THE RECEPTOR TGR5 MEDIATES THE PROKINETIC ACTIONS OF INTESTINAL BILE ACIDS AND IS REQUIRED FOR NORMAL DEFECATION IN MICE

Short Title: The mechanism of bile acid-evoked peristalsis

Farzad Alemi[1], Daniel P. Poole[2], Jonathan Chiu[1], Kristina Schoonjans[3], Fiore Cattaruzza[1], John R. Grider[4], Nigel W. Bunnett[5], Carlos U. Corvera[1]

[1]Department of Surgery, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA; [2]Department of Anatomy and Neuroscience, University of Melbourne, Parkville, VIC 3010, Australia; [3]Laboratory of Integrative and Systems Physiology, Institute of Bioengineering, School of Life Sciences, EPFL, SV, Station 15, CH-1015 Lausanne, Switzerland; [4]Department of Physiology, P.O. Box 980551, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298, USA; [5]Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, VIC 3052, Australia.

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Abbreviations: BA, bile acid; BDNF, brain-derived neurotropic factor; CGRP, calcitonin gene-related peptide; DCA, deoxycholic acid; EC, enterochromaffin; 5-HT, 5-hydroxytryptamine; IPANs, intrinsic primary afferent neurons; IR, immunoreactivity; LCA, lithocholic acid; NFM, neurofilament M; OA, oleanolic acid.

Correspondence: Nigel Bunnett, B.Sc., Ph.D., Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, VIC 3052, Australia. Tel: Office - +61 3 9903 9136; Mobile - +61 407 392 619. Facsimile: +61 3 9903 9581. Email: Nigel.Bunnett@Monash.edu; Carlos U. Corvera, M.D., Department of Surgery, University of California, San Francisco, VA Medical Center, Surgical Service (112), 4150 Clement Street, San Francisco, CA, 94121. Tel: Office - 415 221 4180 ext. 4019. E-mail: Carlos.corvera@ucsfmedctr.org.

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ABSTRACT

Background & Aims: Abnormal delivery of bile acids (BAs) to the colon, due to disease or therapy, causes constipation or diarrhea by unknown mechanisms. The G protein-coupled BA receptor TGR5 (or GPBAR1) is expressed by enteric neurons and endocrine cells, which regulate motility and secretion.

Methods: We analyzed gastrointestinal and colon transit, and defecation frequency and water content, in wild-type, knockout and transgenic mice (*trg5-wt, tgr5-ko and tgr5-tg*, respectively). We analyzed colon tissues for contractility, peristalsis, and transmitter release.

Results: Deoxycholic acid inhibited contractility of colonic longitudinal muscle from *tgr5-wt* but not *tgr5-ko* mice. Application of deoxycholic acid, lithocholic acid, or oleanolic acid (a selective agonist of TGR5) to the mucosa of *tgr5-wt* mice caused oral contraction and caudal relaxation, indicating peristalsis. BAs stimulated release of the peristaltic transmitters 5-hydroxytryptamine and calcitonin gene-related peptide; antagonists of these transmitters suppressed BA-induced peristalsis, consistent with TGR5 localization to enterochromaffin cells and intrinsic primary afferent neurons. *tgr5-ko* mice did not undergo peristalsis or transmitter release in response to BAs. Mechanically induced peristalsis and transmitter release were not affected by deletion of *tgr5*. Whole-gut transit was 1.4-fold slower in *tgr5-ko* than *tgr5-wt* or *tgr5-tg* mice, whereas colonic transit was 2.2-fold faster in *tgr5-tg* mice. Defecation frequency was reduced 2.6-fold in *tgr5-ko* and increased 1.4-fold in *tgr5-tg* mice, compared to *tgr5-wt* mice. Water content in stool was lower (37%) in *tgr5-ko* than *tgr5-tg* (58%) or tgr5-wt mice (62%).

Conclusions: The receptor TGR5 mediates the effects of BAs on colonic motility; TGR5 deficiency causes constipation in mice. These findings might mediate the long-known laxative properties of BAs; TGR5 might be a therapeutic target for digestive diseases.

Keywords: digestion; mouse model; intestine; diarrhea

INTRODUCTION

In addition to their role in the digestion and absorption of dietary fat, bile acids (BAs) control intestinal motility and secretion, and abnormal BA delivery to the intestine due to disease or therapy results in defects in intestinal functions [1, 2]. The mechanisms of these well recognized patho-physiological actions of BAs are unknown. The primary bile acids, cholic and chenodeoxycholic acid, are synthesized from cholesterol in hepatocytes, secreted into canaliculi and stored in the gallbladder. After secretion into the intestine, primary BAs are actively absorbed in the ileum. The small amounts of primary BAs that normally reach the colon are deconjugated and dehydroxylated by bacteria to form the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA), which are passively absorbed. Since BAs are secreted episodically and are efficiently absorbed *via* the enterohepatic circulation, BA concentrations in the systemic and portal circulation fluctuate with feeding [3].

Luminal BAs exert region-specific actions in the intestine. They inhibit motility of the small intestine, which may contribute to the "ileal brake" that slows transit to allow efficient absorption [4, 5]. In contrast, BAs stimulate motility in the large intestine [6], and their effects on colonic transit are related to their prosecretory actions [7, 8]. Disease- or therapy-related alterations in BA concentrations in the intestine have a marked impact on digestive functions. Decreased colonic delivery of free BAs as a result of cholestatic disease or after treatment of lipid disorders with BA sequestrants results in constipation [9, 10]. Conversely, inflammatory bowel disease or ileal resection lead to impaired ileal BA absorption and increased colonic delivery, which can cause diarrhea [11]. Many (20%) cholecystectomy patients develop diarrhea due to continuous delivery of BAs into the intestine [12]. These effects of BAs have therapeutic implications. The ingestion of bile has been used to alleviate constipation for millennia [13], and drugs that target the ileal BA transporter can ameliorate constipation by increasing the colonic delivery of BAs [14]. The mechanisms by which luminal BAs affect intestinal functions are unknown.

Although the cell types that mediate these actions of BAs have not been identified, studies using neurotoxins and neurotransmitter antagonists suggest that BAs control motility and secretion through effects on the enteric nervous system [15]. Endocrine and paracrine mechanisms may also contribute, since DCA acts on enterochromaffin (EC) cells to stimulate release of 5-

hydroxytryptamine (5-HT), a major regulator of secretion and motility [16]. BAs also induce release of glucagon-like peptide-1 [17], an incretin and mediator of the ileal brake [18]. How BAs regulate enteric neurons and enteroendocrine cells is unknown.

We report that a G protein-coupled receptor TGR5 (GPR130, GpBAR1) that is expressed by EC cells and myenteric neurons mediates the effects of BAs on intestinal motility. BAs exert hormonal-like effects by activating nuclear and plasma membrane receptors [19]. Nuclear receptors mediate many of the genomic effects of BAs. TGR5 is a widely distributed plasma membrane BA receptor [20, 21] that controls energy metabolism [22], glucose homeostasis [17, 23], bile composition/secretion [24-27], and inflammation [20, 28, 29]. We observed that TGR5 is expressed by >50% of enteric neurons [30]. We now report that TGR5 mediates the effects of BAs on peristalsis, an evolutionarily conserved reflex that is essential for normal digestion, and that altered TGR5 expression has a major impact on intestinal transit and defecation. We have thus identified the elusive mechanism that underlies the patho-physiological actions of BAs in the intestine that have been recognized for millennia.

MATERIALS AND METHODS

Mice. The generation of *tgr5-ko*, *tgr5-tg* and *tgr5-wt* mice in a C57BL/6 background has been described [23]. C57BL/6 mice were from Charles River (Wilmington, MA). Mice were killed by sodium pentobarbital (200 $mg.kg^{-1}$, i.p.) or CO_2 inhalation and bilateral thoracotomy. Institutional Animal Care and Use Committees approved all procedures.

Contractility. Full thickness segments (1 cm) of proximal colon were mounted in organ baths in physiological saline solution under 1 g tension. Longitudinal contractions were recorded using isotonic transducers, and data were processed and analyzed as described [30]. Tissues were equilibrated for 30 min, and challenged with carbachol (1 μM) to assess viability. Tissues were washed and challenged with DCA (100 μM), UDCA (100 μM) or vehicle (0.1% ethanol or distilled H_2O). The mean amplitude of the basal tension and the frequency of phasic contractions were determined 5 min before and after challenge, and results are normalized to basal values.

Peristalsis. Peristalsis was examined using a flat sheet preparation of proximal colon [31-33]. A 5-cm segment of colon was opened and pinned mucosal side up in a tissue bath. The segment was divided into three compartments by vertical partitions and 1 ml of a Krebs'-bicarbonate medium

was added to each compartment. The mucosa of the central compartment was stimulated by application of DCA, LCA, OA (1-100 μM) or vehicle or by stroking with a fine brush (2-8 strokes, 1 stroke.s^{-1}). Ascending contraction of circular muscle was measured in the orad peripheral compartment and descending relaxation was measured in the caudad peripheral compartment using force-displacement transducers attached to the muscle layers. Results are expressed as grams force above or below baseline tone. In some experiments, GR113808 (1 μM), $CGRP_{8-37}$ (10 μM) or vehicle (control) were added the central compartment 10 min before mucosal application of BAs [31].

Transmitter release. Transmitter release into the central compartment was measured using a flat sheet preparation of distal colon. For measurement of neuropeptide and BDNF release, the medium contained bovine serum albumin (0.1%), amastatin (10 μM) and phosphoramidon (1 μM). For measurement of 5-HT release, the medium contained pargyline (10 μM). The mucosa of the central compartment was stimulated by application of DCA, OA or vehicle for 15 min, and medium was collected for immunoassays of CGRP, 5-HT and BDNF [31, 33, 34]. Results are expressed as ng.g^{-1} wet tissue.

Gastrointestinal transit. Mice were fasted overnight with free access to water. Evans blue (5%) and methyl cellulose (1.5%) solution was administered by gavage (100 μl). The time for expulsion of the first blue pellet was determined.

Colonic transit. Mice were fasted overnight with free access to water. Colonic transit was measured using a bead expulsion test as described [35]. A glass bead (3 mm diameter) was inserted into the colon (2 cm). The time until bead expulsion was measured.

Defecation frequency and water content. Freely feeding mice were observed for 2 hr. and the frequency of pellet expulsion was determined. Fecal water content was measured by comparing the weight of the pellets at the end of the experiment and after drying (24 h, 37°C).

Immunofluorescence. Whole mounts and frozen sections prepared from paraformaldehyde-fixed tissues were processed for indirect immunofluorescence and confocal microscopy as described [30]. Tissues were incubated with primary antibodies (overnight or 48 h, 4°C): rabbit anti-TGR5 P87/88 [30] (1:200), NLS1937 (Novus Biologicals, Littleton, CO) (1:5,000); chicken anti-NFM (Gene Tex Inc., Irvine, CA) (1:500); sheep anti-CGRP (1:1,000); goat anti-5-HT #20079 (Immunostar, Hudson, WI, UK) (1:1,000).

Statistical analysis. Results are expressed as mean±SEM and were compared by Student's t-test (2 comparisons) or ANOVA and Student-Newman-Keuls test (multiple comparisons). $P<0.05$ was considered significant.

RESULTS

BAs regulate contractility of colonic muscle via TGR5. DCA is a TGR5 agonist [21] that inhibits spontaneous phasic contractions of longitudinal muscle of the mouse proximal colon by a neurogenic, nitrergic mechanism [30]. Consistent with this observation, DCA (100 μM) inhibited spontaneous phasic contractions of longitudinal muscle of isolated proximal colon from *tgr5-wild type (wt)* mice, inhibiting both the frequency of contractions and the muscle tension (fold-basal (1.0): frequency (0.25±0.05; tension, 0.78±0.05) (Fig. 1A-C). In marked contrast, DCA had no effect on spontaneous contractility of the colon from *tgr5-knockout (ko)* mice (fold-basal: frequency (0.91±0.05; tension, 0.92±0.02; $P<0.001$ frequency, $P<0.05$ tension to *tgr5-wt*). UDCA (100 μM), a weak agonist of TGR5 that retains the detergent and irritant properties of BAs [21], did not affect contractility of the colon from *tgr5-wt* mice (Fig. 1A). Under basal conditions, the frequency of spontaneous contractions was higher in *tgr5-wt* (contractions/min, 11.67±2.08) than in *tgr5-ko* (7.33±1.48) mice, although the difference was not significant ($P=0.12$, n=6) (tension was set to 1 g). The response to carbachol was similar (1 μM carbachol, 3 min: tension, fold-basal, *tgr5-wt*, 1.44±0.08; *tgr5-ko*, 1.46±0.06, n=6; frequency could not be determined). Thus, the actions of DCA on contractility of colonic longitudinal muscle in the mouse require TGR5 expression.

BAs promote peristaltic contractions of the colon via TGR5. A flat sheet preparation of mouse colon divided into three compartments allows assessment of peristaltic contractions of the oral and caudal compartments after application of stimulants to the mucosal side of the central compartment [31]. In a flat sheet preparation of proximal colon from *tgr5-wt* mice, application of DCA (1, 10, 100 μM) to the mucosa of the central compartment initiated an immediate and concentration-dependent contraction of the oral compartment and relaxation of the caudal compartment (Fig. 2A, B), consistent with activation of the peristaltic reflex, which was also stimulated by mucosal stroking (Fig. 2C) [31]. The effect of the maximal concentration of DCA (100 μM) was slightly less than that elicited by maximal mucosal stroking (83.6±6.9% of 8-

stroke value for ascending contraction, 61.5±4.1% for descending relaxation). Oleanolic acid (OA, 100 μM), a TGR5-selective agonist [36], had a similar effect to DCA (63.6±7.2% of 8-stroke response for ascending contraction, 64.1±6.2% for descending relaxation). In tissue from *tgr5-ko* mice, these effects were absent (1, 10 μM DCA; 100 μM OA) or markedly blunted (100 μM DCA) (Fig. 2A, B). In contrast, the effects of mucosal stroking on oral contraction and caudal relaxation were the same in tissues from *tgr5-wt* and *tgr5-ko* mice (Fig. 2C). Thus, DCA stimulates the ascending contraction and descending relaxation components of the peristaltic reflex of the mouse colon by a TGR5-mediated mechanism. However, TGR5 does not participate in peristalsis induced by mechanical stimulation of the mucosa.

Given the irritant actions of BAs and the finding that repeated administration of high concentrations of DCA (4 mM) can cause colonic inflammation [37], we examined whether the highest concentration of DCA (100 μM) induced damage to the colonic mucosa. Exposure of the mucosa of the isolated colon to 100 μM DCA for 10 min caused no detectable histological damage or neutrophil infiltration to the mucosa (Supporting Information Fig. 1). Moreover, intracolonic administration of DCA (50 μl, 1 or 3 mM) to mice did not induce granulocyte infiltration, determined by assays of myeloperoxidase activity, or plasma extravasation, determined by measurement of Evans blue leak, within 2-3 h (Supporting Information Fig. 2). Thus, the effects of DCA on peristalsis, which were immediate, are unrelated to mucosal damage or inflammation.

5-HT and CGRP mediate BA-induced peristalsis. Chemical and mechanical stimulation of the mucosa can release 5-HT from EC cells, which activates 5-HT_4 receptors on intrinsic primary afferent neurons (IPANs) to release CGRP [31-33]. CGRP stimulates ascending and descending interneurons, and is thus a critical neuronal transmitter of peristalsis. To investigate the contribution of 5-HT and CGRP to BA-induced peristalsis, antagonists of 5-HT_4 receptors (GR113808) or CGRP receptors ($CGRP_{8-37}$) were added to the central compartment of colon from C57BL/6 mice 10 min before mucosal application of BAs. In tissues treated with antagonists, the effects of DCA on ascending contraction and descending relaxation were abolished (1 μM DCA) or markedly blunted (10, 100 μM DCA) (Fig. 3A, B). However, $CGRP_{8-37}$ consistently inhibited DCA-stimulated peristalsis to a greater extent than GR113808 at all DCA concentrations. Thus, $CGRP_{8-37}$ inhibited DCA (10 μM)-stimulated ascending contraction by 86±7% and descending relaxation by 91±5%, whereas GR113808 inhibited ascending

contraction by 33±8% and descending relaxation by 38±7%. Mucosal application of LCA, another secondary BA that activates TGR5 [21], stimulated peristalsis to a similar extent to DCA (Fig. 3C). In tissues treated with GR113808 or $CGRP_{8-37}$, the effects of LCA were also abolished (1 μM LCA) or attenuated (10, 100 μM LCA). $CGRP_{8-37}$ inhibited OA (100 μM)-stimulated ascending contraction by 72±5% and descending relaxation by 67±5%, whereas GR113808 inhibited ascending contraction by 30±10% and descending relaxation by 40±5%. Thus, 5-HT and CGRP mediate the effects of BAs on the peristaltic reflex of the mouse colon.

BAs stimulate the release of 5-HT and CGRP from the colon via TGR5. To confirm the effects of the antagonists, 5-HT and CGRP release were examined. In preparations of distal colon from *tgr5-wt* mice, mucosal application of DCA or OA stimulated a concentration-dependent secretion of 5-HT and CGRP immunoreactivity (IR) into the medium of the middle chamber (Fig. 4A, B). Although basal levels of both transmitters were similar between *trg5-wt* and *trg5-ko* mice, stimulated release of both transmitters was absent (1, 10 μM DCA; 100 μM OA) or blunted (100 μM DCA) in tissues from *tgr5-ko* mice (Fig. 4A, B), in agreement with the defective BA-stimulated peristalsis (Fig. 2) and the inhibitory effects of antagonists (Fig. 3). BDNF is also released by mechanical stimulation of the mucosa and augments peristalsis by enhancing 5-HT and CGRP release in response to mechanical stimuli [34]. Basal BDNF release was the same in *tgr5-wt* (0.85±0.12 ng/g tissue, n=4) and *tgr5-ko* (0.98±0.0.19 ng/g) mice (P>0.05). Neither DCA (100 μM, *tgr5-wt* 0.89±0.20 ng/g; *tgr5-ko* 1.11±0.22 ng/g, n=4) nor OA (100 μM, *tgr5-wt* 1.1±0.21 ng/g; *tgr5-ko* 0.87±0.11 ng/g, n=4) stimulated BDNF release (P>0.05 to basal). However, mechanically-stimulated BDNF release was the same in *tgr5-wt* (8-strokes=1.66±0.24 ng/g, n=4) and *tgr5-ko* (8-strokes=1.84±0.18 ng/g) mice (P>0.05 to basal). The lack of BA-stimulated BDNF release may explain the diminished peristaltic contractions in response to DCA compared to mucosal stroking.

TGR5 is expressed by IPANs and EC cells. To determine the possible site of action of BAs, we localized TGR5 in the intestine using two antibodies directed to the C-terminus [30]. Both antibodies detected human TGR5 heterologously expressed in HEK cells but did not stain non-transfected cells (Supporting Information Fig. 3), and staining of intestinal tissues was abolished by preadsorption with antigen (see [30], indicating specific detection). In sections of colon, TGR5-IR was localized to mucosal epithelial cells and to neurons of the myenteric plexus (Fig. 5A, arrowheads). TGR5-IR colocalized with CGRP-IR in neurons of the myenteric plexus, and

CGRP-IR was also detected in mucosal nerve fibers (Fig. 5A, arrowhead with asterisk). In the mucosa, TGR5-IR colocalized with 5-HT-IR in EC cells (Fig. 5B, arrowheads). Wholemounts of myenteric plexus were studied to define the neurochemical coding and identification of neurons expressing TGR5-IR. TGR5-IR colocalized with CGRP-IR and neurofilament M (NFM)-IR, which identify IPANs with Dogiel type II morphology (Fig. 5C, arrowheads). However, TGR5-IR was also detected in other unidentified neurons (Fig. 5C, arrowhead with asterisk). Thus, TGR5 is expressed by cell types that regulate peristalsis.

TGR5 expression affects gastrointestinal and colonic transit. The observation that TGR5 expression is required for the effects of DCA on contractility and peristalsis raised the possibility that the level of TGR5 expression *per se* may affect gastrointestinal and colonic transit. To evaluate this possibility, Evans blue/methyl cellulose was administered by gavage to *tgr5-wt*, *tgr5-ko* and *tgr5-transgenic (tg*, overexpressing mouse TGR5) mice. Gastrointestinal transit, determined by measuring the time for expulsion of the first blue fecal pellet, was 1.4-fold slower in *tgr5-ko* mice (402±21 min) compared to *tgr5-wt* (283±30 min) and *tgr5-tg* (284±27 min) mice (Fig. 6A, $P<0.01$ *tgr5-ko* to *tgr5-wt* and *tgr5-tg*). BAs can affect gastric emptying as well as motility of the small and large intestines [30]. To specifically examine colonic transit, the times for expulsion of a glass bead from the colon of *tgr5-wt*, *tgr5-ko* and *tgr5-tg* mice was determined. Bead expulsion was accelerated 2.2-fold in *tgr5-tg* mice (879±397 s) compared to *tgr5-wt* (1939±531 s) or *tgr5-ko* (1963±434 s) mice (Fig. 6B, $P<0.05$ *tgr5-tg* to *tgr5-wt* and *tgr5-ko*). Thus, the absence of TGR5 slows gastrointestinal transit, whereas overexpression of TGR5 has no overall effect. TGR5 overexpression accelerates colonic transit, consistent with the pro-secretory and pro-kinetic actions of BAs in the colon.

TGR5 expression affects the frequency of defecation and fecal water content. To determine if the level of TGR5 expression affects defecation, the rate of pellet excretion in freely-feeding *tgr5-wt*, *tgr5-ko* and *tgr5-tg* mice was determined. Measured over a 2 h period, pellet excretion was increased 1.4-fold in *tgr5-tg* mice (7.1±0.9 pellets/2 h; $P<0.05$ to *tgr5-wt*) but decreased 2.6-fold in *tgr5-ko* mice (1.9±0.5 pellets/2 h; $P<0.01$ to *tgr5-wt*) compared to *tgr5-wt* mice (4.9±0.7 pellets/2 h) (Fig. 7A). Fecal water content was lower in *tgr5-ko* mice (37±5%; $P<0.001$ to *tgr5-wt*) compared to *tgr5-tg* mice (57±4%) and *tgr5-wt* (62±2%) mice, consistent with the decreased frequency of defecation in *tgr5-ko* mice (Fig. 7B). Thus, *tgr5-ko* mice are constipated, producing

fewer and drier pellets, in accordance with the constipation observed in patients with cholestatic liver disease.

DISCUSSION

We have defined the mechanism underlying the established patho-physiological motor actions of BAs in the colon. Our results show that BAs activate TGR5, which is expressed by EC cells and IPANs, and release 5-HT and CGRP, the major transmitters of the afferent limb of the peristaltic reflex. In keeping with the prokinetic actions of BAs and TGR5, TGR5 deletion delays gastrointestinal transit whereas TGR5 overexpression accelerates colonic transit. Loss of TGR5 function results in excretion of fewer and drier fecal pellets, indicating constipation. Our results identify TGR5 as the key mediator of BA-induced alterations in colonic motility, and suggest that therapies that target TGR5 could be effective treatments for constipation and diarrhea.

TGR5 mediates the effects of BAs on contractility and peristalsis

Our results show that DCA inhibits spontaneous contractility of longitudinal muscle of the isolated mouse colon by a mechanism that requires TGR5 expression. We have previously reported that TGR5 is expressed by nitrergic inhibitory motoneurons of the myenteric plexus of the colon, and that DCA inhibits contractility by a neurogenic, nitrergic mechanism [30]. Our present findings support the hypothesis that DCA activates TGR5 on inhibitory motoneurons of the colon that release nitric oxide and inhibit contractility.

A major finding is that mucosal application of DCA and LCA, the major secondary BAs of the colon, stimulated ascending contraction and descending relaxation of colonic circular muscle. DCA and LCA induced peristalsis and stimulated release of peristaltic transmitters at concentrations ranging from 1-100 μM. These concentrations are within the physiological range of BAs in the lumen of the human intestine, which spans 10 mM in the proximal small intestine, 2 mM in the terminal ileum and 0.4 mM in the large intestine, where DCA accounts for 34% of BAs [38, 39]. The actions of DCA and LCA on peristalsis were immediate and did not cause detectable damage or inflammation *in vitro* or *in vivo*, either in the short- (10 min) or long- (2-3 h) term. Thus, BAs are physiological stimulants of colonic peristalsis, which is essential for normal transit and digestion. DCA, like mechanical stimulation of the mucosa, stimulated release of 5-HT and CGRP, and antagonism of 5-HT_4 and CGRP receptors attenuated BA-evoked

peristalsis. Remarkably, the effects of DCA on peristalsis and transmitter release were absent or blunted in *tgr5-ko* mice. The involvement of TGR5 is substantiated by the observation that OA, a TGR5-selective agonist [36], stimulated peristalsis and transmitter release *via* TGR5. In contrast, mechanically-evoked peristalsis was unaffected by TGR5 expression, indicating the selectivity of this process for BAs.

Our results suggest that luminal BAs trigger the afferent limb of the peristaltic reflex by activating TGR5 on EC cells and IPANs, which release 5-HT and CGRP, respectively (Supporting Information Fig. 4). We localized TGR5-IR to EC cells and IPANs, suggesting that DCA may regulate both cell types, and BAs also stimulate 5-HT release from isolated human EC cells [16]. Further experimentation is required to determine the relative importance of TRG5 activation on EC cells and IPANs, since 5-HT activates 5-HT_4 receptors on IPANs to stimulate CGRP release [31-33]. However, CGRP antagonism was consistently more effective than $5HT_4$ antagonism in suppressing BA-evoked peristalsis, which supports a major role for CGRP. CGRP activates ascending and descending interneurons that transmit the peristaltic reflex [31]. Activation of ascending excitatory motoneurons causes contraction of circular muscle through release of acetylcholine and substance P, whereas activation of descending inhibitory motoneurons induces relaxation of circular muscle *via* release of nitric oxide and vasoactive intestinal polypeptide [31]. When added to the central compartment of a flat sheet preparation, BAs would activate the sensory limb of the peristaltic reflex rather than directly affecting motor neurons. In contrast, BAs could directly regulate motoneurons of muscle strips to affect spontaneous contractility. However, the inhibition of longitudinal muscle contractility is consistent with the reciprocal regulation of circular and longitudinal muscle during peristalsis. Our conclusion that DCA stimulates peristalsis by a TGR5-mediated mechanism is in accordance with reports that luminal BAs stimulate colonic transit [6, 40].

Whereas DCA stimulated release of 5-HT and CGRP, DCA did not affect BDNF release from the colon. BDNF augments peristalsis by enhancing the release of 5-HT and CGRP to mechanical stimulation of the mucosa [34]. This inability of DCA to release BDNF may account for the smaller magnitude of DCA-evoked compared to mechanically-evoked peristaltic contractions.

The level of TGR5 expression determines intestinal transit and defecation

By studying mice with loss or gain of TGR5 function, we evaluated the importance of TGR5 in the regulation of intestinal transit and defecation. Gastrointestinal transit, determined by measuring the time for excretion of a marker administered by gavage, was 1.4-fold slower in *tgr5-ko* mice compared to *tgr5-wt* and *tgr5-tg* mice, which were identical. From these studies we were unable to define the major region of the gut that was affected by TGR5 deletion. However, BAs inhibit gastric emptying and slow small intestinal transit [5, 30], possibly by activating TGR5 on intestinal L cells to release glucagon-like peptide 1 [17], which slows transit to allow more complete nutrient absorption [18]. Thus, it is likely that TGR5 deletion impairs the prokinetic effects of BAs in the colon, resulting in an overall inhibition of whole gut transit.

In contrast to the inhibition of small intestinal transit, BAs and TGR5 agonists promote peristalsis in the colon and accelerate colonic transit [6, 40]. Although TGR5 deletion did not affect colon transit, determined by measuring the time for bead expulsion, TGR5 overexpression accelerated colonic transit by 2.2-fold, suggesting a major role for TGR5 in this tissue. This difference between propulsion in mice with loss and gain of TGR5 function is not surprising since bead propulsion is probably initiated by mucosal mechanical stimulation and distension-activated sensory pathways rather than the chemosensitive pathway activated by TGR5. Stimulation of the chemosensitive pathway, such as mediated by overexpression of TGR5, would likely enhance the response to mechanical stimulation as has been shown for the potentiation between luminal fatty acids and mechanical stimulation of the mucosa [41]. Loss of TGR5 function reduced the frequency of defecation by 2.6-fold and the fecal water content by 1.7-fold compared to *trg5-wt* mice, consistent with constipation, whereas gain of TGR5 function was associated with a 1.4-fold increase in defecation frequency.

The impact of TGR5 expression on transit and defecation may be related to a role for TGR5 in control of secretion as well as motility. TGR5 is expressed by cholinergic secretomotor neurons of the submucosal plexus [30], and BAs stimulate fluid and mucus secretion by a neuronal, cholinergic mechanism [15, 42]. TGR5 also regulates gall bladder filling [26] and mucosal integrity [43], and TGR5 deficient mice have a reduced BA pool size [26, 27], all of which could influence transit and defecation.

TGR5 as a mediator of and target for digestive diseases

Our results suggest that defects in the colonic delivery of free BAs that are secondary to certain diseases and therapeutic regimens cause constipation or diarrhea due to abnormal activation of TGR5. Decreased colonic delivery of BAs as a result of cholestatic disease or treatment of lipid disorders with BA sequestrants results in constipation [9, 10], possibly due to decreased TGR5 activity. Conversely, excessive delivery of BAs to the colon, which occurs as a result of defective ileal absorption after inflammation or resection, or due to continuous bile secretion after cholecystectomy, can cause diarrhea [11, 12], perhaps by over activation of TGR5. BA have been ingested for millennia to treat constipation [13], and inhibitors of ileal BA transporters relieve constipation by increasing colonic delivery of BAs [14]. Our observations that TGR5 activation promotes colonic peristalsis and that TGR5 overexpression accelerates colonic transit, whereas TGR5 deficiency has the opposite effects and results in constipation, suggest that therapeutic targeting of TGR5 is a new strategy to treat constipation and diarrhea. TGR5 agonists may be therapies for constipation, whereas antagonists may relieve diarrhea. Future studies will evaluate these possibilities.

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REFERENCES

1. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci 2008;65:2461-2483.
2. Bajor A, Gillberg PG, Abrahamsson H. Bile acids: short and long term effects in the intestine. Scandinavian journal of gastroenterology 2010;45:645-664.
3. Angelin B, Bjorkhem I, Einarsson K, et al. Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum. J Clin Invest 1982;70:724-731.
4. Armstrong DN, Krenz HK, Modlin IM, et al. Bile salt inhibition of motility in the isolated perfused rabbit terminal ileum. Gut 1993;34:483-488.
5. Penagini R, Misiewicz JJ, Frost PG. Effect of jejunal infusion of bile acids on small bowel transit and fasting jejunal motility in man. Gut 1988;29:789-794.
6. Snape WJ, Jr., Shiff S, Cohen S. Effect of deoxycholic acid on colonic motility in the rabbit. Am J Physiol 1980;238:G321-325.
7. Camilleri M, Murphy R, Chadwick VS. Dose-related effects of chenodeoxycholic acid in the rabbit colon. Dig Dis Sci 1980;25:433-438.

8. Chadwick VS, Gaginella TS, Carlson GL, et al. Effect of molecular structure on bile acid-induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. J Lab Clin Med 1979;94:661-674.
9. Ghaffari K, Savadkuhi ST, Honar H, et al. Obstructive cholestasis alters intestinal transit in mice: role of opioid system. Life Sci 2004;76:397-406.
10. Knodel LC, Talbert RL. Adverse effects of hypolipidaemic drugs. Med Toxicol 1987;2:10-32.
11. Miettinen TA. The role of bile salts in diarrhoea of patients with ulcerative colitis. Gut 1971;12:632-635.
12. Arlow FL, Dekovich AA, Priest RJ, et al. Bile acid-mediated postcholecystectomy diarrhea. Arch Intern Med 1987;147:1327-1329.
13. Thorner RS. The effect of exclusion of the bile upon gastrointestinal motility. Am J Roentgenol Radium Ther Nucl Med 1955;74:1096-1122.
14. Camilleri M. Pharmacology of the new treatments for lower gastrointestinal motility disorders and irritable bowel syndrome. Clin Pharmacol Ther 2012;91:44-59.
15. Karlstrom L, Cassuto J, Jodal M, et al. Involvement of the enteric nervous system in the intestinal secretion induced by sodium deoxycholate and sodium ricinoleate. Scand J Gastroenterol 1986;21:331-340.
16. Kidd M, Modlin IM, Gustafsson BI, et al. Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile salts, amines, tastants, and olfactants. Am J Physiol Gastrointest Liver Physiol 2008;295:G260-272.
17. Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem Biophys Res Commun 2005;329:386-390.
18. Schirra J, Goke B. The physiological role of GLP-1 in human: incretin, ileal brake or more? Regul Pept 2005;128:109-115.
19. Lefebvre P, Cariou B, Lien F, et al. Role of bile acids and bile acid receptors in metabolic regulation. Physiological reviews 2009;89:147-191.
20. Kawamata Y, Fujii R, Hosoya M, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem 2003;278:9435-9440.
21. Maruyama T, Miyamoto Y, Nakamura T, et al. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 2002;298:714-719.
22. Watanabe M, Houten SM, Mataki C, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 2006;439:484-489.
23. Thomas C, Gioiello A, Noriega L, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab 2009;10:167-177.
24. Keitel V, Cupisti K, Ullmer C, et al. The membrane-bound bile acid receptor TGR5 is localized in the epithelium of human gallbladders. Hepatology 2009;50:861-870.
25. Lavoie B, Balemba OB, Godfrey C, et al. Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels. J Physiol 2010;588:3295-3305.
26. Li T, Holmstrom SR, Kir S, et al. The G protein-coupled bile acid receptor, TGR5, stimulates gallbladder filling. Mol Endocrinol 2011;25:1066-1071.
27. Maruyama T, Tanaka K, Suzuki J, et al. Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. J Endocrinol 2006;191:197-205.

28. Pols TW, Nomura M, Harach T, et al. TGR5 Activation Inhibits Atherosclerosis by Reducing Macrophage Inflammation and Lipid Loading. Cell Metab 2011;14:747-757.
29. Wang YD, Chen WD, Yu D, et al. The G-Protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-kappaB) in mice. Hepatology 2011;54:1421-1432.
30. Poole DP, Godfrey C, Cattaruzza F, et al. Expression and function of the bile acid receptor GpBAR1 (TGR5) in the murine enteric nervous system. Neurogastroenterol Motil 2010;22:814-825, e227-818.
31. Grider JR. Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. J Pharmacol Exp Ther 2003;307:460-467.
32. Grider JR, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. Gastroenterology 1998;115:370-380.
33. Grider JR, Kuemmerle JF, Jin JG. 5-HT released by mucosal stimuli initiates peristalsis by activating 5-HT4/5-HT1p receptors on sensory CGRP neurons. The American journal of physiology 1996;270:G778-782.
34. Grider JR, Piland BE, Gulick MA, et al. Brain-derived neurotrophic factor augments peristalsis by augmenting 5-HT and calcitonin gene-related peptide release. Gastroenterology 2006;130:771-780.
35. Poole DP, Pelayo JC, Cattaruzza F, et al. Transient receptor potential ankyrin 1 is expressed by inhibitory motoneurons of the mouse intestine. Gastroenterology 2011;141:565-575, 575 e561-564.
36. Sato H, Genet C, Strehle A, et al. Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. Biochem Biophys Res Commun 2007;362:793-798.
37. Traub RJ, Tang B, Ji Y, et al. A rat model of chronic postinflammatory visceral pain induced by deoxycholic acid. Gastroenterology 2008;135:2075-2083.
38. Hamilton JP, Xie G, Raufman JP, et al. Human cecal bile acids: concentration and spectrum. Am J Physiol Gastrointest Liver Physiol 2007;293:G256-263.
39. Northfield TC, McColl I. Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine. Gut 1973;14:513-518.
40. Kirwan WO, Smith AN, Mitchell WD, et al. Bile acids and colonic motility in the rabbit and the human. Gut 1975;16:894-902.
41. Grider JR, Piland BE. The peristaltic reflex induced by short-chain fatty acids is mediated by sequential release of 5-HT and neuronal CGRP but not BDNF. American journal of physiology. Gastrointestinal and liver physiology 2007;292:G429-437.
42. Camilleri M, Murphy R, Chadwick VS. Pharmacological inhibition of chenodeoxycholate-induced fluid and mucus secretion and mucosal injury in the rabbit colon. Dig.Dis.Sci. 1982;27:865-869.
43. Cipriani S, Mencarelli A, Chini MG, et al. The Bile Acid Receptor GPBAR-1 (TGR5) Modulates Integrity of Intestinal Barrier and Immune Response to Experimental Colitis. PLoS One 2011;6:e25637.

FIGURE LEGENDS

Figure 1. TGR5-dependent regulation of colonic contractility. Recordings were made of spontaneous phasic contractions of longitudinal muscle of isolated proximal colon. **A.** Representative recordings from *tgr5-wt* and *tgr5-ko* mice. In *tgr5-wt* mice, UDCA (100 μM) had no effect, whereas DCA (100 μM) immediately inhibited spontaneous phasic contractions. In *tgr5-ko* mice, DCA was inactive. **B, C.** Mean results of the effects of DCA on frequency (B) and tension (C) normalized to basal values (1.0). DCA reduced the frequency and tension in *tgr5-wt* but not in *tgr5-ko* mice. * P< 0.05, *** P<0.001 to *tgr5-wt*.

Figure 2. TGR5-dependent stimulation of colonic peristalsis. Peristaltic contractions of isolated proximal colon were recorded from *tgr5-wt* and *tgr5-ko* mice. **A.** Representative recordings of ascending contraction and descending relaxation to mucosal application of DCA (100 μM), which stimulated peristalsis in *tgr5-wt* mice, and had diminished effects in *tgr5-ko* mice. **B, C.** Mean results of ascending contraction and descending relaxation (grams force above or below baseline tone) in response to mucosal application of graded concentrations of DCA (1-100 μM) (B) or graded mechanical stimulation of the mucosa (2-8 strokes). Compared to responses in *tgr5-wt* mice, peristaltic contractions to DCA were abolished or attenuated in *tgr5-ko* mice, whereas responses to mechanical stimulation were unaffected by TGR5 expression. **P<0.005 to *tgr5-wt*.

Figure 3. Contributions of 5-HT and CGRP to colonic peristalsis. Peristaltic contractions of isolated proximal colon were recorded from C57BL/6 mice. **A.** Representative recordings of ascending contraction and descending relaxation showing that GR113808 or $CGRP_{8-37}$ attenuated DCA (100 μM)-stimulated peristalsis. **B, C.** Mean results of ascending contraction and descending relaxation to graded concentrations of DCA (B) or LCA (C) (1-100 μM). GR113808 or $CGRP_{8-37}$ attenuated DCA- and LCA-stimulated peristalsis, although $CGRP_{8-37}$ was more effective. *P<0.05, **P<0.005, ***P<0.001 to vehicle.

Figure 4. TGR5-dependent release of peristaltic transmitters from proximal colon. Release of 5-HT-IR (**A**) or CGRP-IR (**B**) into the central compartment of the isolated proximal colon from *tgr5-wt* and *trg5-ko* mice after mucosal application of DCA (1-100 μM) or OA (100 μM). Whereas DCA and OA stimulated 5-HT and CGRP release in *tgr5-wt* mice, responses were absent or attenuated in *tgr5-ko* mice. *P<0.05, **P<0.01 to basal.

Figure 5. Localization of TGR5-IR to myenteric neurons and EC cells of mouse colon. TGR5 was detected in C57BL/6 mice using antibody NLS1937 (A) or P87/88 [30] (B, C) with

similar results. **A.** In sections of whole thickness colon, TGR5-IR was detected in neurons of the myenteric plexus (MP) within the muscularis externa (ME) and in mucosal epithelial cells (arrowheads). CGRP-IR colocalized with TGR5-IR in myenteric neurons, and was also found in nerve fibers in the mucosa (arrowhead asterisk). **B.** In sections of the colonic mucosa, TGR5-LI colocalized with 5-HT-IR in EC cells (arrowheads), and was also detected in epithelial cells. **C.** Analysis of the neurochemical coding of neurons in wholemounts of the myenteric plexus revealed colocalization of TGR5-IR, CGRP-IR and NFM-IR in IPANS (arrowheads), although TGR5-IR was also detected in other neuronal subtypes (arrowhead asterisk). Scale bars: A 100 μm, B, C 20 μm.

Figure 6. Gastrointestinal and colonic transit. A. Whole gut transit of Evans blue dye. After gavage of dye, the time for expulsion of the first blue pellet was longer in *tgr5-ko* mice than *tgr5-wt* or *tgr5-tg* mice, indicating delayed transit. **B.** Expulsion of a glass bead from the colon. After insertion of a glass bead into the colon, the time for expulsion was less in *tgr5-tg* mice than in *tgr5-wt* or *tgr5-ko* mice, indicating accelerated colonic transit. **P< 0.01, ***P<0.001 to *tgr5-wt*.

Figure 7. Defecation frequency and water content. A. Defecation frequency was diminished in *tgr5-ko* mice but increased in *trg5-tg* mice compared to *tgr5-wt* mice. **B.** Fecal water content was diminished in *tgr5-ko* mice compared to *tgr5-wt* or *tgr5-tg* mice. **P< 0.01, ***P<0.001 to *tgr5-wt*.

Figure 1

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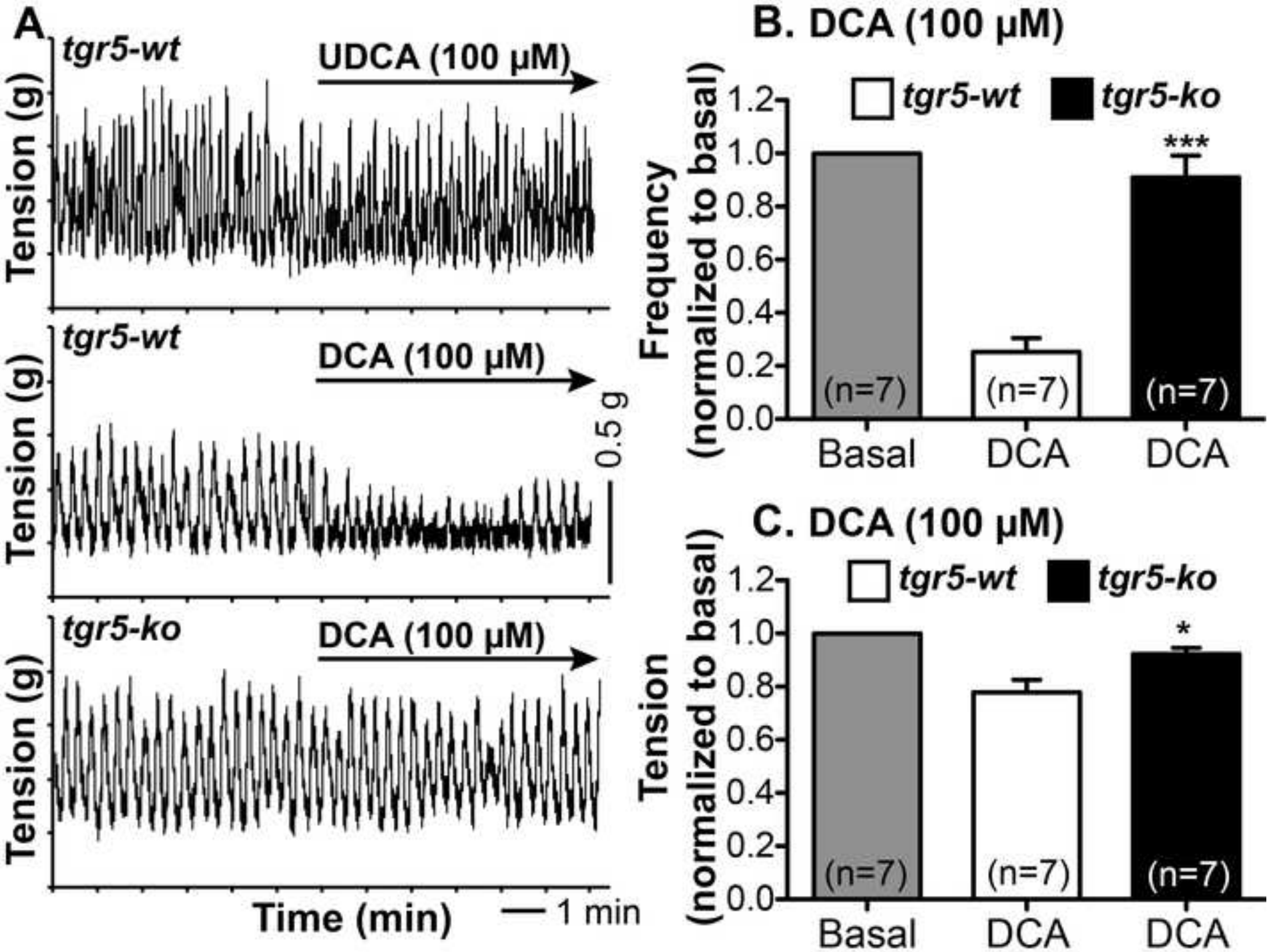

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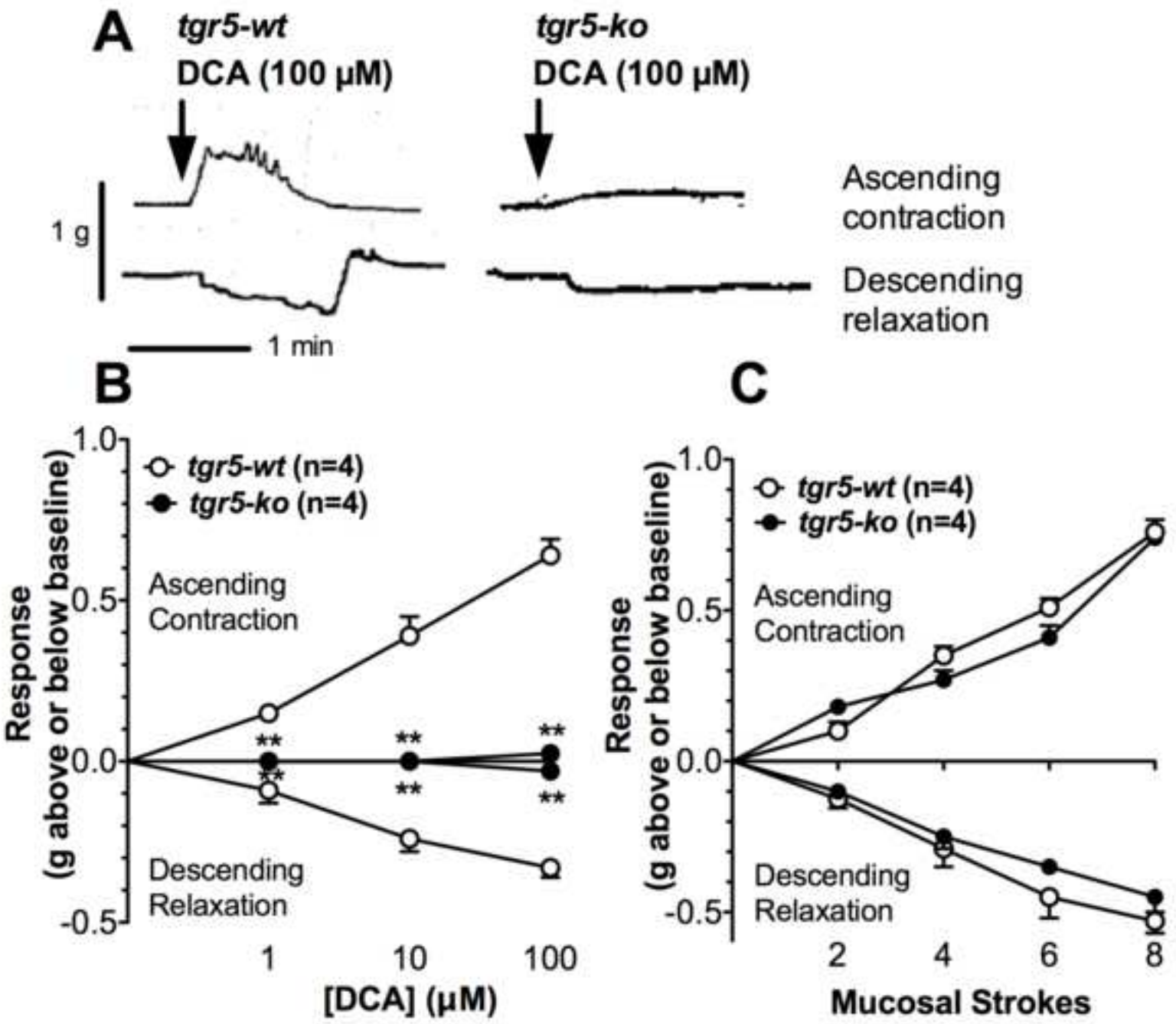

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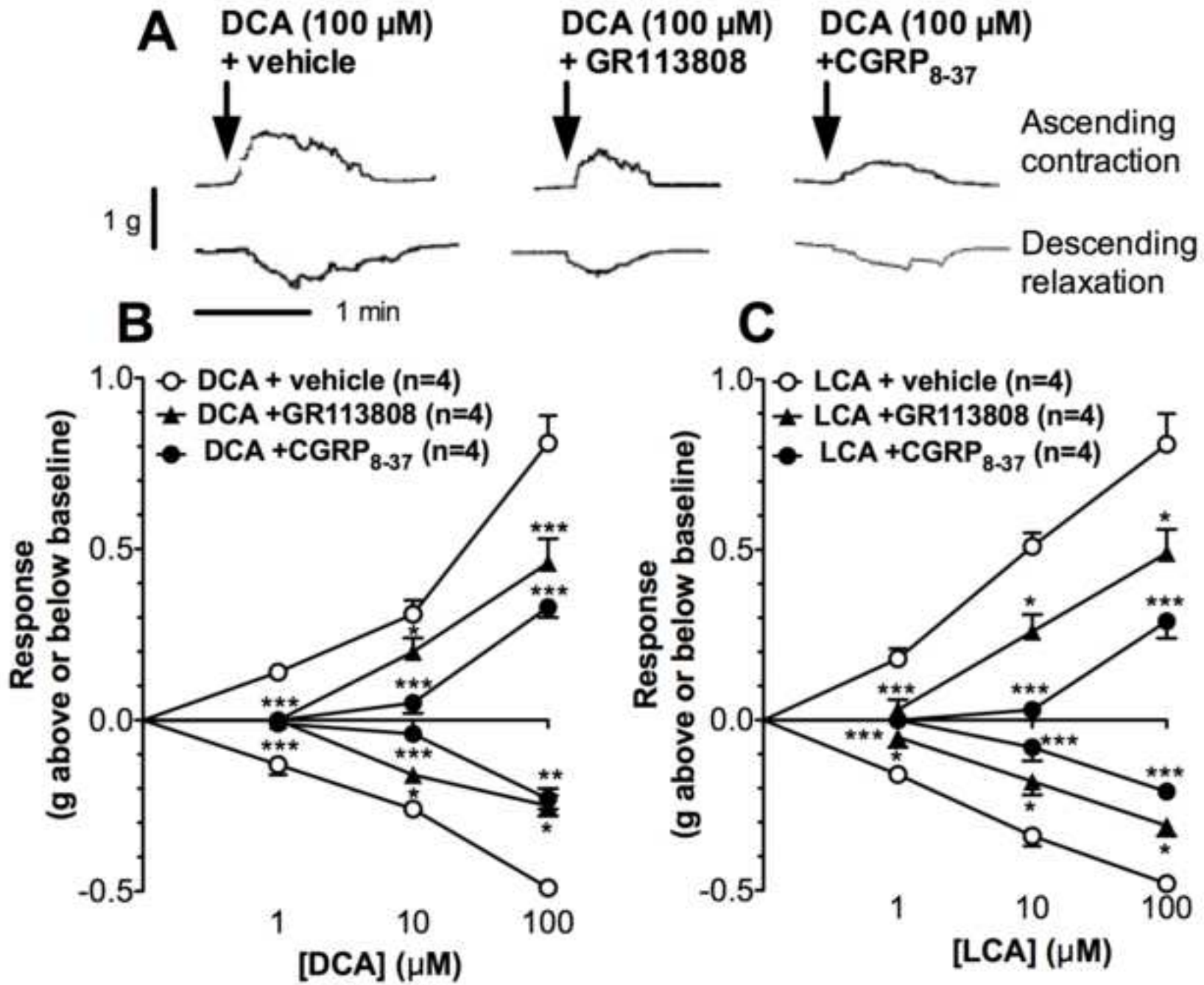

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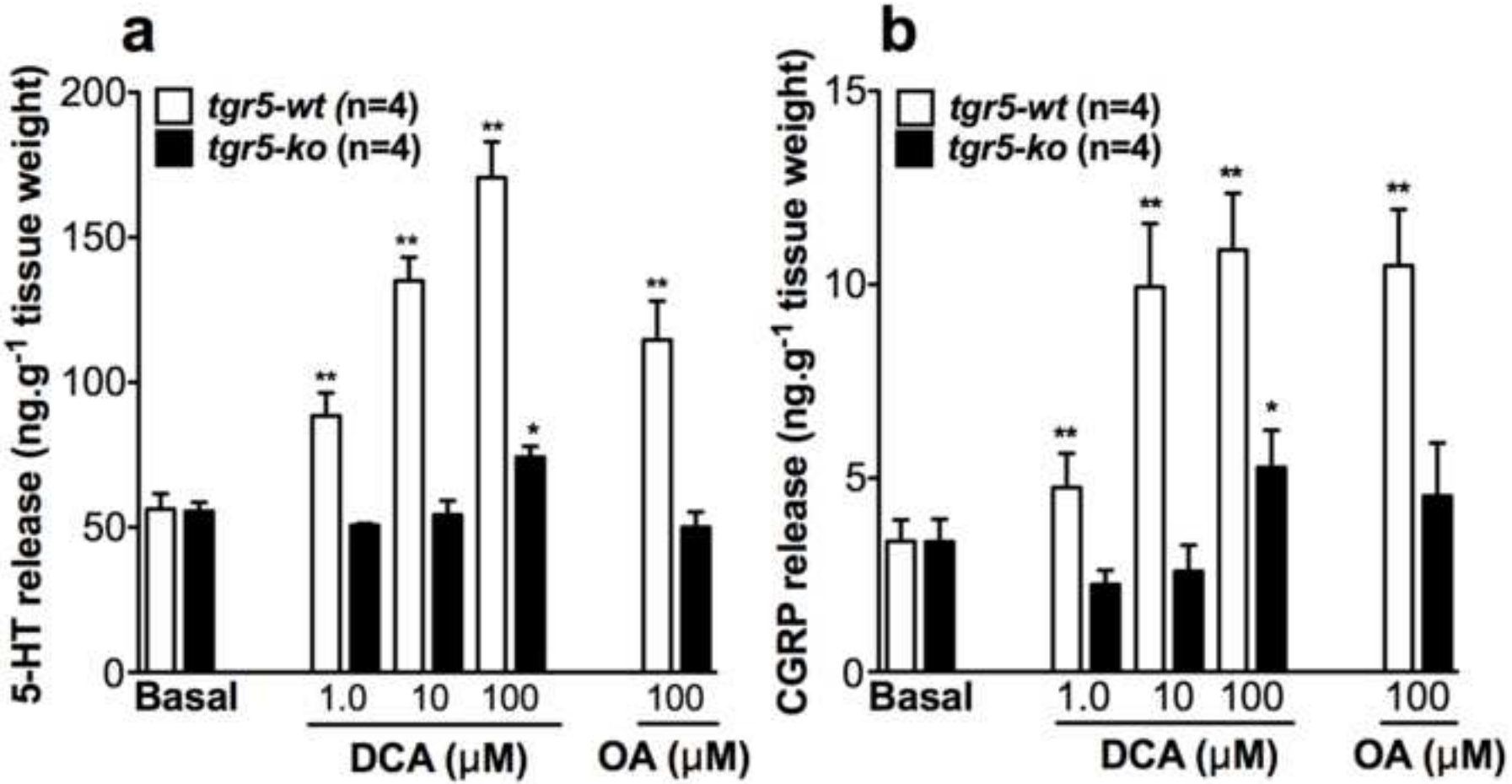

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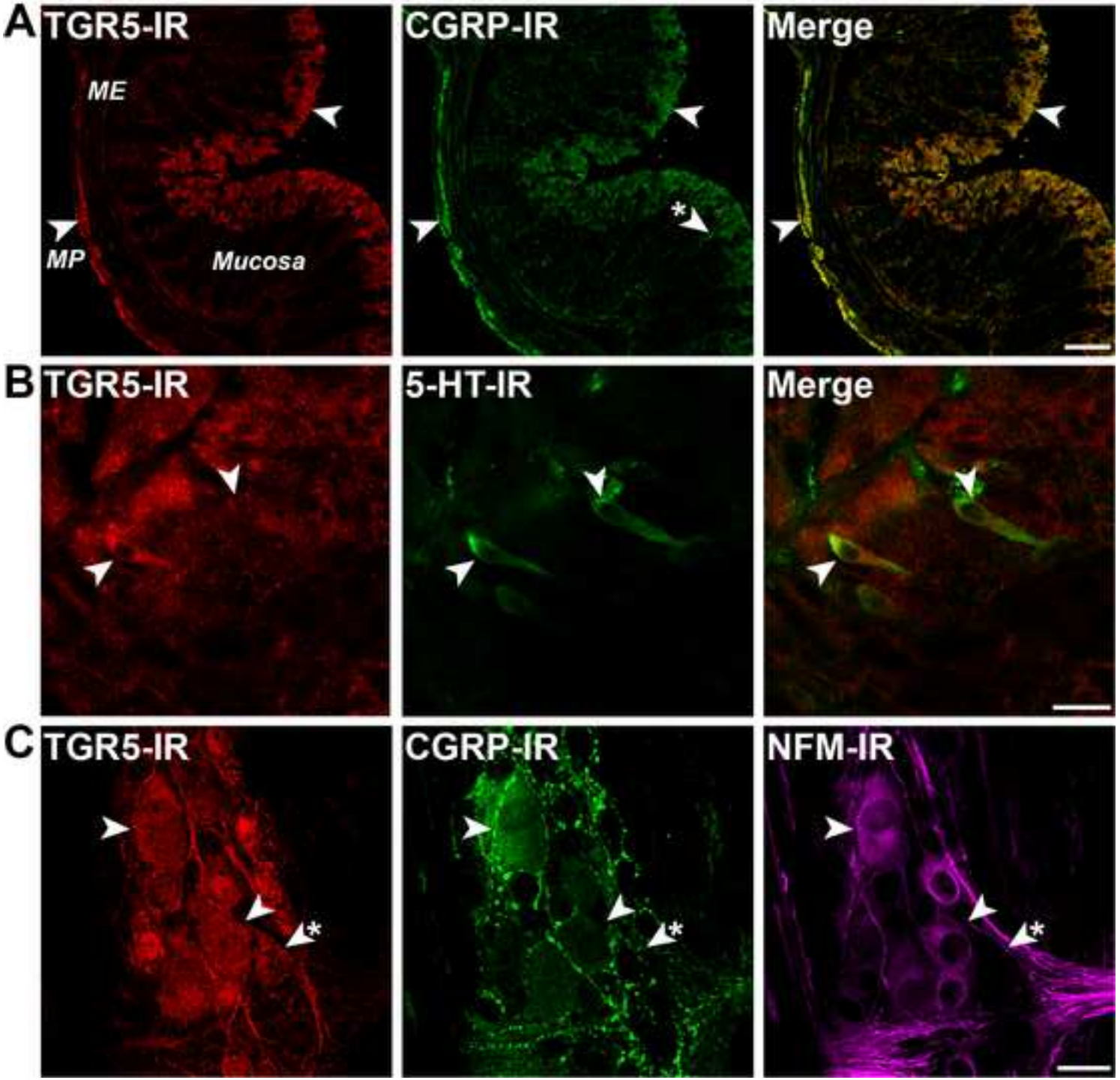

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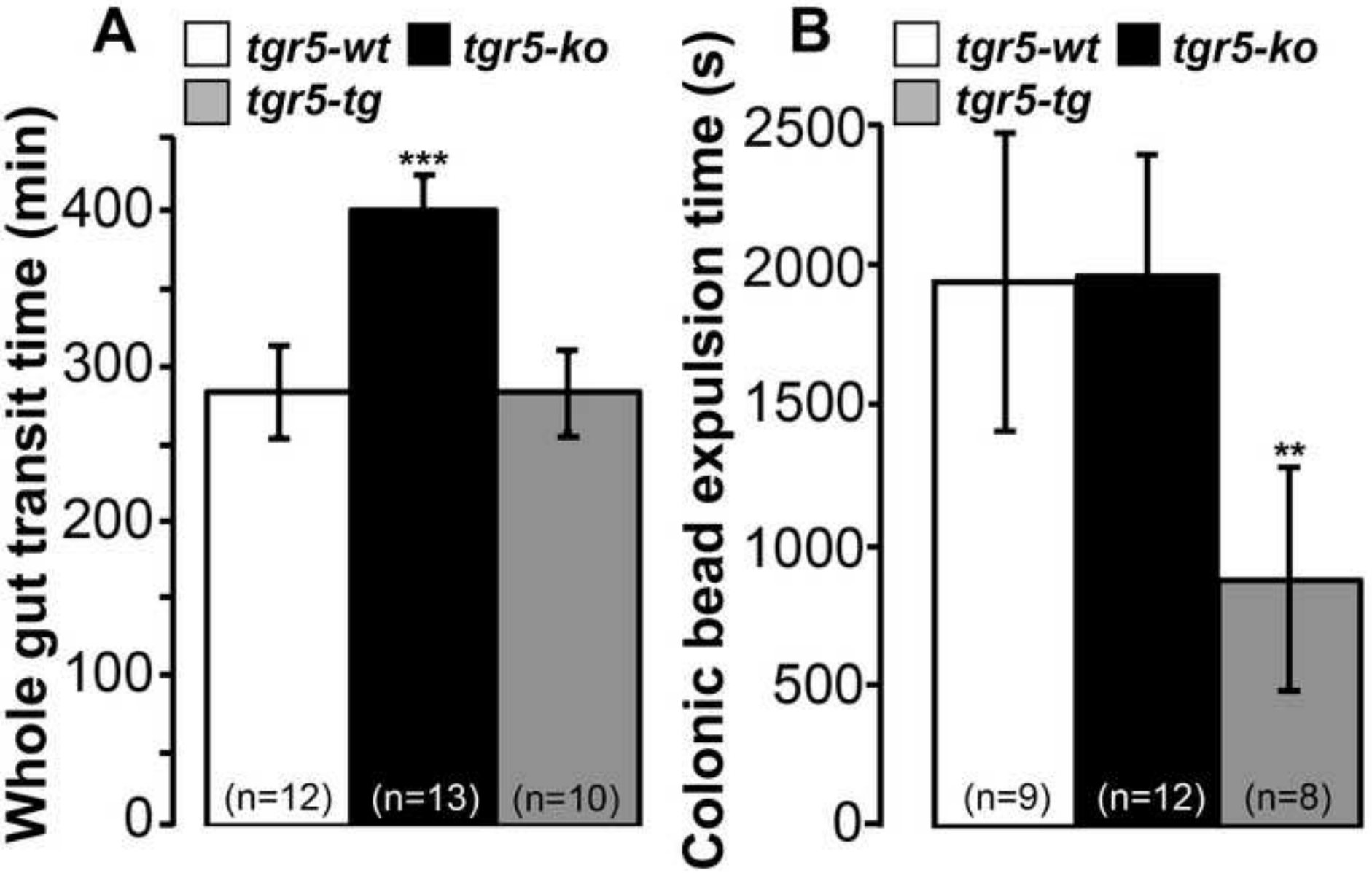

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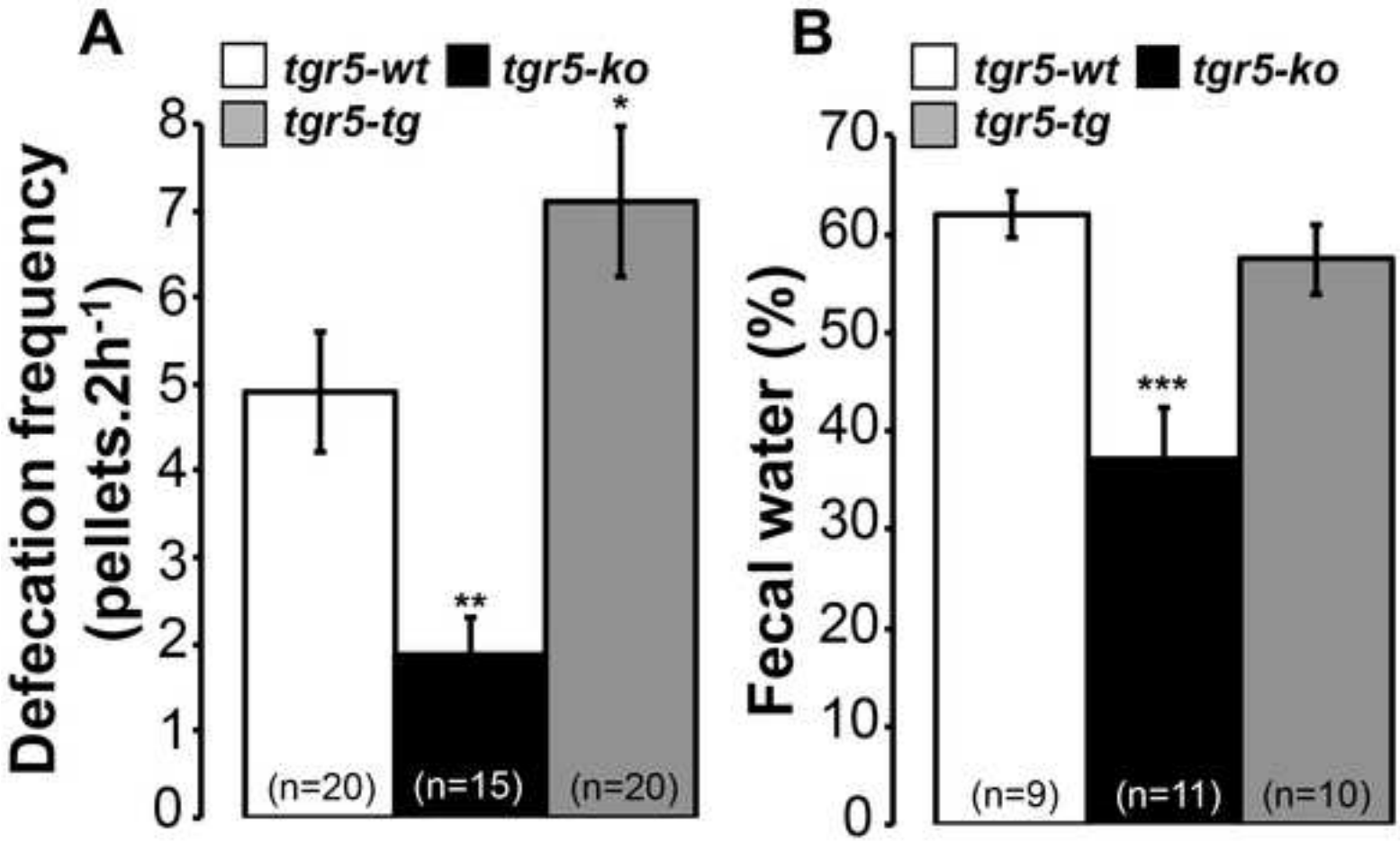

Author/s:
Alemi, F; Poole, DP; Chiu, J; Schoonjans, K; Cattaruzza, F; Grider, JR; Bunnett, NW; Corvera, CU

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