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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Agonist-Evoked Ca^{2+} Signaling in Enteric Glia Drives Neural Programs That Regulate Intestinal Motility in Mice

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SUMMARY

Enteric glia monitor neurotransmission in the gut but the significance of glial activity has remained unclear. We found that selectively triggering glial activity has the potential to drive excitatory neural programs that control gut motility.

BACKGROUND & AIMS: Gastrointestinal motility is regulated by enteric neural circuitry that includes enteric neurons and glia. Enteric glia monitor synaptic activity and exhibit responses to neurotransmitters that are encoded by intracellular calcium (Ca^{2+}) signaling. What role evoked glial responses play in the neural regulation of gut motility is unknown. We tested how evoking Ca^{2+} signaling in enteric glia affects the neural control of intestinal motility.

METHODS: We used a novel chemogenetic mouse model that expresses the designer receptor hM3Dq under the transcriptional control of the glial fibrillary acidic protein (GFAP) promoter (*GFAP::hM3Dq* mice) to selectively trigger glial Ca^{2+} signaling. We used in situ Ca^{2+} imaging and immunohistochemistry to validate this model, and we assessed gut motility by measuring pellet output and composition, colonic bead expulsion time, small intestinal transit time, total gut transit time, colonic migrating motor complex (CMMC) recordings, and muscle tension recordings.

RESULTS: Expression of the hM3Dq receptor is confined to GFAP-positive enteric glia in the intestines of *GFAP::hM3Dq* mice. In these mice, application of the hM3Dq agonist clozapine-*N*-oxide (CNO) selectively triggers intracellular Ca^{2+} responses in enteric glia. Glial activation drove neurogenic contractions in the ileum and colon but had no effect on neurogenic relaxations. CNO enhanced the amplitude and frequency of CMMCs in ex vivo preparations of the colon, and CNO increased colonic motility in vivo. CNO had no effect on the composition of fecal matter, small intestinal transit, or whole gut transit.

CONCLUSIONS: Glial excitability encoded by intracellular Ca^{2+} signaling functions to modulate excitatory enteric circuits. Selectively triggering glial Ca^{2+} signaling might be a novel strategy to improve gut function in motility disorders. *(Cell Mol Gastroenterol Hepatol 2015;1:631–645; http://dx.doi.org/10.1016/j.jcmgh.2015.08.004)*

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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.[1] 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).[2] However, the exact roles of many classes of cells in the gut wall is still highly debated.[3–5]

One class of cells that have gained particular interest recently are the enteric glia.[5] 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[6–11] and express receptors for all major classes of enteric neurotransmitters.[12] New data showing that enteric glia are activated by specific neural pathways[13] and during physiologic patterns of ENS activity [9] raise the possibility that glial activity may be involved in the modulation of ENS circuits in addition to supporting neuronal health.[14–16] In support, impairing glial functions with a metabolic toxin[17] or the selective genetic ablation of glial channels involved in intercellular communication impairs gut motility.[18] 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

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Abbreviations used in this paper: **ADP, adenosine diphosphate; BCH, bethanechol; CMMC, colonic migrating motor complex; DMEM, Dulbecco's modified Eagle medium; DREADD, designer receptors exclusively activated by designer drugs; CNO, clozapine-*N*-oxide; EFS, electrical field stimulation; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; HA, hemagglutinin; LMMP, longitudinal muscle myenteric plexus; $PGF_{2\alpha}$, prostaglandin F2α; tTA, tetracycline-controlled transactivator protein; WT, wild type.**

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couple to Gq and downstream intracellular signaling cascades that lead to elevations in intracellular Ca^{2+}.[8] These intracellular Ca^{2+} responses are largely considered central to many glial functions and are currently used as the main readout of glial activity[10] but the significance of glial Ca^{2+} responses remains a matter of great debate.[19] 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.[20,21] 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[22] 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. Ken McCarthy (University of North Carolina at Chapel Hill) and were bred for experiments as heterozygotes at Michigan State University.[23] The *GFAP::tTA* mice (lines 67, 78, and 110) 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.[25] 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

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_1549585
	Invitrogen, Carlsbad, CA	1:250	AB_87935
Anti-human Hu C/D	Molecular Probes, Eugene, OR	1:200	AB_1500232
Rabbit anti-S100beta	Abcam, Cambridge, MA	1:2000	AB_882426
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_10584483
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.[26] 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 loaded in the dark for 45 minutes at 37°C (5% CO_2, 95% air) with 4 μM Fluo-4 AM, 0.02% Pluronic F-127 and 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.[18] 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 ($PGF_{2\alpha}$). Relaxations were induced when the contractile response to $PGF_{2\alpha}$ 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.[27] 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.[18]

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.[18] 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 H_2O with 0.5% methylcellulose to the appearance of red dye in fecal pellets.[18]

Upper Gastrointestinal Transit

Upper gastrointestinal transit was assessed as described previously.[28] Briefly, mice received a gavage of 0.2 mL of a 6% carmine red solution in H_2O 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 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21.2 $NaHCO_3$, 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 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ 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

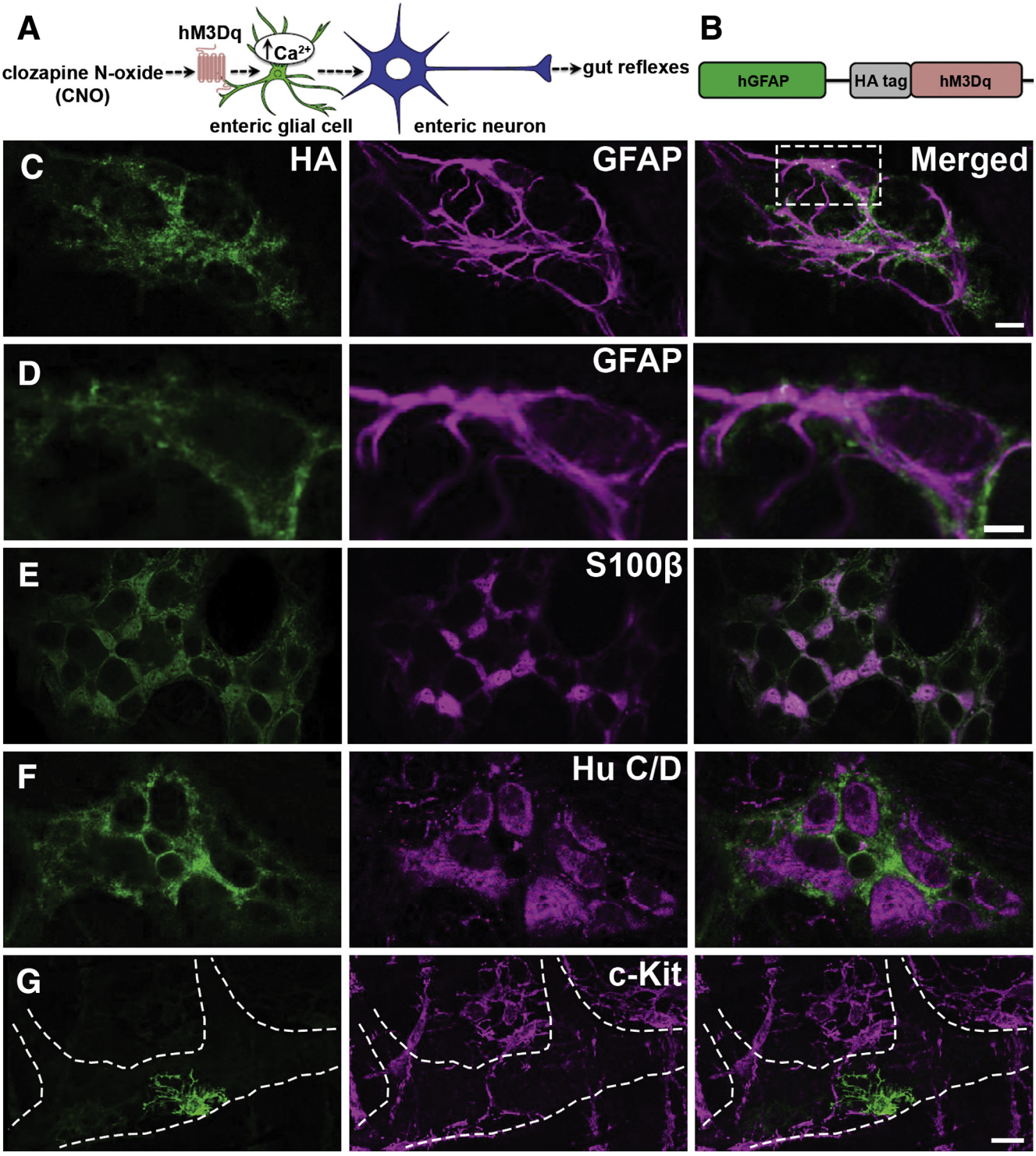

Figure 1. Selective expression of hM3Dq receptors by enteric glia in the intestine of *GFAP::hM3Dq* mice. (*A*) Model of experimental paradigm used in this study. (*B*) Schematic of transgene containing hemagglutinin (HA)-tagged hM3Dq driven by the glial fibrillary acidic protein (GFAP) promoter. (*C–G*) 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^{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^{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 Jolia, 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[6,11] and physiologic patterns of neural activity in the ENS[9] through GPCR pathways that evoke intracellular Ca^{2+} responses.[6,8] Yet what role, if any, glial signaling downstream of intracellular Ca^{2+} responses plays in the neural control of gut functions is unknown. We addressed this issue directly by using a chemogenetic approach[22] to selectively trigger glial Ca^{2+} responses and examining the effect on neuromuscular transmission in the gut measured with in vivo and ex vivo motility assays (Figure 1*A*).

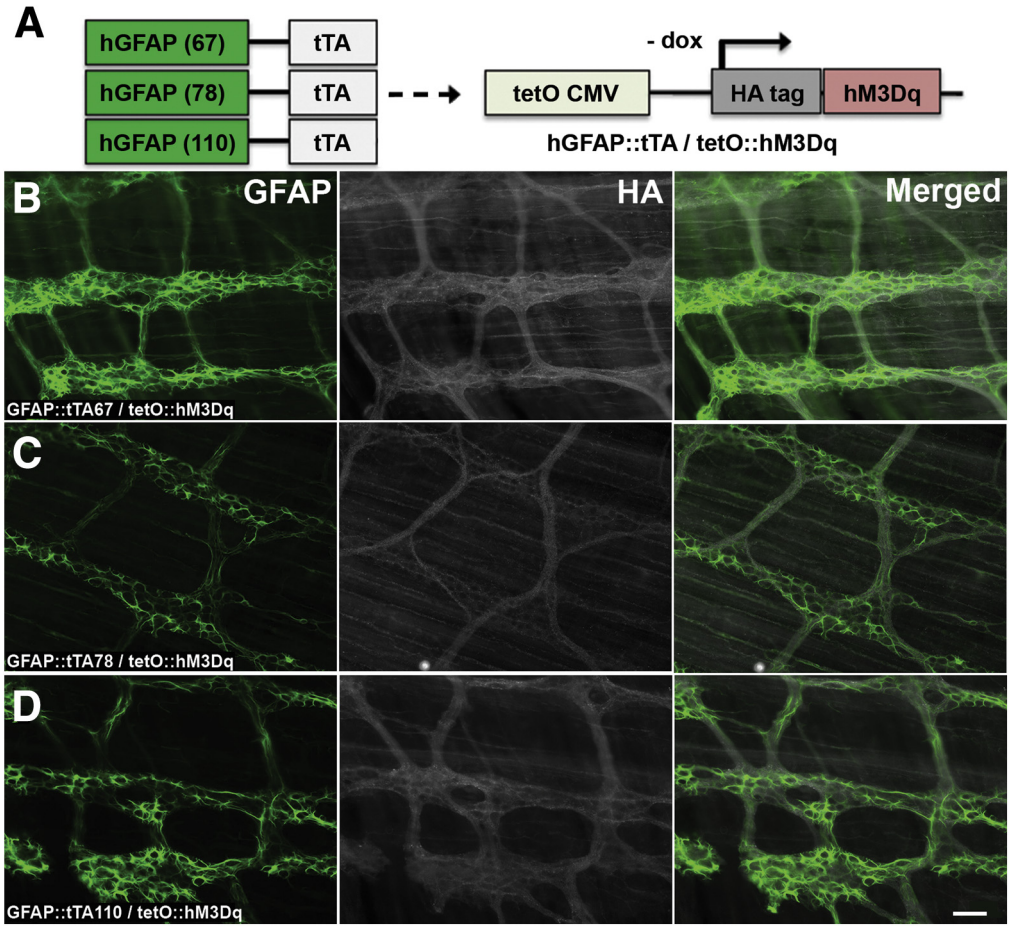

Figure 2. Ectopic expression of hM3Dq receptors in the intestines of *GFAP::tTA/tetO::hM3Dq* transgenic mice. (*A*) Breeding scheme to generate double transgenic mice that express the tetracycline-controlled transactivator protein (tTA) driven by the glial fibrillary acidic protein (GFAP) promoter and 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 μM, 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* promoter[23] (Figure 1*B*) and three double transgenic lines that express the tetracycline-controlled transactivator protein (tTA) under regulatory control of the *GFAP* promoter[24] and the tetracycline-responsive promoter element (TRE; tetO) upstream of the hM3Dq receptor[29] (Figure 2*A*). 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 1*C–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 1*F*), interstitial cells of Cajal (Figure 1*G*), or smooth muscle cells (Figure 1*G*). 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

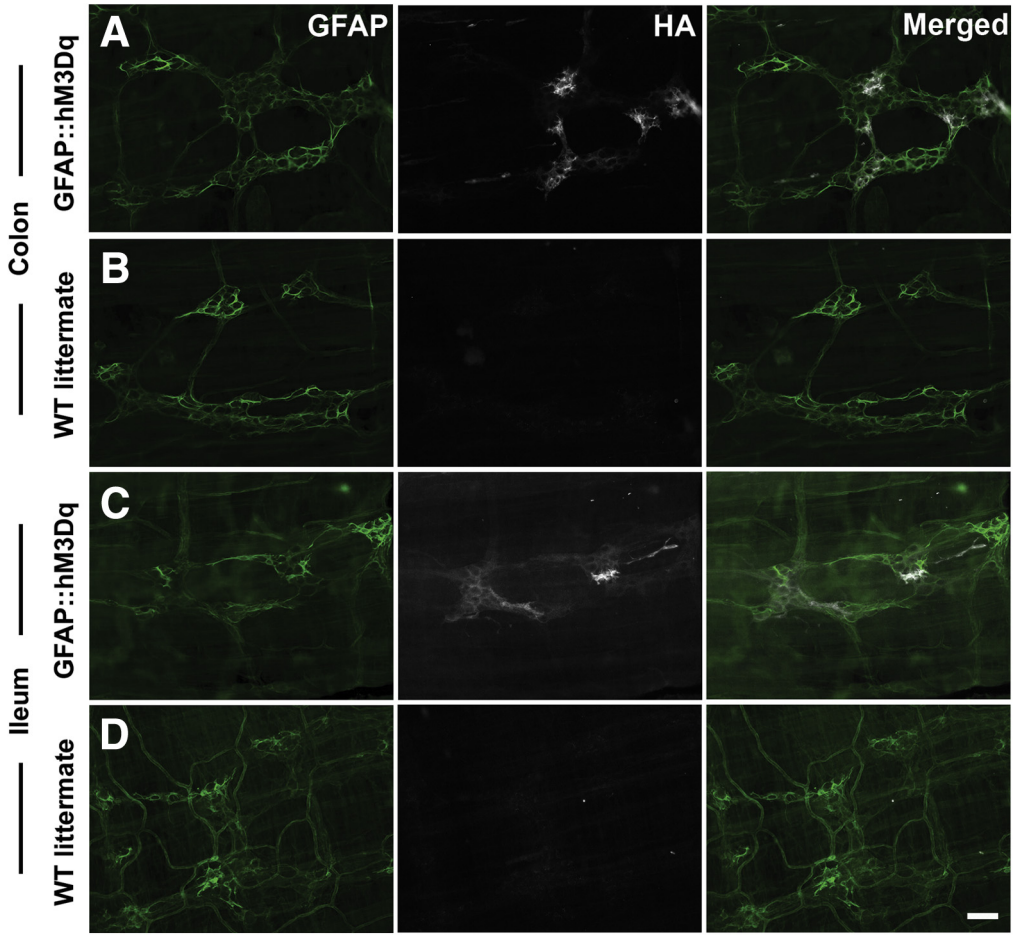

Figure 3. Expression of hM3Dq receptors is confined to enteric glia in the intestines of *GFAP::hM3Dq* mice. (*A–D*) Epifluorescence images of immunoreactivity for enteric glia (glial fibrillary acidic protein [GFAP] *shown in green*) and the hemagglutinin (HA)-tagged hM3Dq protein (*grayscale*) in whole-mount 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 μM, 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 μM)[23] in tissue from *GFAP::hM3Dq* mice selectively induces reliable intracellular Ca^{2+} responses within enteric glia (Figure 4*A*, *C*, and *D* and Video 1) but has no effect on cellular Ca^{2+} levels in tissue from WT littermates (Figure 4*B*). Enteric glia from *GFAP::hM3Dq* mice and WT littermates exhibited comparable purine-evoked Ca^{2+} responses (adenosine diphosphate [ADP], 100 μM, 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 4*C*). 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

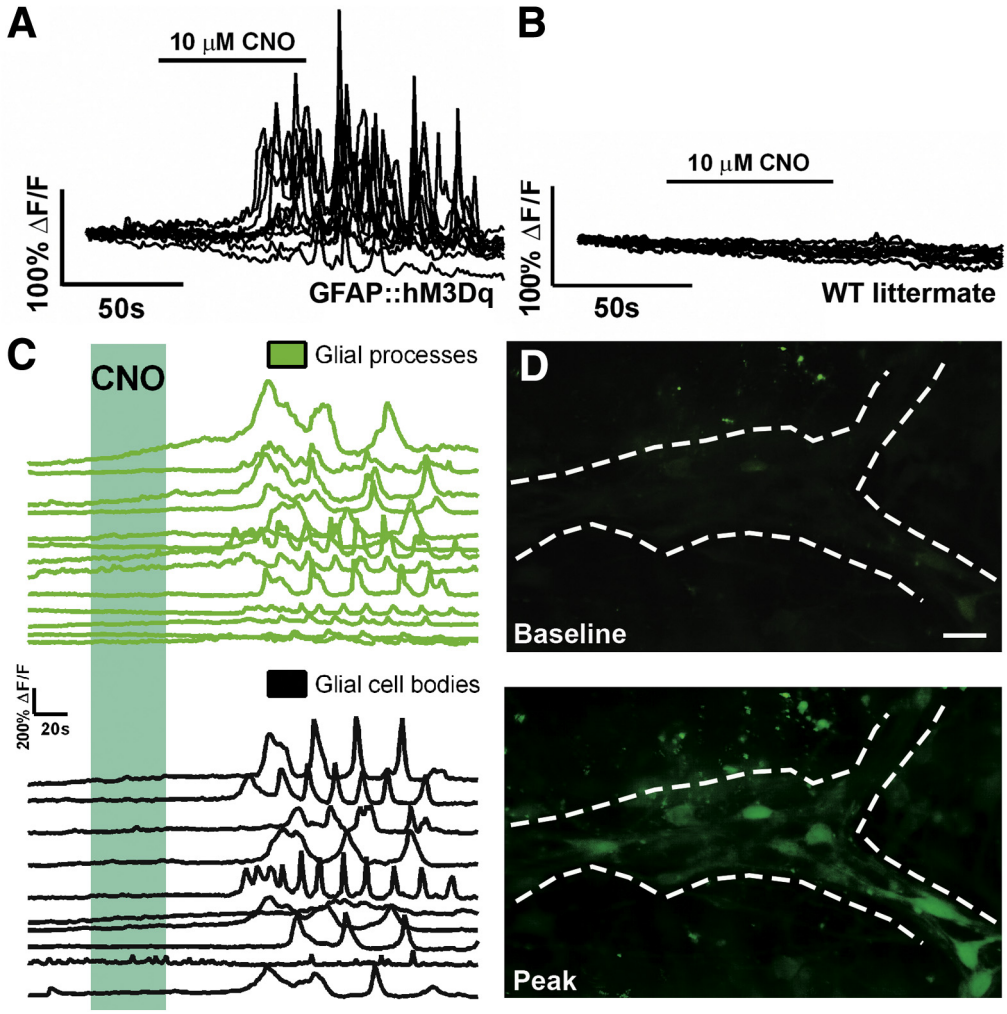

Figure 4. Activation of glial hM3Dq receptors reliably evokes intracellular calcium (Ca^{2+}) transients. (*A*, *B*) Representative traces of intracellular Ca^{2+} levels in myenteric glia in ganglia from (*A*) *GFAP::hM3Dq* transgenic mice and (*B*) wild-type (WT) littermates in response to the hM3Dq agonist clozapine-*N*-oxide (CNO, 10 μM). 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 μM. Traces are representative of recordings in n = 6 ganglia from at least three mice of each genotype.

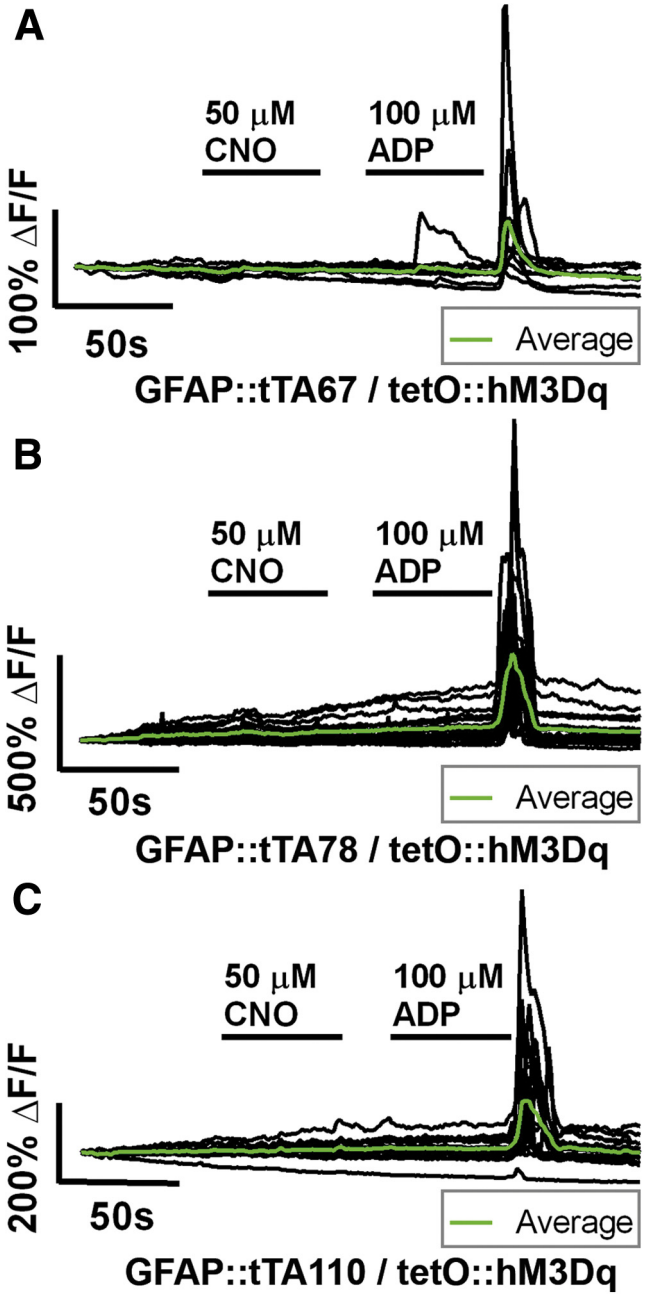

Figure 5. Representative calcium (Ca^{2+}) imaging of myenteric glia in experimental lines (*A*) *GFAP::tTA67/tetO::hM3Dq*, (*B*) *GFAP::tTA78/tetO::hM3Dq*, and (*C*) *GFAP::tTA110/tetO::hM3Dq*. Each panel shows the activity of all individual glial cells (*black traces*) within a myenteric ganglion (averaged response of all glia within ganglion *overlaid in green*). In all cases, application of clozapine-*N*-oxide (CNO; 30 seconds, 50 μM) failed to elicit Ca^{2+} activity in glial cells. These same cells exhibited robust elevations in intracellular Ca^{2+} in response to adenosine diphosphate (ADP; 30 seconds, 100 μM).

intracellular Ca^{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^{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^{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^{2+} responses with CNO in tissue from *GFAP::hM3Dq* mice (Figure 6).

Application of CNO (10 μM) elicited large contractions in segments of ileum and colon from *GFAP::hM3Dq* mice (Figure 6*A* and *B*) but had no effect on tissue from WT littermates (Figure 6*C* and *D*). Contractions elicited by CNO were equal in magnitude to those driven by the direct stimulation of smooth muscle with bethanechol (Figure 6*E* and *F*, BCH, 10 μM) and equal in magnitude to those driven by maximal stimulation of ENS neurons with EFS (20 Hz, 20 V; Figure 6*E* and *F*). Contractions driven by CNO were entirely dependent upon neuronal activation because they were absent in the presence of tetrodotoxin (Figure 6*A* 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 6*E* and *F*).

EFS induced neurogenic relaxations in segments of ileum and colon precontracted with $PGF_{2\alpha}$ (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 7*A–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).[30,31] Enteric glial cell Ca^{2+} responses are entrained with neuronal activity during the CMMC,[9] but their significance remains unclear. Therefore, we tested how the activation of glial Ca^{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

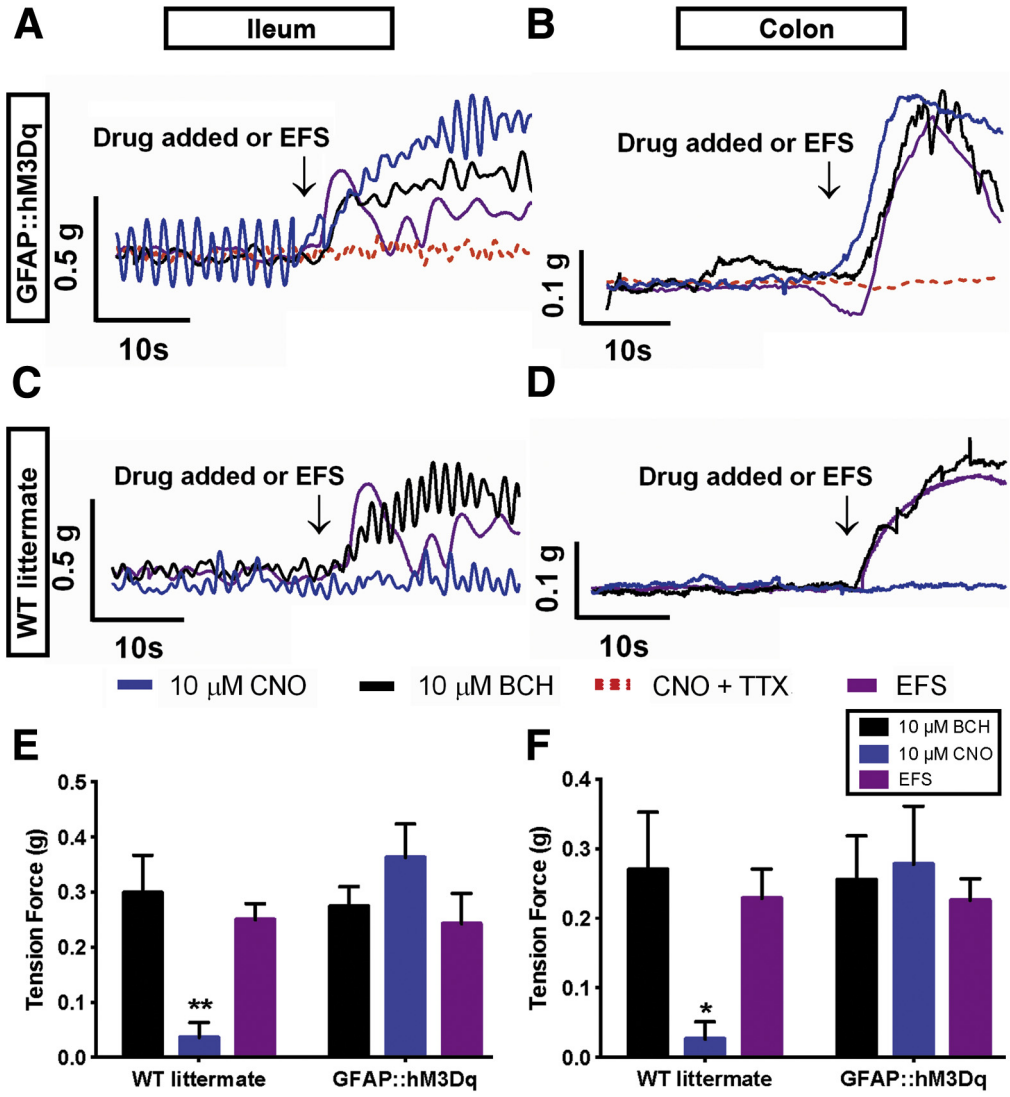

Figure 6. Selective activation of glial calcium (Ca^{2+}) responses elicits neurogenic contractions of intestinal smooth muscle. (*A–D*) Representative isometric muscle tension recordings of segments of ileum and colon from (*A, B*) *GFAP::hM3Dq* transgenic mice and (*C, D*) WT littermates. Note that clozapine-*N*-oxide (CNO; 10 μM, *blue traces*), bethanechol (BCH; 10 μM, *black traces*), and electrical field stimulation (EFS; 20 V, 0.1 ms, 20 Hz, *purple traces*) (*A, B*) elicit comparable contractions in the intestines of *GFAP::hM3Dq* transgenic mice and (*C, D*) that CNO has no effect on the contractility of tissue from wild-type (WT) littermates. Contractions elicited by CNO in *GFAP::hM3Dq* mice are abolished in the presence of tetrodotoxin (TTX, 0.3 μM, *red dashed trace* in *A, B*). (*E, F*) Summary data of contractions measured in response to CNO, BCH, and EFS in tissue from *GFAP::hM3Dq* transgenic mice and WT littermates (n = 5–7, $^*P < .05$, $^{**}P < .01$, two-way analysis of variance).

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 8*A, 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 Ca^{2+} signaling functions to enhance excitatory neural circuits that control the CMMC in mice.

Stimulation of Enteric Glial Cell Ca^{2+} Signaling Augments Gastrointestinal Motility in Vivo

Our results show that glial mechanisms downstream of Ca^{2+} signaling can activate neural circuits that drive neurogenic contractions of intestinal smooth muscle and that glial Ca^{2+} 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 Ca^{2+} 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 10*A*). Despite enhancing the number of pellets produced, we found that the composition of water and fecal matter in the pellets produced remained comparable (Figure 10*B*).

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 10*C*). However, the activation

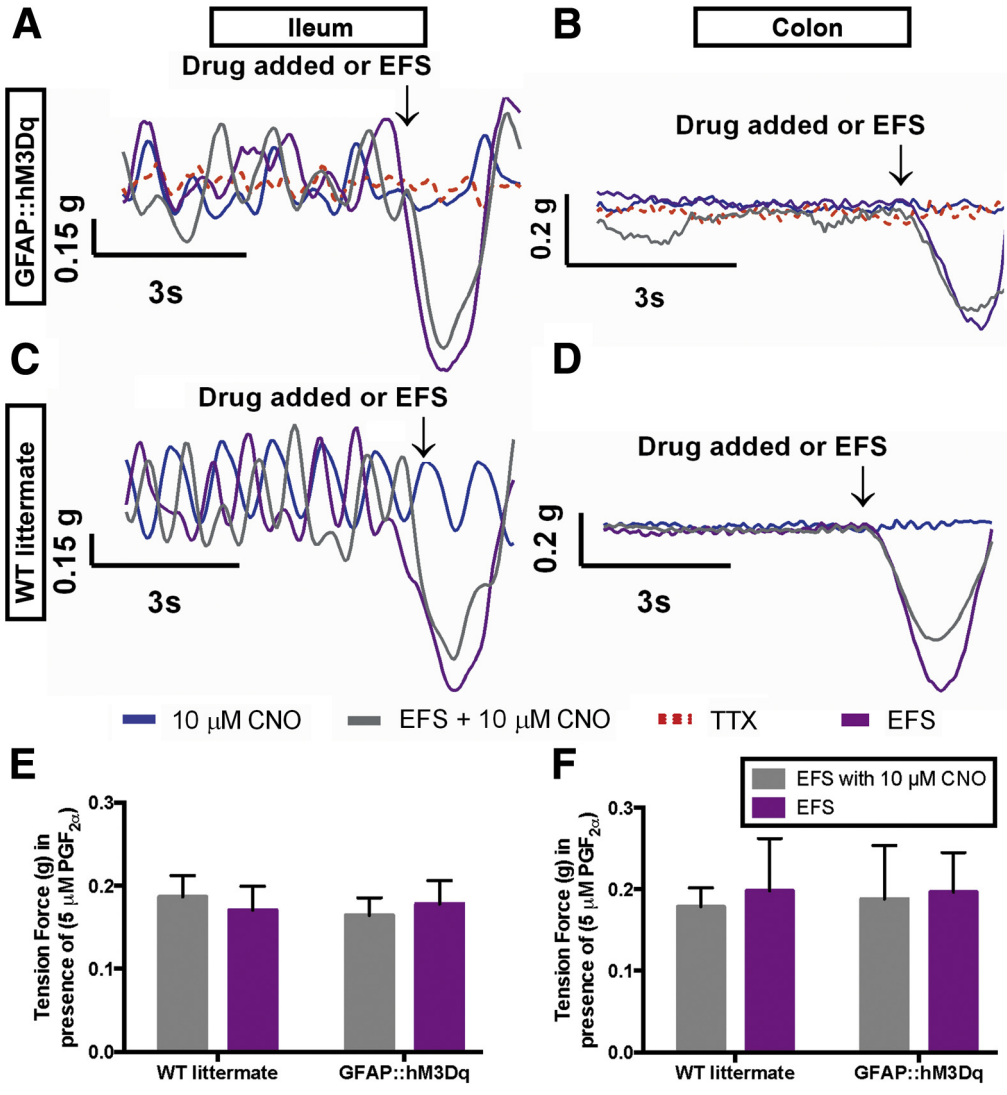

Figure 7. Glial stimulation has no effect on neurogenic relaxations. (*A, B*) Representative traces showing neurogenic relaxations elicited by electrical field stimulation (EFS) (20 V; 0.1 ms; 20 Hz, purple traces) in prostaglandin F2α-precontracted, longitudinally oriented segments of ileum (*A, C*) and colon (*B, D*) from *GFAP::hM3Dq* transgenic mice (*A, B*) and wild-type (WT) littermates (*C, D*). All relaxations are abolished in the presence of tetrodotoxin (TTX, 0.3 μM, *red dashed traces* in *A-B*). Clozapine-*N*-oxide (CNO) alone (10 μm; *blue traces* in *A–D*) does not stimulate relaxations in precontracted segments of intestine and has no effect on neurogenic relaxations driven by EFS in WT or *GFAP::hM3Dq* mice (*gray traces* in *A–D*). (*E, F*) Summary data of relaxations measured in response to EFS in the presence and absence of CNO in tissue from *GFAP::hM3Dq* transgenic mice and WT littermates (n = 5–7, $P > .05$, two-way analysis of variance).

of glial Ca^{2+} signaling did not significantly alter whole intestinal transit time or small intestinal transit velocity in *GFAP::hM3Dq* mice (Figure 10*D* and *E*). Together, these results indicate that glial excitation triggering Ca^{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,[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[33] and are thought to play more significant roles in the metabolic support of these neuronal circuits.[2] However, recent experimental findings showing that enteric glia display a form of excitability encoded by elevations in intracellular Ca^{2+} suggest that this division many not be as straightforward as once thought.[6–9,11] Indeed, results showing that glial Ca^{2+} responses are evoked by a variety of neuromodulators[11] including those released by neurons during physiologic pattern of ENS activity[9] have raised the controversial possibility that glial cells actively participate in information transfer in enteric circuits. Yet the outcomes of glial Ca^{2+} signaling have thus far remained unclear despite intense interest in observing glial Ca^{2+} signaling in response to various mediators.[10]

Our goal in the present study was to clarify the significance of evoked glial Ca^{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^{2+} responses.[23] Our results show that evoked glial Ca^{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

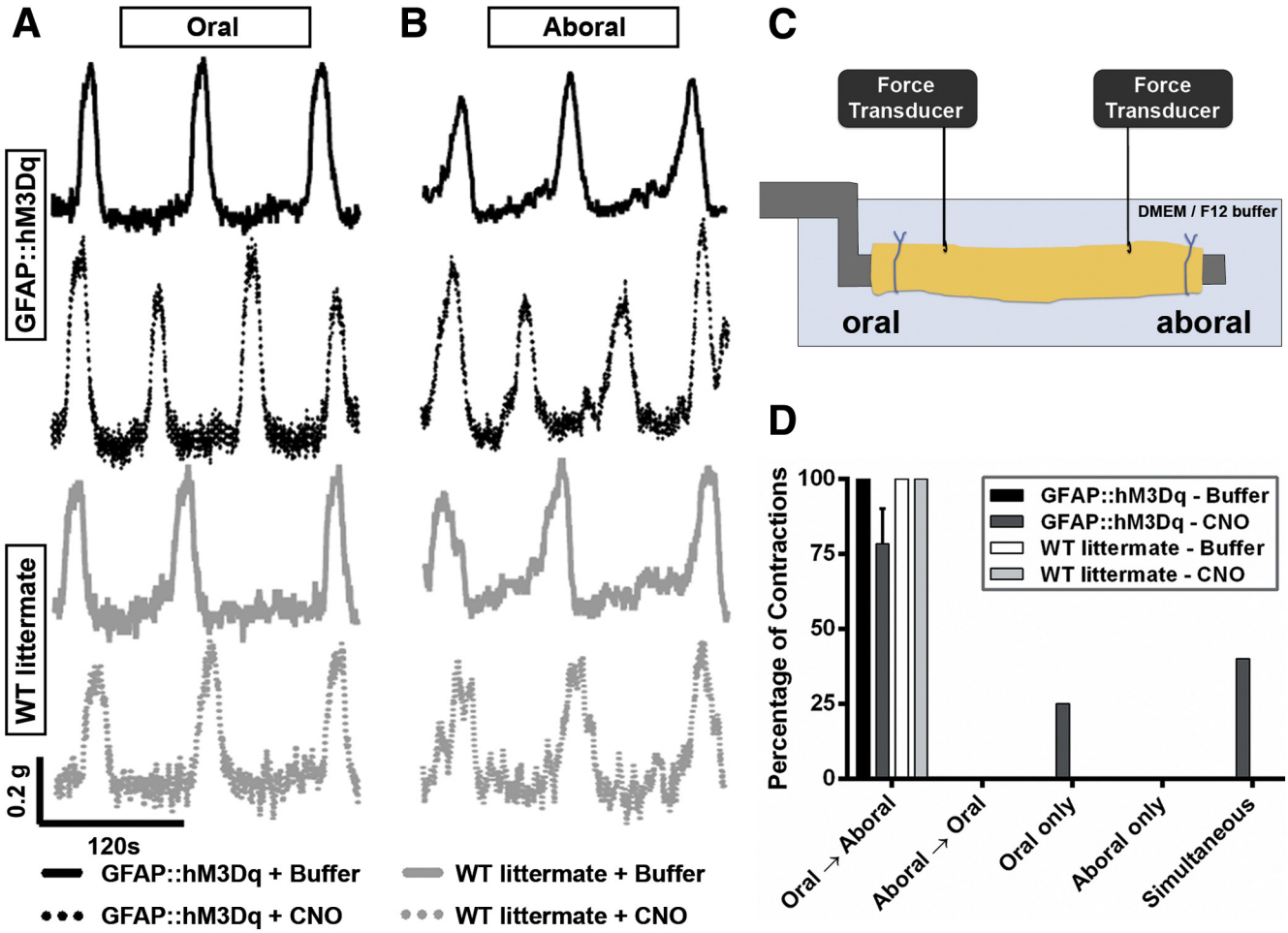

Figure 8. Selective activation of glial calcium (Ca^{2+}) transients enhances the colonic migrating motor complex (CMMC) in *GFAP::hM3Dq* transgenic mice. Representative (*A*) oral and (*B*) aboral recordings of CMMCs in *GFAP::hM3Dq* mice (*black traces*) and WT littermates (*gray traces*) under basal 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).

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^{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^{2+} signaling remain a matter of great controversy.[19] 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"[34] and the active control of extracellular potassium (K^+) ion concentration.[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[36] or by decreasing the inhibiting tone by decreasing extracellular K^+[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

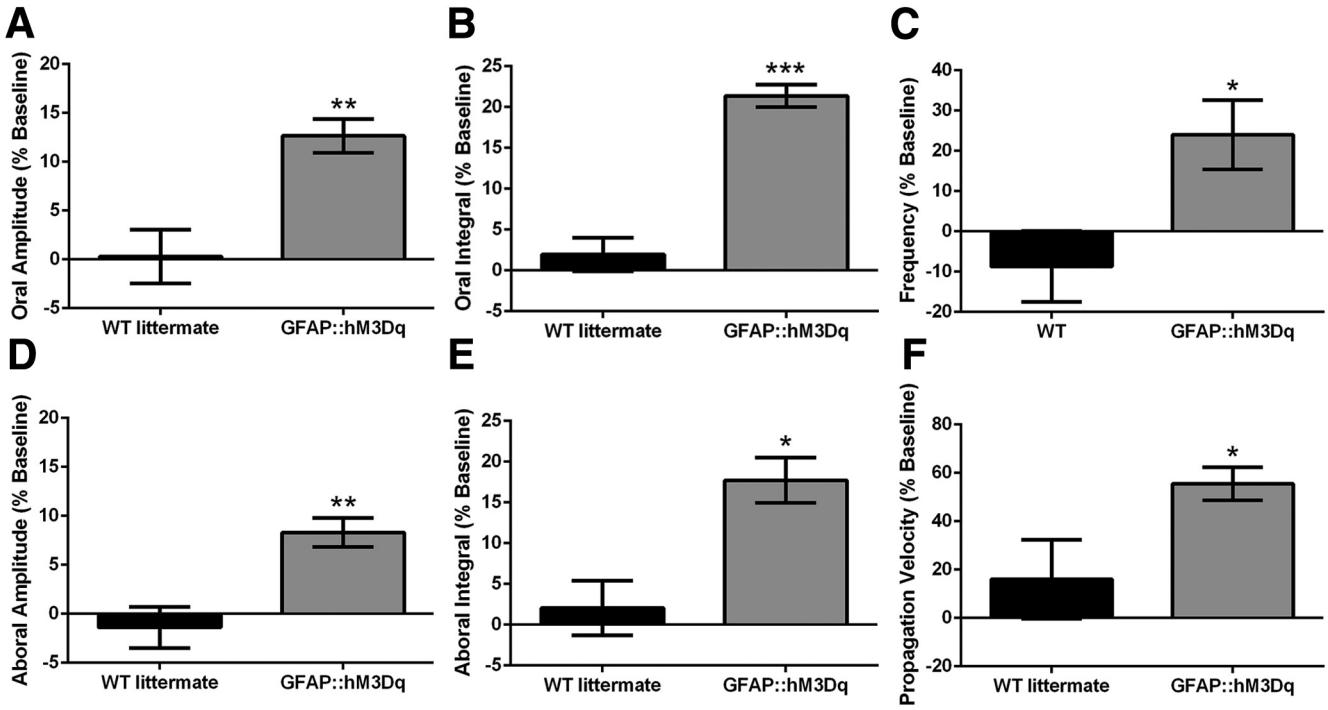

Figure 9. Activation of glial calcium (Ca^{2+}) signaling enhances key aspects of colonic migrating motor complexes (CMMCs). (*A–F*) Summary data of CMMC characteristics including oral contraction (*A*) amplitude and (*B*) integral, (*C*) CMMC frequency, aboral contraction (*D*) amplitude and (*E*) integral, 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).

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.[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

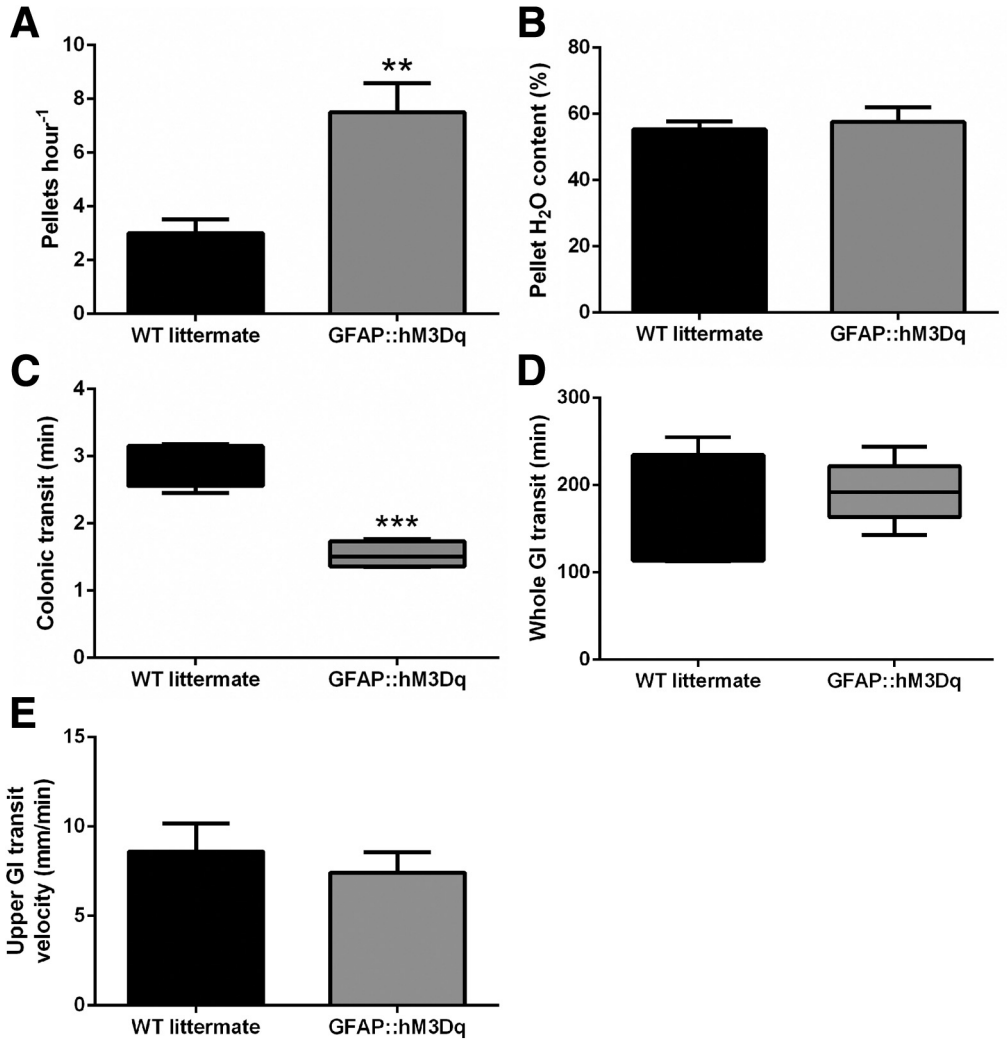

Figure 10. Selective activation of enteric glial calcium (Ca^{2+}) signaling in vivo enhances gastrointestinal motility. Effect of the hM3Dq agonist clozapine-*N*-oxide (0.25 mg kg^{-1}, intraperitoneal) on (*A*) endogenous pellet production, (*B*) fecal pellet composition, (*C*) colonic transit time, and (*D*) whole-gastrointestinal transit time and (*E*) upper gastrointestinal transit velocity in *GFAP::hM3Dq* transgenic mice and WT littermates (n = 4–5, $^{**}P < .01$, $^{***}P < .001$, two-tailed *t*-test).

constipation and other functional gastrointestinal disorders where the glial network is disrupted.[42] In these conditions, modifying glial activity could prove to be an extremely effective and novel therapeutic strategy to restore gut motility.

References

1. Wood JD, Alpers DH, Andrews PL. Fundamentals of neurogastroenterology. Gut 1999;45(Suppl 2):II6–II16.
2. Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012; 9:294.
3. Keating DJ, Spencer NJ. Release of 5-hydroxytryptamine from the mucosa is not required for the generation or propagation of colonic migrating motor complexes. Gastroenterology 2010;138:659–670, 670.e1–2.
4. Kurahashi M, Mutafova-Yambolieva V, Koh SD, et al. Platelet-derived growth factor receptor-α-positive cells and not smooth muscle cells mediate purinergic hyperpolarization in murine colonic muscles. Am J Physiol Cell Physiol 2014;307:C561–C570.
5. Neunlist M, Rolli-Derkinderen M, Latorre R, et al. Enteric glial cells: recent developments and future directions. Gastroenterology 2014;147:1230–1237.
6. Gulbransen BD, Sharkey KA. Purinergic neuron-to-glia signaling in the enteric nervous system. Gastroenterology 2009;136:1349–1358.
7. Gomes P, Chevalier J, Boesmans W, et al. ATP-dependent paracrine communication between enteric neurons and glia in a primary cell culture derived from embryonic mice. Neurogastroenterol Motil 2009;21: 870–e62.
8. Kimball BC, Mulholland MW. Enteric glia exhibit P2U receptors that increase cytosolic calcium by a phospholipase C-dependent mechanism. J Neurochem 1996; 66:604–612.
9. Broadhead MJ, Bayguinov PO, Okamoto T, et al. Ca^{2+} transients in myenteric glial cells during the colonic migrating motor complex in the isolated murine large intestine. J Physiol 2012;590:335–350.
10. Boesmans W, Martens MA, Weltens N, et al. Imaging neuron-glia interactions in the enteric nervous system. Front Cell Neurosci 2013;7:183.

11. Boesmans W, Cirillo C, van den Abbeel V, et al. Neurotransmitters involved in fast excitatory neurotransmission directly activate enteric glial cells. Neurogastroenterol Motil 2013;25:e151–e160.
12. Gulbransen BD. Enteric Glia. San Rafael, CA: Morgan & Claypool Life Sciences, 2014:1–70.
13. Gulbransen BD, Bains JS, Sharkey KA. Enteric glia are targets of the sympathetic innervation of the myenteric plexus in the guinea pig distal colon. J Neurosci 2010; 30:6801–6809.
14. Aube A-C, Cabarrocas J, Bauer J, et al. Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. Gut 2006;55:630–637.
15. Abdo HH, Derkinderen P, Gomes P, et al. Enteric glial cells protect neurons from oxidative stress in part via reduced glutathione. FASEB J 2010;24:1082–1094.
16. Abdo HH, Mahé MM, Derkinderen P, et al. The omega-6 fatty acid derivative 15-deoxy-$\Delta^{12,1}$-prostaglandin J2 is involved in neuroprotection by enteric glial cells against oxidative stress. J Physiol 2012;590: 2739–2750.
17. Nasser Y, Fernandez E, Keenan CM, et al. Role of enteric glia in intestinal physiology: effects of the gliotoxin fluorocitrate on motor and secretory function. Am J Physiol Gastrointest Liver Physiol 2006;291:912–927.
18. McClain JL, Grubišić V, Fried D, et al. Ca^{2+} responses in enteric glia are mediated by connexin-43 hemichannels and modulate colonic transit in mice. Gastroenterology 2014;146:497–507.e1.
19. Volterra A, Liaudet N, Savtchouk I. Astrocyte Ca^{2+} signalling: an unexpected complexity. Nat Rev Neurosci 2014;15:327–335.
20. Sternson SM, Roth BL. Chemogenetic tools to interrogate brain functions. Neuroscience 2014;37:387–407.
21. Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. Annu Rev Neurosci 2011; 34:389–412.
22. Armbruster BN, Li X, Pausch MH, et al. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci USA 2007;104:5163–5168.
23. Agulhon C, Boyt KM, Xie AX, et al. Modulation of the autonomic nervous system and behaviour by acute glial cell Gq protein-coupled receptor activation in vivo. J Physiol 2013;591:5599–5609.
24. Lin W, Kemper A, McCarthy KD, et al. Interferon-gamma induced medulloblastoma in the developing cerebellum. J Neurosci 2004;24:10074–10083.
25. Gulbransen BD, Bashashati MM, Hirota SAS, et al. Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis. Nat Med 2012;18:600–604.
26. Fried DE, Gulbransen BD. In situ Ca^{2+} imaging of the enteric nervous system. J Vis Exp 2015, e52506–e52506.
27. Devries MP, Vessalo M, Galligan JJ. Deletion of P2X2 and P2X3 receptor subunits does not alter motility of the mouse colon. Front Neurosci 2010;4:22.
28. Grubišić V, Kennedy AJ, Sweatt JD, et al. Pitt-Hopkins mouse model has altered particular gastrointestinal transits in vivo. Autism Res 2015, Published online, http://dx.doi.org/10.1002/aur.1467.
29. Alexander GM, Rogan SC, Abbas AI, et al. Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 2009; 63:27–39.
30. Heredia DJ, Dickson EJ, Bayguinov PO, et al. Colonic elongation inhibits pellet propulsion and migrating motor complexes in the murine large bowel. J Physiol 2010; 588:2919–2934.
31. Heredia DJ, Dickson EJ, Bayguinov PO, et al. Localized release of serotonin (5-hydroxytryptamine) by a fecal pellet regulates migrating motor complexes in murine colon. Gastroenterology 2009;136:1328–1338.
32. Dogiel AS. Über den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Säugetiere [article in German]. Arch Anat Physiol Leipz 1899;5:130–158.
33. Hanani M, Francke M, Hartig W, et al. Patch-clamp study of neurons and glial cells in isolated myenteric ganglia. Am J Physiol Gastrointest Liver Physiol 2000; 278:G644–G651.
34. Cao X, Li L-P, Wang Q, et al. Astrocyte-derived ATP modulates depressive-like behaviors. Nat Med 2013; 19:773–777.
35. Wang F, Smith NA, Xu Q, et al. Astrocytes modulate neural network activity by Ca^{2+}-dependent uptake of extracellular K^{+}. Sci Signal 2012;5:ra26.
36. Zhang W, Segura BJ, Lin TR, et al. Intercellular calcium waves in cultured enteric glia from neonatal guinea pig. Glia 2003;42:252–262.
37. Costagliola A, Van Nassauw L, Snyders D, et al. Voltage-gated delayed rectifier Kv1-subunits may serve as distinctive markers for enteroglial cells with different phenotypes in the murine ileum. Neurosci Lett 2009; 461:80–84.
38. Boesmans W, Lasrado R, Vanden Berghe P, et al. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. Glia 2015; 63:229–241.
39. Bush TG, Savidge TC, Freeman TC, et al. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. Cell 1998;93: 13–13.
40. Grundy D, Brookes S. Neural Control of Gastrointestinal Function. San Rafael, CA: Morgan & Claypool Life Sciences, 2011:1–134.
41. MacEachern SJ, Patel BA, Keenan CM, et al. Inhibiting inducible nitric oxide synthase in enteric glia restores electrogenic ion transport in mice with colitis. Gastroenterology 2015;149:445–455.e3.
42. Bassotti G, Villanacci V, Maurer CA, et al. The role of glial cells and apoptosis of enteric neurones in the neuropathology of intractable slow transit constipation. Gut 2006; 55:41–46.

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Conflicts of interest
The authors disclose no conflicts.

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