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A sexually dimorphic effect of cholera toxin: rapid changes in colonic motility mediated via a 5-HT_3 receptor dependent pathway in female C57Bl/6 mice

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Running title: Sex and estrus cycle dependent effect of CT on motility

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Key words: Cholera toxin, mouse, colon, motility, gender dimorphism, serotonin, enterochromaffin cells, estrus cycle

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Key points

- Cholera causes more than 100,000 deaths each year as a result of severe diarrhea, vomiting and dehydration due to the actions of cholera toxin; more females than males are affected.
- Cholera toxin induces hypersecretion via release of mucosal serotonin and over-activation of enteric neurons, but its effects on gastrointestinal motility are not well characterized.
- We found that cholera toxin rapidly and reversibly reduces colonic motility in female mice in estrus, but not in males or females in proestrus, an effect mediated by 5-HT in the colonic mucosa and by 5-HT_3 receptors.
- We show that the number of mucosal enterochromaffin cells containing 5-HT changes with the estrus cycle in mice.
- These findings indicate that cholera toxin's effects on motility are rapid and depend on the estrus cycle and therefore can help us better understand differences in responses in males and female patients.

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Abstract

Extensive studies of the mechanisms responsible for the hypersecretion produced by cholera toxin (CT) have shown that this toxin produces a massive over-activation of enteric neural secretomotor circuits. The effects of CT on gastrointestinal motility, however, have not been adequately characterised. We investigated effects of luminal CT on neurally mediated motor activity in *ex vivo* male and female mouse full length colon preparations. We used video recording and spatiotemporal maps of contractile activity to quantify colonic migrating motor complexes (CMMCs) and resting colonic diameter. We compared effects of CT in female colon from wild type and mice lacking tryptophan hydroxylase (TPH1KO). We also compared CMMCs in colons of female mice in estrus with those in proestrus. In female (but not male) colon, CT rapidly, reversibly, and concentration-dependently, inhibits CMMC frequency and induces a tonic constriction. These effects were blocked by granisetron (5-HT_3 antagonist) and were absent from TPH1KO females. CT effects were prominent at estrus but absent at proestrus. The number of EC cells containing immunohistochemically demonstrable serotonin (5-HT) was 30% greater in female mice during estrus than during proestrus or in males. We conclude that CT inhibits CMMCs via release of mucosal 5-HT, which activates an inhibitory pathway involving 5-HT_3 receptors. This effect is sex- and estrus cycle-dependent and is probably due to an estrus cycle-dependent change in the number of 5-HT containing EC cells in the colonic mucosa.

Abbreviations: WT, wild type; KO, knock out; GI, gastrointestinal; CT, Cholera Toxin; CMMC, colonic migrating motor complex; TPH1, tryptophan hydroxylase; ENS, enteric nervous system; 5-HT3, serotonin type 3 receptor; TTX, tetrodotoxin; EC, enterochromaffin

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Introduction

Sex based differences in gastrointestinal (GI) disorders are well known and widespread (Palomba *et al.*, 2011). GI complications are often reported to be correlated with fluctuations in sex steroid hormone levels such as pregnancy, menopause and different stages of the menstrual cycle. The prevalence of constipation increases and GI motility decreases during pregnancy (Datta *et al.*, 1974; Wald *et al.*, 1982; Lawson *et al.*, 1985; Truswell, 1985). Nutrient transit is also slower during the luteal phase of the ovarian cycle than during the follicular phase (Wald *et al.*, 1981; Heitkemper & Jarrett, 1992). In addition, more women than men (2:1) are affected by functional bowel disorders (Mathias & Clench, 1998). Indeed, even infectious diseases can show sex based differences. For example, a limited number of studies report a higher incidence of cholera in females (Fauveau *et al.*, 1991; Tornheim *et al.*, 2010)

Cholera causes more than 100,000 deaths each year as a result of severe diarrhea, vomiting and dehydration due to the actions of cholera toxin (CT) (WHO, 2013). CT is an exotoxin produced by the *Vibrio cholerae* bacterium and induces hypersecretion in the GI tract via activation of the enteric nervous system (ENS) (Cowles & Sarna, 1990a; Lundgren & Jodal, 1997; Bornstein *et al.*, 2012). Few studies, however, have investigated the effects of CT on GI motility. Historically, *in vivo* studies in animal models suggest that motility is increased following exposure to CT (Finkelstein *et al.*, 1964; Banwell & Sherr, 1973; Cowles & Sarna, 1990b). Juvenile rabbits infected with cholera show increased GI transit (Finkelstein *et al.*, 1964). Similarly, increased migrating action potential complexes following administration of CT in anaesthetised rabbits has also been reported (Banwell & Sherr, 1973). In contrast, exposure to CT reduced propagating contractions in the small intestine after a meal in conscious dogs (Cowles & Sarna, 1990b); nevertheless, no change was observed in GI transit times in patients with cholera (Banwell *et al.*, 1970) and there is little data relating to whether increases in motility induced by CT are secondary to secretion changes.

Kordasti *et al*. (2006) and Fung *et al*. (2010) assessed CT induced changes in small intestinal motility in animal models *in vivo* and *in vitro* respectively. Both reported that CT increases small intestinal motility with the *in vitro* effects being very rapid, within the first 15 minutes of exposure, but the time course of onset of changes *in vivo* was not assessed. Interestingly, this effect was further increased in each case by blocking 5-HT_3 receptors. Another potential site of impact of CT is the colon, which is a major site of fluid reabsorption (Bornstein *et al.*,

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2012), a process that depends on the rate of transit of fecal matter; however, whether CT has a direct effect on colonic motility *in vitro* has not been assessed.

We investigated the effects of CT on colonic migrating motor complexes in isolated colon of female and male mice to determine whether effects are sex-specific and whether mucosal 5-HT plays a role in mediating the effects of CT.

Materials and Methods

Ethical Approval and Animals

Mice were killed by cervical dislocation; this and other procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee (approval no: 1011897) according to guidelines of the National Health and Medical Research Council of Australia. The investigators understand the ethical principles under which the journal operates and confirm that this work complies with this animal ethics checklist. Mice were obtained from the Animal Research Centre Canning Vale, WA, Australia) and maintained on-site by the Biological Research facility in the Departments of Physiology and Pharmacology at The University of Melbourne. Mice were housed in individually ventilated cages. They were given water and fed sterilized Walter and Eliza Hall Institute mouse breeder cubes ad libitum along with sunflower seeds once weekly to supplement their diet. Adult mice female or male (C57Bl/6, > 22 g, 8-10 weeks old) were used.

Vaginal smears

Female mice were maintained under a light-dark cycle of 0600 h to 1800 h. Vaginal smears were performed between 0800 h and 0900 h. Determination of the stage in the estrus cycle was carried out as previously reported (Tran *et al.*, 2012). Briefly, a blunted sterile Pasteur pipette filled with approximately 50 µl of sterile distilled water was inserted into the vaginal canal and the contents were slowly flushed back into the pipette. The contents were then transferred to glass microscope slides (Livingstone international Pty Ltd, Australia), air dried and stained (Shandon™ Wright-Giemsa Stain Kit, Thermo Scientific™, Australia). The cytology of smear samples was examined using a light microscope to determine the stage of the estrus cycle for each animal. Proestrus smears contained large numbers of nucleated

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epithelial cells while estrus smears predominantly contained cornified, non-nucleated epithelial cells.

Plasma Estrogen Measurement

Following cervical dislocation, blood samples were obtained by cardiac puncture and centrifuged for 15 min at 10,000 g in order to separate plasma. Plasma samples were stored at -80°C and estrogen concentrations were measured using an ELISA assay kit (Estradiol EIA Kit, Cayman Chemical Company, USA) as per Tran et al (Tran *et al.*, 2012).

Tissue Preparation (motility)

Whole colon was removed from freshly killed mice and placed in an organ bath containing 15 ml of warmed (36°C) physiological saline solution (composition, mM: NaCl 118, KCl 4.6, NaH_2PO_4 1, NaHCO3 25, $MgSO_4$ 1.2, D-glucose 11, $CaCl_2$ 2.5; bubbled with 95% O_2: 5%CO_2). Physiological saline was continuously superfused through the organ bath at a flow rate of approximately 6 ml min^{-1}. Segments were connected to an adjustable pressure head via oral and anal cannulas, as previously described (Gwynne *et al.*, 2004; Roberts *et al.*, 2007). The oral end of the tissue was connected to a reservoir of physiological saline, the anal end to an outflow tube that provided a back pressure of 3-4 cm H_2O.

Video imaging of colonic motor patterns

Video imaging analysis of colonic motility was conducted as described in (Swaminathan *et al.*, 2015) (Roberts *et al.*, 2007). Briefly, colonic motility was recorded *in vitro* using a Logitech camera (QuickCam Pro 4000; I-Tech, Ultimo, NSW, Australia) mounted directly above the organ bath. In-house software (Scribble 2.0) and a purpose-built Matlab (2013b) plugin, Analyse 2.0, were used to convert recorded video segments (15 min duration) to spatiotemporal maps where the diameter of the colon is mapped (as a heat map) along the length of the segment as a function of time. The x axes of the spatiotemporal maps represent increasing time, with length of colonic segment along the y axes. The diameter along the colon is color-coded, such that blue-green pixels indicate relaxed tissue and yellow-red pixels identify constricted regions (Fig 1).

Two indices of neurally mediated colonic motor activity were analyzed: colonic migrating motor complexes (CMMCs) defined as spontaneous constrictions originating at the oral end

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of the colon that propagate more than half of the length of the tissue and the resting colonic diameter (diameter of the colon between CMMCs when the colon is quiescent) .

CMMC frequency, the speed of CMMC propagation and resting gut diameter were measured using Analyse2 software as previously reported (Gwynne *et al.*, 2004). Briefly, CMMC frequency was manually counted from spatiotemporal maps. Resting colonic diameter, an index of compliance when luminal pressure is constant, was estimated as the mean diameter between contractions measured at a point lying 66% of the colonic length from the oral end of the preparation (Fig 1 E1-2). This location was chosen to give a constant reference point for comparison between preparations. The speed of CMMC propagation was calculated by measuring the slopes of individual CMMCs on the spatiotemporal maps.

Experimental protocol for motility studies

After a 30 min equilibration period, a 1 h control period (four x 15 minute videos) was recorded. In specific experiments, CT was applied to the lumen by itself or together with tetrodotoxin (TTX) (bath) or granisetron (lumen or bath application). In other experiments, TTX (bath) or granisetron (lumen or bath) were added on their own. In each experiment, tissue was exposed to drugs/toxin for 1 h. Drugs/toxin were then washed out during the following hour using physiological saline. CT (0.125 µg/ml, 1.25 µg/ml and 12.5 µg/ml; Sigma Aldrich, St Louis MO, USA) was applied to the lumen of the colonic preparation via the oral cannula. The 5-HT_3 receptor antagonist, granisetron (1 µM, gift from SmithKline Beecham Pharmaceuticals, Philadelphia, PA, USA) was applied to the lumen or added to the reservoir from which the superfusate for the organ bath containing the tissue was drawn in different experiments. TTX (1µM, Alomone, Jerusalem, Israel) was added to the organ bath. Each tissue preparation served as its own control prior to drug/toxin application. The effect of luminal application of physiological saline alone was studied in 'time control' experiments.

Comparisons of the effects of drugs and toxins were made using data obtained from the last three 15 minute duration maps (45 minutes in total) prior to changes in solutions for each condition to avoid wash-in effects of changes to either the bathing or luminal solutions. To assess the timing of onset of CT effects during estrus and proestrus, CMMCs were compared over the final 15 min map for control and the first 15 minutes in the presence of CT.

Immunohistochemistry

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Tissue from the mid colon region (immediately anal to the mucosal striations of the proximal colon) from estrus, proestrus and male mice was dissected from freshly killed mice, cleared of luminal content, and fixed in 4% formaldehyde (from paraformaldehyde) for 80 mins at room temperature. Tissues were cleared of fixative (3 × 10 min PBS washes) before cryoprotecting in 30% sucrose in PBS solution overnight at 4 °C. Tissues were then mounted in cryo-moulds, with Tissue-Tek® OCT-compound (Optimal Cutting Temperature, ProSciTech, Kirwan, Queensland, Australia) medium and snap frozen in liquid nitrogen. Tissue was then cut in 18 µm thickness sections using a Cyostat (Microm HM 525, Fronine Laboratory Supplies, Riverstone, NSW, Australia). Sections were placed on Super Frost PLus™ slides (Menzel-Glaser®, Gerhard Menzel GmbH – Saarbrückener, Braunschweig, Germany) and air dried for 10-15 min before permeabilizing with 0.1% Triton X-100 (ProSciTech, Thuringowa QLD, Australia) together with 10% Casblock (Zymed, Invitrogen, Carlsbad, CA, USA) for 30 min. Sections were then incubated with primary antisera against the pan neuronal marker Hu (ANNA-1, 1:10,000, a gift from Dr Lennon, USA (Hotta *et al.*, 2013; Fung *et al.*, 2014)) and 5-HT (goat anti-5-HT, 1:400, #20079 Immunostar, Hudson, WI 54016, USA) overnight at 4°C. After being washed (3 × 10 min; PBS), preparations were incubated in secondary antisera, donkey anti-Human Alexa Fluor 594 (1: 750; Jackson ImmunoResearch Inc, West Grove, PA, USA) and donkey anti-sheep Alexa Fluor 488 (1:400; Molecular Probe # A11615) for 150 min at room temperature. The preparations were washed again (3 × 10 min; PBS) before mounting in Dakocytomation fluorescent mounting medium (Carpentaria). Images were captured using a confocal microscope (Zeiss LSM510 fluorescence microscope, Gladesville, NSW, Australia) and Zeiss LSM software, (version 4.2.0.121 Australia). Digital images for 5-HT labelling were quantitatively analyzed using Image J software (NIH Bethesda, USA).

Statistical Analysis

Data were analyzed by two-way ANOVA or *t* test as appropriate. *N* is the number of animals from which measures were taken, and statistical significance was set at $P < 0.05$. Data are presented as mean ± standard error of the mean (S.E.M.)

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Results

Cholera toxin (CT) depresses colonic motility in female mice during estrus

CT, added to the lumen, reduced CMMC frequency in a dose dependent manner in randomly selected females (Fig. 1A, B, D). Within 15 min of CT application, CMMC frequency was reduced compared to the control period at all concentrations of the toxin. CT at 0.125 µg/ml caused a 40% reduction in CMMCs. CT at 1.25 µg/ml rapidly reduced CMMC frequency by 65% compared to the control period. Administration of 12.5 µg/ml CT further depressed CMMC frequency by 84% (descriptive statistics are provided in Table 1). Despite the effect of CT on CMMC frequency, the speed of propagation of the CMCCs was unaffected by luminal CT (Fig. 1C, Table 2; $P > 0.05$, 2 way ANOVA). No change in CMMC frequency was observed under baseline conditions (i.e. in time control experiments; Fig. 1B). Application of 1.25 µg/ml CT resulted in robust effects in female colon (Fig. 1D) and this concentration was therefore used in subsequent studies.

CT constricted the colon in a concentration-dependent manner in female mice (Fig. 1E, Table 1). In control solutions, the mean colonic diameter between CMMC contractions ranged from 4.3 ± 0.02 mm to 4.8 ± 0.15 mm in different experimental series (all estimates from 5 - 12 preparations). For ease of comparison, all diameter measurements in individual maps were normalised by dividing the measurement for that map by the value measured for the first control map taken from that preparation. Colonic diameter was reduced within 15 min of exposure to CT at all concentrations used, with 12.5 µg/ml producing the largest constriction. Application of 1.25 µg/ml CT caused a 30% reduction in colonic diameter (Table 1). Both the reduction in CMMC frequency and the tonic constriction were reversed during washout of CT (Fig. 1).

To assess the neural contribution to the effects of CT, tetrodotoxin (1 µM; TTX) was added to the bath. This abolished CMMCs in randomly selected female mice (Fig. 2A). CMMCs were similarly absent in the presence of TTX and CT (Table 1). TTX caused a colonic constriction

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(Fida *et al.*, 1997) indistinguishable from the constriction induced by CT (1.25µg/ml, Fig. 2B, Table 1). Addition of TTX together with CT produced no further constriction of the colon (Fig. 2, Table 1) suggesting that the constriction produced by CT depends on neural activity.

Cholera toxin does not alter CMMC frequency or colonic diameter in male mouse colon

There were no significance differences in either CMMC frequency or colonic diameter between male (4.8 ± 0.04 mm, n = 7) and female (4.4 ± 0.1 mm, n = 12) colon under control conditions. Luminal CT (1.25µg/ml) did not alter CMMC frequency in male C57Bl/6 colon preparations nor did it change resting diameter (Fig. 3, Table 1). At a higher concentration (12.5 µg/ml) CT caused a small reduction in CMMC frequency (37%, Table 1), but did not affect colonic diameter.

Blockade of 5-HT$_3$ receptors abolishes the effect of luminal CT in female mouse colon

The hypersecretion evoked by CT is depressed or abolished by blockade of 5-HT$_3$ receptors with antagonists like granisetron, while this antagonist enhances the increased motility in the small intestine produced by luminal CT (Kordasti *et al.*, 2006; Fung *et al.*, 2010). Accordingly we investigated the role of 5-HT$_3$ receptors in the actions of CT on CMMCs and colonic diameter by adding granisetron (1 µM) either to the bathing solution or together with CT to the lumen.

Granisetron, whether luminal or bath applied, blocked the CT-induced reductions in CMMCs and resting colonic diameter in female colon (Fig. 4, Table 1). This suggests that 5-HT plays a key role in the actions of CT that suppress CMMCs and also that constrict the colon. In the absence of CT, luminal granisetron produced a small reduction in CMMC frequency (Fig. 4, Table 1), but bath application of granisetron alone did not alter CMMC frequency from control values (Fig. 4, Table 1). Neither route of administration altered colonic diameter in the absence of luminal CT.

CT inhibits colonic motility in female mice via release of mucosal 5-HT.

There are two possible sources of the 5-HT involved in CT-induced motility effects; mucosal EC cells and a subset of myenteric neurons each synthesise this monoamine. To test the idea that EC cells provide the 5-HT, we measured CMMC frequency and colonic diameter in

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female transgenic *tph1* knockout (TPH1KO) mice. Tryptophan hydroxylase 1 (TPH1) is the rate-limiting enzyme regulating the synthesis of 5-HT in EC cells. Because TPH2 is rate-limiting in central and enteric serotonergic neurons (Walther *et al.*, 2003), TPH1KO mice selectively lack the ability to produce mucosal 5-HT (Li *et al.*, 2011; Heredia *et al.*, 2013). The frequency of CMMCs under control conditions in female TPH1KO mice was lower than that in their WT female littermates (Fig 5C, Table 1), a result similar to data on male TPH1KO mice reported by Heredia et al (Heredia *et al.*, 2013). Importantly, in female mice, CT (1.25 mg/ml) significantly reduced the number of CMMCs in WT animals (Table 1; Fig. 5A,C), but had no effect on randomly selected TPH1KO colon (Table 1; Fig. 5B,C).

CT strongly reduced the diameter of the colons of WT female mice (Table 1; Fig. 5A, D), but had no significant effect on the diameter of the colons of TPH1KO female mice (Table 1; Fig. 5B,D). These observations support the idea that CT induces 5-HT secretion from EC cells to constrict the colon and inhibit CMMCs.

The effects of luminal CT depend on the estrus cycle in female mice

In female mice, estrogen levels fluctuate over the 4-day estrus cycle from a high plasma estrogen stage (proestrus) to a low estrogen stage (estrus). To examine whether the motility effects of CT in female mice are dependent upon the estrus cycle, proestrus and estrus mice were selected by vaginal cytology screening and the stage of the estrus cycle was further confirmed by measuring plasma estrogen levels in a subset (n = 12) of the mice. As expected, all plasma samples assayed from mice with the cytological characteristics of the estrus stage had lower estrogen levels than samples from mice in proestrus (mean plasma estrogen concentration during estrus was 28.5 ± 2.4 pg/ml, n = 6; during proestrus it was 61.6 ± 1.8 pg/ml; n = 6, p = 0.001).

Changes in motility in estrus and proestrus females in the presence of CT (1.25 µg/ml) were investigated. CT reduced CMMC frequency in estrus females by 60% over that of the control period. In contrast, CT had no effect on CMMC frequency in proestrus females (Fig. 6A,B; Table 1). In addition, CT (1.25 µg/ml) reduced the resting colonic diameter in estrus females, but not the diameter of the proestrus colon. (Fig. 6D, Table 1).

Mucosal 5-HT depends on the estrus cycle

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Our data show that the ability of CT to inhibit CMMC frequency and constrict the colon female mice is restricted to the estrus period. This result might be due to estrus-related changes within the enteric neural circuitry that alter the efficacy of 5-HT-activated neural pathways or, more simply, due to a change in the mucosal level of 5-HT. We tested this second possibility by comparing the numbers of 5-HT-immunoreactive cells in the mucosal layer of transverse sections of colons from female mice in estrus or proestrus and from male mice. Two clearly distinct classes of immunoreactive cells were identified. One class was confined to the epithelial cell layer of the mucosa and had the typical morphologies of enterochromaffin (EC) cells including, in many cases, long "axon"-like processes (Fig. 7A)(Cremon *et al.*, 2011) that were similar to the "neuropods" of enteroendocrine cells (Bohórquez *et al.*, 2015). The other class was found in the lamina propria and were probably mast cells (Mawe & Hoffman, 2013).

During estrus there were significantly greater numbers of each class of 5-HT-immunoreactive cells than during proestrus, or in males (Fig. 7). The number of EC cells per mm of epithelium in estrus female colon was 35 ± 3, but was 27 ± 1 at proestrus and 26 ± 2 in male colon ($P < 0.05$ in each case).

Discussion

The data presented here indicate that there are substantial differences between female and male mice in the effects of luminal CT on neurally regulated contractile activity of the isolated colon. These differences depend on mucosal 5-HT, presumably acting via 5-HT_3 receptors, and on the estrus cycle, disappearing at proestrus. Mucosal 5-HT-containing EC cells also vary with the estrus cycle with substantially more being present in the colonic mucosa of females at estrus than in the mucosa of females at proestrus or in the colonic mucosa of males. The sexually dimorphic actions of CT, therefore, may result from greater release of mucosal 5-HT in females (except during proestrus) than in males.

Luminal CT rapidly and reversibly reduces spontaneous neurogenic contractile activity in female mouse colon. Infusion of CT into the lumen of the colon from randomly selected female mice or from female mice at estrus reduced CMMC frequency within the first 15 minutes of exposure. In most cases, a substantial tonic constriction was seen within 200 s of exposure indicating that the effects of CT on colonic motility were much more rapid in these animals than hypersecretory effects that have been reported in many previous studies.

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Hypersecretion is seen after 90 - 120 minutes of incubation with CT and persists for several hours after the toxin is flushed from the lumen (Field *et al.*, 1972; Argenzio & Whipp, 1981; Turvill *et al.*, 1999; Banks *et al.*, 2005; Kordasti *et al.*, 2006). Both effects of CT on contractility were reversible within an hour when the toxin was flushed from the colonic lumen. As both the prolonged hypersecretion and the relatively rapid motility effects described here are mediated by mucosal 5-HT (see below) acting on enteric neural circuits, the reasons for the different time courses of the two effects are unclear and need further investigation.

A further difference from earlier *in vivo* studies is that both inhibition of CMMC generation and the tonic constriction were seen with as little as 0.125 µg/ml, while studies of CT induced secretion have used much larger concentrations (e.g. 40 µg/ml (Kordasti *et al.*, 2006); 12.5 µg/ml (Turvill *et al.*, 1999); 25 µg/ml (Banks *et al.*, 2005)). Interestingly, a previous study reporting rapid effects of CT on motility also found effects at 1.25 µg/ml toxin (Fung *et al.*, 2010). A small effect on CMMC frequency was observed in male colon at 12.5 µg/ml, which is comparable to the concentrations used in previous studies of secretion. As such studies are often confined to males, our results suggest that an analysis of CT induced hypersecretion in females may be rewarding.

Blocking neural activity with TTX, which blocks most voltage-dependent sodium channels, abolished the CMMCs as expected and produced a tonic constriction of the colon that was indistinguishable from that produced by CT. When TTX and CT were administered together the constriction produced was not increased over levels due to either toxin alone. This was despite our observation of infrequent CMMCs in CT constricted colon indicating that the tonic constriction is not maximal. These data indicate that the tonic constriction produced by luminal CT depends on neural activity.

Our data show that effects of CT on colonic contractile activity are sexually dimorphic and depend on the estrus cycle in female mice. Although low concentrations of CT produced both reduced CMMC activity and a tonic constriction of colon from randomly selected females, no effect of the toxin was seen in males until we used 2 orders of magnitude more toxin (12.5 µg/ml). At this high concentration, there was a 35% reduction in CMMC frequency (note, the reduction in females at 12.5 µg/ml was 86%, while at 0.125 µg/ml it was 40%) and no tonic constriction was detected. To test whether this difference between the sexes might be due to circulating sex steroids, we assessed the effects of CT (1.25 µg/ml) on colon taken

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from female mice in estrus and compared these with the effects on colon taken from mice at proestrus. We confirmed these phases both via vaginal smears and measurement of plasma estradiol levels. There was a striking difference between the two phases with estrus colon showing reduced CMMC frequency and tonic constriction with CT in the lumen, a treatment that was completely ineffective in proestrus colon. Indeed, the behaviour of proestrus colon was indistinguishable from male colon.

Three lines of evidence support the conclusion that the effects of CT on contractile activity in female colon depend on mucosal 5-HT and 5-HT_3 receptors. The strongest evidence comes from analysis of female TPH1KO mice; TPH1 is the rate-limiting enzyme for biosynthesis of 5-HT in EC cells (Walther *et al.*, 2003; Li *et al.*, 2011; Margolis *et al.*, 2014). In these mice, CMMC frequency under control conditions was significantly lower than in the colons of their wild type (WT) littermates, as has also been reported for male TPH1KO mice (Heredia *et al.*, 2013). Importantly, CT in the lumen of colon from TPH1KO females and selected without consideration of the estrus cycle had no effect on either CMMC frequency or resting colonic diameter. This stands in marked contrast to the effects of CT in their WT female littermates. These data strongly suggest that EC cell 5-HT regulates CMMC frequency. When mucosally released 5-HT is not excessive, it enhances CMMC frequency. Very high levels of released 5-HT, such as those seen in female mice during estrus, inhibit CMMC initiation, but not their propagation once initiated (see Table 2, Fig 1,5).

The second line of evidence is that blockade of 5-HT_3 receptors abolished the effects of CT in females selected without regard to the estrus cycle. This result obtained no matter whether the antagonist, granisetron, was added to the organ bath or delivered simultaneously with CT in the lumen. Luminal granisetron produced a small, but significant, reduction in CMMC frequency in the absence of CT (suggesting that 5-HT_3 receptors participate in EC cell-driven CMMC initiation), but abolished the effects of CT. Taken together, these data suggest that there are two distinct 5-HT_3 receptor-mediated pathways that are activated by mucosal 5-HT: a pathway that enhances CMMC generation with low (perhaps physiological) levels of tonic 5-HT release and an inhibitory pathway activated when 5-HT release is excessive, as is in estrus females provoked by CT. Interestingly, 5-HT_3 receptors also appear to mediate the tonic constriction that is produced by CT induced mucosal 5-HT release, which could either be due to inhibition of tonic firing of inhibitory motor neurons or increased tonic firing of excitatory motor neurons. In either case, this effect appears to be distinct from the effects of

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CT on CMMC generation, because CMMCs can be seen superimposed on the tonic constriction (Fig 1).

The third line of evidence is our observation that the number of 5-HT containing cells in the mucosa of female mouse colon is lower at proestrus, when CT has no effect on contractile activity, than at estrus. Together with the TPH1 knockout data and the effects of granisetron, both in randomly selected females, this suggests that the probability that CT will inhibit CMMCs generation depends on the amount of 5-HT available to be released by the toxin. Our observation of a small effect of CT at 12.5 μg/ml on CMMCs in males is consistent with this conclusion and suggests that a higher concentration of toxin may compensate for the lower number of EC cells in male mice. A corollary of this is that EC cell numbers would be expected to be higher in females than in males, except at proestrus, because random selection would sample equal numbers of estrus, metestrus, diestrus and proestrus females. This, in turn, implies that the lower number of EC cells at proestrus is due to an active process, perhaps reflecting high estrogen levels, as activation of estrogen receptor β enhances apoptosis of mucosal epithelial cells and reduces their proliferation (Wada-Hiraiki *et al*., 2006). These changes parallel other changes in the mucosal epithelium during the estrus cycle including alterations in potassium channel expression (Alzamora *et al*,. 2011) and colonic permeability via altered expression of occludin and junctional adhesion molecule-A (Braniste *et al*., 2009).

Our results show that mucosal 5-HT plays a role in the initiation of CMMCs in female mice, because CMMC frequency is lower in female TPH1KO females and in female WT colon when granisetron is in the lumen. The data also indicate, however, that CMMCs can be initiated in the absence of mucosal 5-HT, because CMMCs are still seen in the colons of female TPH1-knockout mice. Heredia *et al.* (2013) observed CMMCs in male TPH1KO mice; however, the properties of these CMMCs differed from those in the colons of WT males. This present study did not explore the mechanisms that initiate CMMCs or colonic propulsion, which have been the subject of debate in the Journal of Physiology CrossTalk series (Smith & Gershon, 2015; Spencer *et al.*, 2015). Nevertheless, the most likely explanation for our observations is that low levels of 5-HT released from the mucosa act via 5-HT_3 receptors to facilitate generation of CMMCs, perhaps resulting from distension produced at the oral cannula or pressure on EC cells.

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The effects of CT that we have identified suggest that the role of mucosal 5-HT in females is much more complex than simply enhancing or initiating CMMCs and related propulsive motility patterns. CT produces a massive release of 5-HT from the mucosa (Farthing, 2002; Lundgren, 2002) and our findings indicate that this 5-HT acts via 5-HT_3 receptors to suppress or obscure CMMCs, rather than to enhance them. A similar mechanism may also operate in the guinea-pig jejunum, where CT produces a rapid increase in propulsive activity that is enhanced by blockade of 5-HT_3 receptors (Fung *et al.*, 2010), and rat jejunum where increased contractile activity resulting from CT treatment is enhanced by granisetron, *in vivo* (Kordasti *et al.*, 2006). This suggests that physiological levels of 5-HT release enhance motility via one neural pathway, while pathophysiological levels of 5-HT suppress motility possibly by over-activating at least one other pathway, in effect a neural spasm, that interferes with the coordinated neural activity required to produce a CMMC. Notably, high levels of CT also depress CMMC generation in male mouse colon (see above) and the motility effects of CT in rat jejunum (Kordasti *et al.*, 2006) were recorded in males.

CT in female colon also produced a tonic constriction via 5-HT_3 receptor activation. This may have been due to inhibition of tonic firing in inhibitory motor neurons, consistent with the similar magnitude effect seen in the presence of TTX, or increased firing of excitatory motor neurons. As this constriction was not seen in TPH1-knockout mice, it was probably due to the release of mucosal 5-HT rather than activity of 5-HT neurons in the myenteric plexus. However, our data cannot rule out any role for neuronal 5-HT in the pathways responsible for either the CMMCs or the constriction. It has been proposed that neural 5-HT_3 receptors are constituently active, so blocking such receptors would modify neural activity in the absence of 5-HT (Sia *et al.*, 2013); however, bath applied granisetron did not modify either CMMC generation or colonic diameter in the absence of luminal CT. This indicates that it is unlikely that 5-HT_3 receptors are constitutively active in this preparation and that such a mechanism cannot explain the effects of this antagonist on CT induced changes in contractile activity in female colon.

In summary, CT in the lumen suppresses CMMC generation and produces a tonic constriction in a concentration and estrus cycle dependent fashion in *ex vivo* colon from female mice. This effect is rapid and reversible, in contrast to the hypersecretion induced by this toxin, which is much slower suggesting that the altered motility is not secondary to the hypersecretion. It depends on the presence of mucosal 5-HT and on activation of 5-HT_3

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receptors, presumably on the mucosal terminals of intrinsic sensory neurons (Bertrand *et al.*, 2000). The dependence of the effect of CT on the estrus cycle probably results from cycle dependent changes in the number of 5-HT-immunoreactive EC cells in the mucosa, but the mechanism responsible for this requires further investigation.

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Additional Information

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Author contributions: G.B. performed the experiments, J.C.B. conceived and designed the experiments, E.L.H. J.C.B. and G.B. wrote the manuscript. M.D.G. provided the TPH1KO mice, conceptual input, and assisted in writing the manuscript. All authors approved the final version of the manuscript. Some experiments were carried out by G.B. in the laboratory of M.D.G. at Columbia University.

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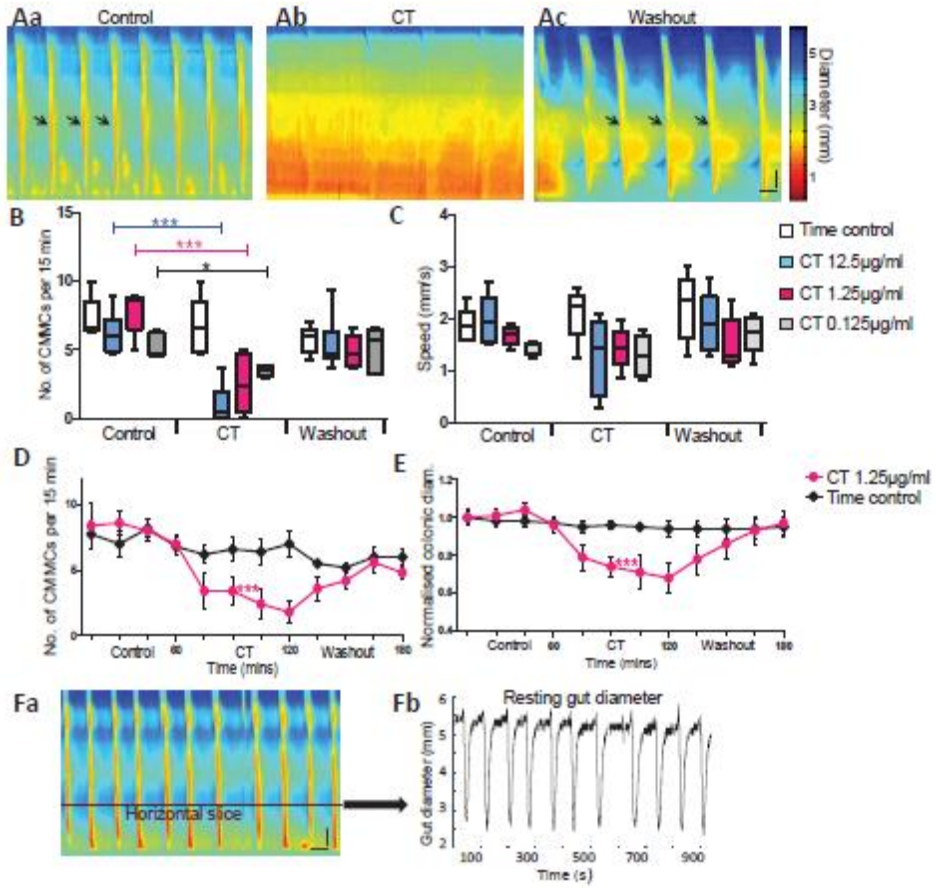
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Figure Legends

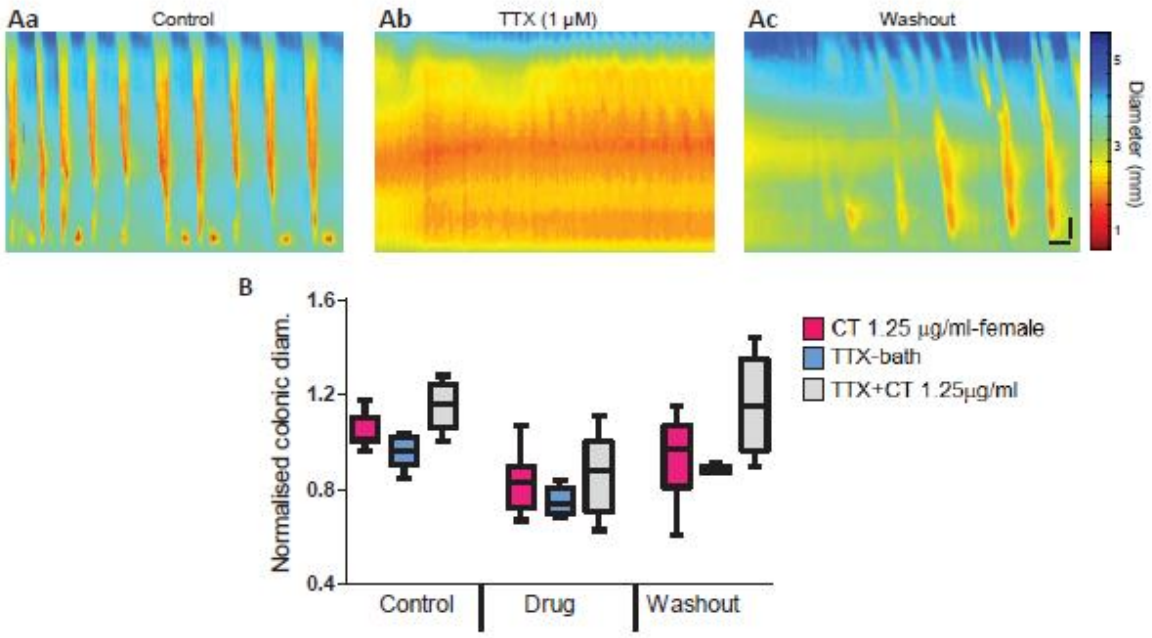
Fig. 1. Representative spatiotemporal maps showing CMMCs (arrows) **in randomly selected females**

Control (**Aa**), CT (1.25 µg/ml) (**Ab**) and washout (**Ac**). **B:** Number of CMMCs per 15 mins recording (mean of four recordings over a one hour time period) vs. control, CT exposure and washout periods (horizontal scale bar 5mm, vertical scale bar 60s). CT inhibits CMMCs in a concentration dependent manner (CT concentrations: 0.125 µg/ml, 1.25 µg/ml and 12.5 µg/ml). CT exerts a rapid effect on **(D)** CMMC frequency and (**E**) resting diameter in the colon of randomly selected female C57Bl/6 mice. Time control data show no significant difference in CMMC frequency or colonic diameter (**D). Fa-Fb:** Resting colonic diameter was taken from a plot of the diameter (F2) of the colon at a consistent point 66% along the length of the colon in each map (F1). ***P* < 0.05, ** *P* < 0.01, ***P* < 0.001,** in **D** and **E** *P* value is for comparison of CT vs time control over the entire curve.



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Fig. 2. TTX abolishes CMMCs and constricts the colon of randomly selected females. **Aa-c:** Spatiotemporal maps showing effects of TTX (1 µM) on CMMCs (Ab) and the beginnings of reversal of the TTX induced abolition (Ac). Aa shows control CMMCs in this preparation. TTX abolished CMMCs **(**horizontal scale bar 60s, vertical scale bar 5mm**). B:** Box plots showing normalised colonic diameter for preparations treated with CT (1.25 µg/ml) (red), 1 µM TTX (blue) and TTX plus 1.25 µg/ml CT (grey). CT, TTX and TTX plus CT all constricted the colon during the exposure period (drug), but their effects were indistinguishable.



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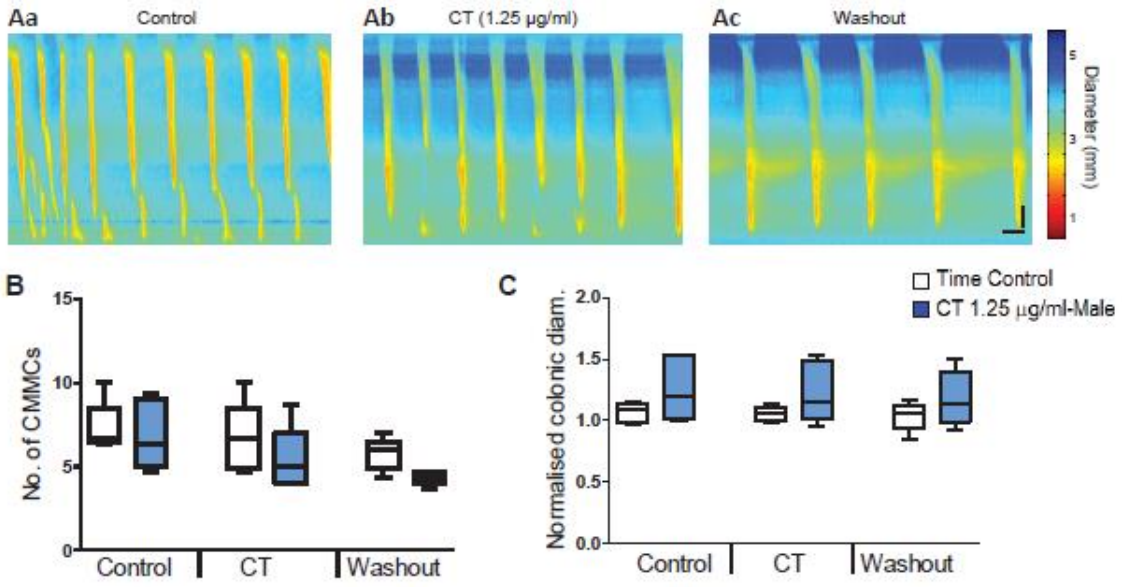


Fig. 3. CT (1.25 µg/ml) does not alter CMMC frequency or resting colonic diameter in male mice.

Aa-c: Representative spatiotemporal maps showing CMMC frequency during control, luminal CT (1.25 µg/ml) exposure and washout conditions (horizontal scale bar 60s, vertical scale bar 5mm). CT application did not alter CMMC frequency (**B**) or colonic resting gut width (**C**) in male colon. Time controls (motility in the absence of CT) showed no change in CMMC frequency or resting gut width over the 3h recording period (**B** and **C**).

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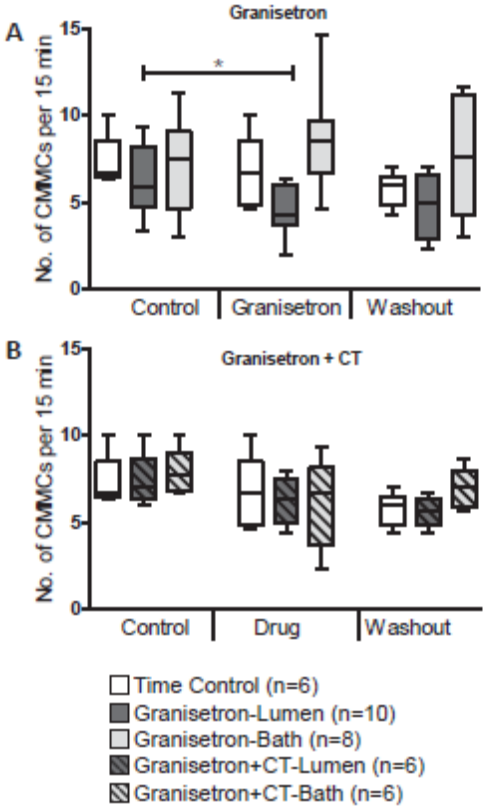


Fig. 4. Granisetron (5-HT_3 antagonist) blocks the CT-induced reduction in CMMCs in C57BL/6 female mouse colon.

A: Bath application of granisetron (GR) did not affect the number of CMMCs (box plots) compared to control, while luminal application of GR reduced the number of CMMCs compared to control period ($p = 0.028$). **B:** Both luminal and bath application of GR prevented the reduction in the number of CMMCs (box plots) induced by CT on C57BL/6 female mouse colon (i.e. no change from time control $p > 0.05$ – control/CT+GR) (horizontal scale bar 60 s, vertical scale bar 5 mm).

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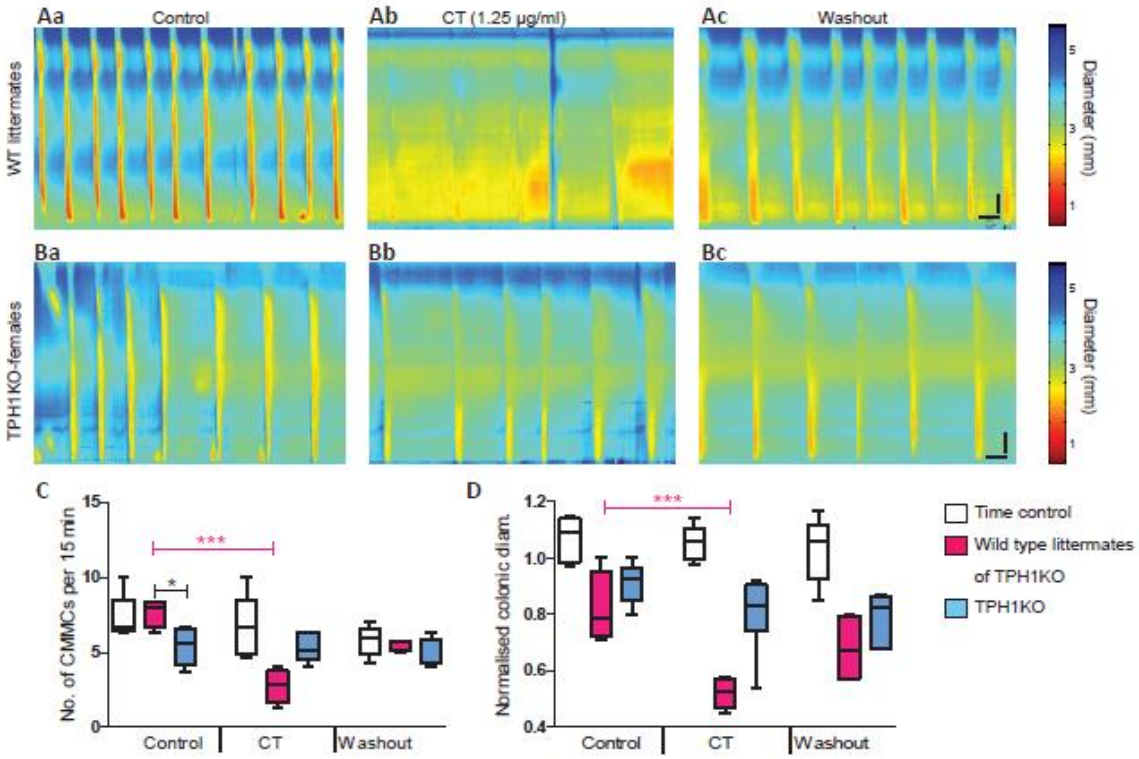


Fig. 5. CT inhibits colonic motility in female mice via mucosal 5-HT.

Aa-c and Ba-c: Spatiotemporal maps showing normal CMMCs during the control period in both WT littermate and TPH1KO females, respectively; CT (1.25 µg/ml) reduced the CMMC frequency and the resting gut width in wild type littermates while having no effect on TPH1KO female mice (horizontal scale 60s, vertical scale bar 5mm). **C:** Box plots comparing numbers of CMMCs in time control (white), WT (pink) and TPH1KO (Pale blue) in control, with CT in lumen and after CT washout (*$P < 0.05$, ***$P < 0.001$). D: Box plots comparing normalise colonic diameter for same groups of preparations.

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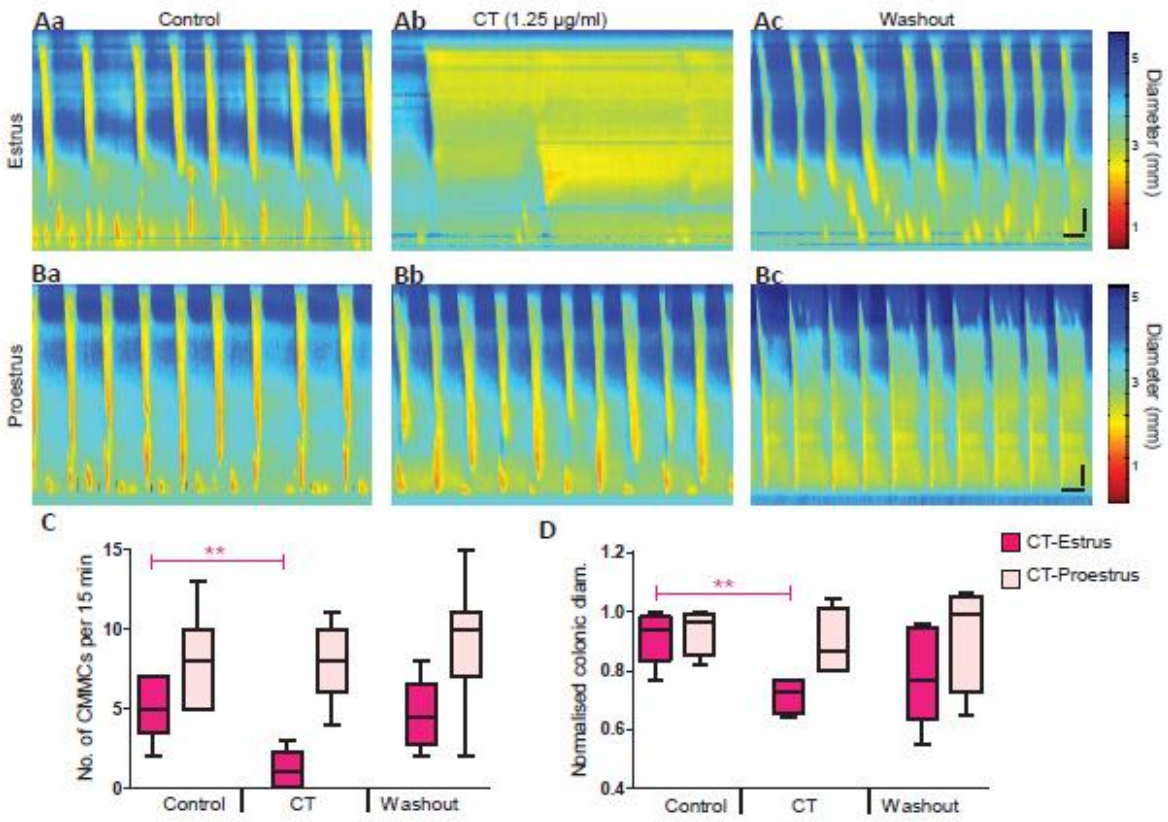


Fig. 6. Effect of CT (1.25 µg/ml) in proestrus and estrus females.

Spatiotemporal maps showing motility in estrus (**Aa-c**) and proestrus (**Ba-c**) females during exposure and washout of 1.25 µg/ml of CT (horizontal scale bar 60s, vertical scale bar 5mm). **C:** Box plots of numbers of CMMCs in 15 minute maps showing that CT significantly reduced the number of CMMCs in estrus female mice ($P < 0.001$), but had no significant effect on CMMC frequency in proestrus females ($P > 0.05$). **D:** Box plots showing that luminal application of CT significantly reduced resting colonic diameter in estrus females ($P < 0.01$), but not in proestrus females ($P > 0.05$).

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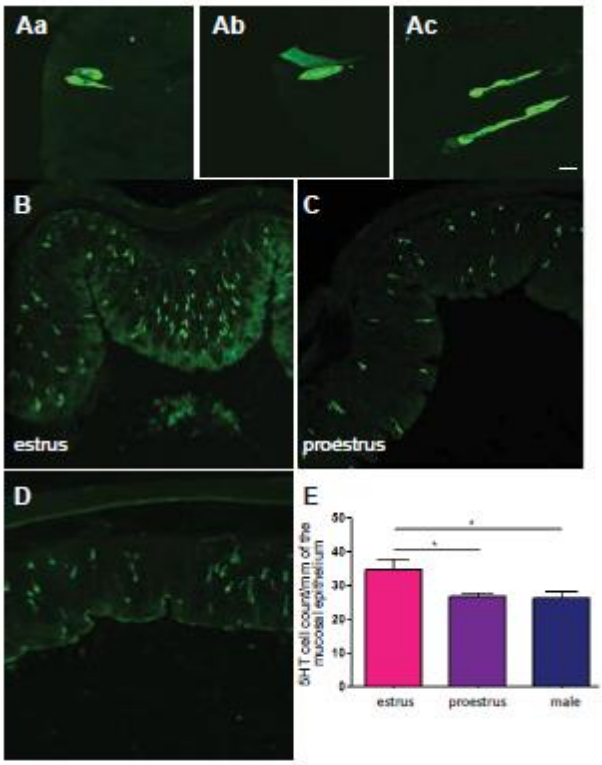


Fig. 7. Expression of 5-HT in EC cells in the mucosal epithelium differs between estrus, proestrus and male mid colon.

A-D: Cross sections of the mid colon showing 5-HT immunoreactivity in EC cells and cells in the lamina propria of the mucosa from an estrus female (**B**), a proestrus female (**C**) and a male (**D**) mouse. **Aa-c** show the different morphologies of EC cells including the axon-like neuropods. **E**: Mean EC cell count per mm of mucosal epithelium showing estrus female colon had significantly more 5-HT-immunoreactive EC cells (35 ± 3) than proestrus females (27 ± 1) or males (26 ± 2). Data are mean ± SEM, $P = 0.0138$ and n = 5 for each. Scale bars for **Aa-c** and **B-D:** 20 µm and 60 µm, respectively.

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Table 1. Descriptive statistics describing the number of CMMCs and normalised resting colonic diameter. Data are mean ± SEM

Experiment	CMMCs Number/15 min		*P*	Normalized colonic diameter		*P*	n
	Control	Treatment		Control	Treatment		
Time control	7 ± 1	7 ± 1	0.336	1.07 ± 0.04	1.05 ± 0.03	0.818	6
CT (0.125µg/ml)	5 ± 0.4	3 ± 0.8	0.04	0.97 ± 0.02	0.73 ± 0.01	<0.0001	5
CT (1.25µg/ml)	8 ± 1	3 ± 1	<0.0001	0.98 ± 0.01	0.75 ± 0.02	<0.0001	12
CT (12.5µg/ml)	7 ± 0.6	1 ± 0.5	<0.0001	0.96 ± 0.03	0.63 ± 0.02	<0.0001	6
TTX	7 ± 0.2	0	<0.0001	0.96 ± 0.03	0.75 ± 0.02	0.001*	5
TTX + CT	7 ± 2.4	0	<0.0001	1.1 ± 0.04	0.8 ± 0.07	0.016*	5
Male + CT (1.25µg/ml)	7 ± 0.7	6 ± 0.7	0.31	1.27 ± 0.09	1.24 ± 0.09	0.85	7
Male + CT (12.5µg/ml)	8 ± 0.2	5 ± 03	0.000	1.01 ± 0.01	0.98 ± 0.02	0.206	6
GR in bath	7 ±0.9	8 ± 1.1	0.38	1.00 ± 0.01	0.99 ± 0.01	0.133	8
GR in lumen	6 ± 0.7	5 ± 0.4	0.014	0.98 ± 0.01	0.96 ± 0.01	0.117	10
CT+GR bath	8 ± 0.6	6 ± 1.1	0.21	1.00 ± 0.01	0.99 ± 0.01	0.062	6
CT+GR lumen	7 ± 0.7	6 ± 0.6	0.24	1.00 ± 0.01	0.96 ± 0.01	0.058	6
WT + CT	8 ±0.5	3 ± 0.5	0.001	0.82 ± 0.06	0.52 ± 0.03	0.011	5
TPH1KO +CT	5 ± 0.5	5 ± 0.4	0.87	0.91 ± 0.03	0.80 ± 0.05	0.127	6
Estrus + CT	5 ± 0.8	1 ± 0.5	0.003	0.91 ± 0.04	0.7 ± 0.02	0.004	6
Proestrus + CT	8 ± 1.2	7 ± 1.0	1.00	0.93 ± 0.03	0.89 ± 0.04	0.534	6

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Table 2. Propagation speed of CMMCs in colons from randomly selected (i.e. chosen without regard for stage of estrus cycle) female mice Data are mean ± SEM.

CT concentration (µg/ml)	Propagation Speed (mm/s): randomly selected females		
	0-60 (control)	60-120 (CT/saline)	120-180 (Washout)
12.5	2.0 ± 0.2	1.3 ± 0.3	1.9 ± 0.3
1.25	1.7 ± 0.1	1.4 ± 0.2	1.5 ± 0.2
0.125	1.5 ± 0.01	1.3 ±0.2	1.7 ± 0.2
control	1.9 ± 0.2	2.1 ± 0.2	2.2 ± 0.3

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