivity as acceptable evidence for hM3Dq protein expression and localization. In GFAP::hM3Dq transgenic mice, we observed robust immunoreactivity for HA-tagged hM3Dq that was confined to GFAP-immunoreactive and S100beta-immunoreactive enteric glia within the myenteric plexus of the ileum and colon (Figure 1C–E and Figure 3). HA-immunoreactivity was distributed across the surface of all observable GFAP-immunoreactive and S100beta-immunoreactive enteric glial cells and was never observed in non-GFAP-immunoreactive cells such as neurons (Figure 1F), interstitial cells of Cajal (Figure 1G), or smooth muscle cells (Figure 1G). Likewise, HA immunoreactivity was never observed in tissue from wild type (WT) littermates of GFAP::hM3Dq transgenic mice (Figure 3). In contrast to the GFAP::hM3Dq mice, we observed only weak HA-immunoreactivity within the myenteric plexus of the intestines of GFAP::hM3Dq mice. (A–D) Epi-fluorescence images of immunoreactivity for enteric glia (glial fibrillary acidic protein [GFAP] shown in green) and the hemagglutinin (HA)-tagged hM3Dq protein (grayscale) in whole-mount longitudinal muscle myenteric plexus (LMMP) preparations from the colon (A, B) and ileum (C, D) of GFAP::hM3Dq transgenic mice (A, C) and wild-type (WT) littermates (B, D). Note that HA-immunoreactivity is confined to GFAP-immunoreactive glia in GFAP::hM3Dq mice. Images are representative of labeling in a minimum of 3 animals. Scale bar in D: 60 \(\mu\)M, and applies to all panels.
three lines of GFAP::tTA/tetO::hM3Dq double-transgenic mice tested, and the pattern of HA labeling more closely resembled that of nerve varicosities than glial cells (Figure 2). This likely reflects ectopic expression due to differing transgene insertion sites into the genome in these lines. These data are consistent with previous work showing that these specific lines of GFAP::tTA mice yield neuronal expression in the cerebellum, forebrain, and spinal cord.\(^{24}\)

Next, we tested the functionality of hM3Dq receptors expressed by enteric glia by imaging intracellular Ca\(^{2+}\) transients evoked by the hM3Dq agonist CNO (Figure 4). Our results show that stimulation of hM3Dq with CNO (10 \(\mu\)M)\(^{23}\) in tissue from GFAP::hM3Dq mice selectively induces intracellular Ca\(^{2+}\) responses within enteric glia (Figure 4A, C, and D and Video 1) but has no effect on cellular Ca\(^{2+}\) levels in tissue from WT littermates (Figure 4B). Enteric glia from GFAP::hM3Dq mice and WT littermates exhibited comparable purine-evoked Ca\(^{2+}\) responses (adenosine diphosphate [ADP], 100 \(\mu\)M, data not shown), suggesting that the expression of endogenous receptor pathways is not significantly altered in this transgenic line.

Glial responses to CNO in GFAP::hM3Dq mice are characterized by large Ca\(^{2+}\) transients in glial cell bodies (mean peak \(\Delta F/F = 1.35 \pm 0.18\), \(n = 60\) glial cells from six ganglia, three animals) and many oscillating Ca\(^{2+}\) transients throughout glial processes (Figure 4C). In contrast to GFAP::hM3Dq mice, we never observed glial Ca\(^{2+}\) transients in response to CNO in tissue from any of the three GFAP::tTA/tetO::hM3Dq lines of mice tested (Figure 5). However, enteric glia in tissue from GFAP::tTA/tetO::hM3Dq mice still exhibited robust purine-evoked Ca\(^{2+}\) transients in glial process and cell bodies (Figure 5).

Figure 4. Activation of glial hM3Dq receptors reliably evokes intracellular calcium (Ca\(^{2+}\)) transients. (A, B) Representative traces of intracellular Ca\(^{2+}\) levels in myenteric glia in ganglia from (A) GFAP::hM3Dq transgenic mice and (B) wild-type (WT) littermates in response to the hM3Dq agonist clozapine-N-oxide (CNO, 10 \(\mu\)M). Each trace represents an individual glial cell and all glial cells within an individual ganglion are shown. (C) Representative traces showing that activation of glial hM3Dq receptors with CNO (blue shaded area) in GFAP::hM3Dq mice elicits intracellular Ca\(^{2+}\) transients in glial processes (green lines, top) and cell bodies (black lines, bottom). (D) Representative images of Fluo-4 fluorescence in a myenteric ganglion from a GFAP::hM3Dq mouse at rest (baseline, top image) and after stimulation with CNO (peak, bottom image). Scale bar: 10 \(\mu\)M. Traces are representative of recordings in \(n = 6\) ganglia from at least three mice of each genotype.
intracellular Ca\textsuperscript{2+} responses (ADP, Figure 5). Together, these results show that GFAP::hM3Dq mice are a suitable model system to study the effects of selectively evoking glial Ca\textsuperscript{2+} responses and we proceeded to use this line for all additional studies.

Enteric Glial Cell Calcium Signaling Driven by Gq-G Protein-Coupled Receptors Triggers Neurogenic Smooth Muscle Contractions

Calcium responses in enteric glia are mainly reported in myenteric circuits controlling gut contractility. Thus, we initially tested whether the activation of glial Ca\textsuperscript{2+} signaling alone has any effect on intestinal contractility. To this end, we conducted isometric muscle tension recordings in longitudinally oriented segments of intestine and selectively triggered glial Ca\textsuperscript{2+} responses with CNO in tissue from GFAP::hM3Dq mice (Figure 6).

Application of CNO (10 \mu M) elicited large contractions in segments of ileum and colon from GFAP::hM3Dq mice (Figure 6A and B) but had no effect on tissue from WT littermates (Figure 6C and D). Contractions elicited by CNO were equal in magnitude to those driven by the direct stimulation of smooth muscle with bethanechol (Figure 6E and F, BCH, 10 \mu M) and equal in magnitude to those driven by maximal stimulation of ENS neurons with EFS (20 Hz, 20 V; Figure 6E and F). Contractions driven by CNO were entirely dependent upon neuronal activation because they were absent in the presence of tetrodotoxin (Figure 6A and B, TTX). BCH and EFS elicited equal contractions in bowel segments from GFAP::hM3Dq and WT mice, indicating that the transgenic mice do not have any gross abnormalities in the excitability of enteric neurons and/or the ability of intestinal smooth muscle to contract (Figure 6E and F).

EFS induced neurogenic relaxations in segments of ileum and colon precontracted with PGF\textsubscript{2\alpha} (Figure 7). CNO had no effect on neurogenic relaxations in either WT or GFAP::hM3Dq mice (Figure 7). In addition, CNO did not stimulate neurogenic relaxations in the absence of EFS (Figure 7A–D). Together, these results show that glial stimulation predominantly affects excitatory motor circuits controlling gut contractions and that glial stimulation has little or no effect on inhibitory circuits regulating gut relaxations.

Stimulation of Enteric Glial Cell Calcium Signaling Enhances Colonic Migrating Motor Complexes

Fecal pellet propulsion in mice is driven by an enteric neural reflex that manifests as a rhythmic migrating motor pattern in the isolated large intestine called the colonic migrating motor complex (CMMC).\textsuperscript{30,31} Enteric glial cell Ca\textsuperscript{2+} responses are entrained with neuronal activity during the CMMC,\textsuperscript{30,31} but their significance remains unclear. Therefore, we tested how the activation of glial Ca\textsuperscript{2+} signaling affects the ongoing pattern of the CMMC in the isolated colon (Figure 8).

We observed spontaneous, regular CMMCs in both GFAP::hM3Dq transgenic mice and WT littermates as contractions migrating from proximal to distal colon that were separated by periods of quiescence. Baseline CMMC activity was comparable between GFAP::hM3Dq mice and WT littermates, and we did not observe any differences in contractile activity or overall propagation characteristics of CMMCs between genotypes in the absence of CNO. Upon
application of CNO (10 μM), we observed a significant alteration in the properties of the CMMCs in GFAP::hM3Dq mice but no significant changes in the CMMCs of WT littermates (Figure 8A, B, and D). Overall, we observed a significant enhancement of all contractile parameters of the CMMC after glial activation including increased CMMC frequency (by 25.8%), propagation velocity (by 55.5%), amplitude (oral by 12.6%, aboral by 13.5%), and integral (oral by 21.4%, aboral by 17.7%) (Figure 9). These results show that glial Ca2+ signaling functions to enhance excitatory neural circuits that control the CMMC in mice.

**Stimulation of Enteric Glial Cell Ca2+ Signaling Augments Gastrointestinal Motility in Vivo**

Our results show that glial mechanisms downstream of Ca2+ signaling can activate neural circuits that drive neurogenic contractions of intestinal smooth muscle and that glial Ca2+ signaling enhances neural circuits that drive CMMCs. We hypothesized these effects observed in ex vivo preparations would correspond to an overall enhancement of gut motility in vivo. To this end, we triggered glial Ca2+ signaling in vivo with a single injection of CNO in GFAP::hM3Dq mice and assessed several measures of gut motility (Figure 10). After administration of CNO, we observed a nearly threefold increase in the number of fecal pellets produced per hour by GFAP::hM3Dq mice as compared to their WT littermates (Figure 10A). Despite enhancing the number of pellets produced, we found that the composition of water and fecal matter in the pellets produced remained comparable (Figure 10B).

To more accurately measure colonic transit, we measured the latency to expel a small plastic bead inserted into the distal colon. Similar to the enhancement of endogenous pellet production, the administration of CNO decreased the time required to expel the bead by nearly two-thirds in GFAP::hM3Dq mice as compared to their WT littermates (Figure 10C). However, the activation
of glial Ca\textsuperscript{2+} signaling did not significantly alter whole intestinal transit time or small intestinal transit velocity in GFAP::hM3Dq mice (Figure 10D and E). Together, these results indicate that glial excitation triggering Ca\textsuperscript{2+} signaling activates excitatory neural programs in the colon.

**Discussion**

Enteric glial cells have been recognized as the sole companions of neurons within enteric ganglia since the earliest studies of the ENS\textsuperscript{32} and our current understanding of the neural control of gut reflexes stems from the assumption that there is a clear division of labor between neurons and glia. Indeed, glial cells are widely considered silent in terms of the synaptic physiology underlying gut reflexes\textsuperscript{33} and are thought to play more significant roles in the metabolic support of these neuronal circuits.\textsuperscript{7} However, recent experimental findings showing that enteric glia display a form of excitability encoded by elevations in intracellular Ca\textsuperscript{2+} suggest that this division may not be as straightforward as once thought.\textsuperscript{6-9,11} Indeed, results showing that glial Ca\textsuperscript{2+} responses are evoked by a variety of neuromodulators\textsuperscript{11} including those released by neurons during physiologic pattern of ENS activity\textsuperscript{1} have raised the controversial possibility that glial cells actively participate in information transfer in enteric circuits. Yet the outcomes of glial Ca\textsuperscript{2+} signaling have thus far remained unclear despite intense interest in observing glial Ca\textsuperscript{2+} signaling in response to various mediators.\textsuperscript{10}

Our goal in the present study was to clarify the significance of evoked glial Ca\textsuperscript{2+} signaling in the regulation of intestinal motility. To this end, we used a novel transgenic chemogenetic mouse model to selectively evoke glial Gq-GPCR signaling leading to intracellular Ca\textsuperscript{2+} responses.\textsuperscript{23} Our results show that evoked glial Ca\textsuperscript{2+} signaling has a strong, excitatory effect on enteric motor circuits. Glial excitation enhanced ongoing patterns of ENS activity such as the CMMC and glial excitation per se was sufficient to drive
neurogenic contractions of the intestine. The latter outcome is quite astounding because it suggests that glial excitation may play an important role in the initiation of specific motor programs in the gut. Given the present results, it is conceivable that glial excitation initiated by neuronal signaling functions to ‘call up’ certain motor programs and distributes this message through the glial network. Whether the motor programs of digestion are stored in glial networks is certainly still a hypothetical question, but it will be an intriguing issue addressed in future work.

Precisely how glial Ca\textsuperscript{2+} signaling is translated into neuronal excitation in the ENS is unknown and will likely remain an active area of research for quite some time. Likewise, the downstream effects of astrocytic Ca\textsuperscript{2+} signaling remain a matter of great controversy. Several models have been put forth to account for the effects of glia on central networks. Given that enteric glia share many similarities with astrocytes, we speculate that enteric glia modulate enteric neuronal networks using similar mechanisms. The most promising of these include the release of "gliotransmitters"\textsuperscript{34} and the active control of extracellular potassium (K\textsuperscript{+}) ion concentration.\textsuperscript{35}

Either mechanism, or both, could contribute to the effects we observed upon glial activation in the gut by directly activating excitatory circuits with excitatory gliotransmitters\textsuperscript{36} or by decreasing the inhibiting tone by decreasing extracellular K\textsuperscript{+}\textsuperscript{37} and hyperpolarizing inhibitory neurons. We feel that the most likely explanation for our current data is that glial stimulation drives the release of an excitatory mediator that stimulates one, or all of the following classes of myenteric neurons: excitatory ascending interneurons, excitatory motorneurons, or intrinsic primary afferent neurons. Excitation of any one or combination of these neuron classes could produce the observed result on neurogenic contractions. Importantly, we did not observe any noticeable effect of glial excitation on neurogenic relaxations. This outcome strongly suggests that glia regulate neurotransmission on a synapse-by-synapse basis and not by the diffuse release of neuroactive compounds that broadly affect all types of

Figure 8. Selective activation of glial calcium (Ca\textsuperscript{2+}) transients enhances the colonic migrating motor complex (CMMC) in GFAP::hM3Dq transgenic mice. Representative (A) oral and (B) aboral recordings of CMMCs in GFAP::hM3Dq mice (black traces) and WT littermates (gray traces) under basal conditions (solid lines) and after glial activation with clozapine-N-oxide (CNO) (dashed lines). (C) Model of experimental setup for CMMC recordings. (D) Frequency distribution of CMMC propagation initiation point in wild-type and GFAP::hM3Dq transgenic mice in the presence or absence of CNO (n = 3–5, two-way analysis of variance).
neurons. Thus, mechanisms downstream of glial Ca\(^{2+}\) signaling appear to not necessarily set the tone of neurotransmission but rather to play an active role in the modulation of specific neuronal circuits controlling the contractile aspect of gut motility. However, more work is clearly needed to decipher the exact mechanisms involved.

Interestingly, our in vivo data suggest that the role of glial Ca\(^{2+}\) signaling could differ between gut regions. For example, we observed a marked increase in colonic transit but no change in whole gut transit or small intestinal transit. However, our ex vivo data show that the activation of glial Ca\(^{2+}\) signaling evokes similar contractions in segments of ileum and colon. One explanation for these results is that there is significant glial heterogeneity along the length of the gastrointestinal tract.\(^{38,39}\) Thus, it is conceivable that the different populations of enteric glia have different roles in synaptic transmission. However, a more likely explanation lies in the fact that in vivo motility patterns and neural innervation differ significantly between the ileum and colon.\(^{11,40}\) The fact that glial excitation elicited similar contractions in segments of ileum and colon in vitro strongly suggests that glial excitation has a similar effect on enteric circuits in both organs. In vivo, motility patterns in the small intestine are heavily influenced by central pathways, and the concomitant activation of enteric and central glia could confound clear results in this region of gut. In contrast, extrinsic innervation plays less of a role in colonic motility, and the effects we observe in this organ likely reflect a more pure stimulation of enteric glial cells.

In addition to differing between gut regions, the role of glial Ca\(^{2+}\) signaling appears to differ significantly between the myenteric and submucosal plexuses. In our hands, we did not observe a major effect of glial stimulation on the fluid content of fecal matter. In agreement, MacEachern et al\(^{41}\) found that the gliotoxin fluoroacetate had no effect on electrogenic ion transport in the colon. Based on their findings, these investigators concluded that enteric glia do not play a role in the regulation of electrogenic ion transport in the gut under physiologic conditions. Our current data would support this conclusion in that we show that glial Ca\(^{2+}\) signaling does not play a major role in the regulation of fluid exchange in the colon on an acute time scale. However, many other glial signaling mechanisms that do not rely on fluxes of intracellular Ca\(^{2+}\) may play important roles in the regulation of fluid exchange, and investigating these alternate signaling pathways will be important to understand the integrated function of glia in the intestine.

Together, our results provide a framework for understanding the consequences of glial excitation in the form of Ca\(^{2+}\) signaling in past and future work. Importantly, our results show that the activation of glia can have profound effects on gut physiology. This is particularly important when considering disorders such as chronic

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**Figure 9.** Activation of glial calcium (Ca\(^{2+}\)) signaling enhances key aspects of colonic migrating motor complexes (CMMCs). (A–F) Summary data of CMMC characteristics including oral contraction (A) amplitude and (B) integral, (C) CMMC frequency, aboral contraction (D) amplitude and (E) integral, and (F) propagation velocity. All data are expressed as percentage change from baseline after the addition of clozapine-N-oxide. (n = 3–5, *P < .05, **P < .01, ***P < .001, two-tailed t-test).
constipation and other functional gastrointestinal disorders where the glial network is disrupted. In these conditions, modifying glial activity could prove to be an extremely effective and novel therapeutic strategy to restore gut motility.

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