Deoxycholic acid activates colonic afferent nerves via 5-HT_3 receptor dependent and independent mechanisms

Running title: Deoxycholic acid activates colonic afferents

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Abbreviations

5-HT (5-hydroxytryptamine), DCA (deoxycholic acid), DRG (dorsal root ganglion), EC (enterochromaffin), FXR (farnesoid X receptor), GLP-1 (glucagon-like peptide-1), GPBAR1/TGR5 (G protein-coupled bile acid receptor), HT (high threshold), IBS (irritable bowel syndrome), LT (low threshold), NG (nodose ganglion), RMP (resting membrane potential), TRPA1 (transient receptor potential ankyrin receptor).

Abstract

Increased bile acids in the colon can evoke increased epithelial secretion resulting in diarrhea but little is known whether colonic bile acids contribute to abdominal pain. This study aimed to investigate the mechanisms underlying activation of colonic extrinsic afferent nerves and their neuronal cell bodies by a major secondary bile acid, deoxycholic acid (DCA). All experiments were performed on male C57BL/6 mice. Afferent sensitivity was evaluated using *in vitro* extracellular recordings from mesenteric nerves in the proximal colon (innervated by vagal and spinal afferents) and distal colon (spinal afferents only). Neuronal excitability of cultured dorsal root ganglion (DRG) and nodose ganglion (NG) neurons was examined with perforated patch clamp. Colonic 5-HT release was assessed using ELISA, and 5-HT immunoreactive enterochromaffin (EC) cells were quantified. Intraluminal DCA increased afferent nerve firing rate concentration-dependently in both proximal and distal colon. This DCA-elicited increase was significantly inhibited by a 5-HT_3 antagonist in the proximal colon but not in the distal colon, which may be in part due to lower 5-HT immunoreactive EC cell density and lower 5-HT levels in the distal colon following DCA stimulation. DCA increased the excitability of DRG neurons, whereas it decreased the excitability of NG neurons. DCA potentiated mechanosensitivity of high threshold spinal afferents independent of 5-HT release. Together, this study suggests that DCA can excite colonic afferents via direct and indirect mechanisms but the predominant mechanism may differ between vagal and spinal afferents. Furthermore, DCA increased mechanosensitivity of high threshold spinal afferents and may be a mechanism of visceral hypersensitivity.

Key words:

Bile acid, spinal afferent, vagal afferent, 5-HT, hypersensitivity

New & Noteworthy

- DCA directly excites spinal afferents, and to a lesser extent, indirectly via mucosal 5-HT release.
- DCA potentiates mechanosensitivity of high threshold spinal afferents independent of 5-HT release.
- DCA increases vagal afferent firing in proximal colon via 5-HT release but directly inhibits the excitability of their cell bodies.

Introduction

Bile acids are classically known for their roles in facilitating the digestion and absorption of dietary lipids. Primary bile acids are synthesized from cholesterol and conjugated with glycine or taurine in the liver. They are stored in the gall bladder, and released into the small intestine upon digestion of a meal. Most bile acids are reabsorbed by active transport in the ileum, although a small proportion, 5%, enters the colon where bacteria deconjugate and dehydroxylate primary bile salts to form secondary bile acids, that can be partly passively absorbed into the enterohepatic circulation (7). Deoxycholic acid (DCA), a secondary bile acid converted from cholic acid, is normally the predominant colonic bile acid (26).

Emerging evidence has suggested that bile acids also have complex hormonal actions both within and outside the intestinal tract, particular through the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (GPBAR1), also known as TGR5 (29). FXR is a nuclear receptor that mediates the genomic actions of bile acids and plays a key role in activating pathways that maintain bile acid homeostasis (10). TGR5 is a transmembrane receptor that couples to Gαs and cAMP signaling pathways. TGR5 has been implicated in mediating the actions of bile acids on secretion, motility, sensory transduction and inflammation (7).

Disruptions in the synthesis, excretion and recycling of bile acids are implicated in the onset of many diseases of the intestine, its accessory organs and beyond (18). Postprandial bile acid concentration is approximately 10 mM in human proximal small intestine, 2 mM in the distal ileum, and 0.6 mM in the cecum (17). Bile acids at high

physiological concentrations cause oxidative stress, DNA damage, apoptosis and cancer (31). Elevated levels of bile acids in the colon may also have profound influence on epithelial function and motility, including increased Cl^- secretion, enhanced permeability and increased intestinal transit (18), and this may have important implications in GI disorders. For example, increased bile acid delivery to the colon is observed in a subpopulation of irritable bowel syndrome with predominant diarrhea (IBS-D) patients; this is associated with altered bowel movements and accelerated colonic transit time, implying a partial mechanism for symptom generation in non-constipated IBS patients (4, 34, 39).

Previous work has shown that DCA can directly excite dorsal root ganglion (DRG) neuron cell bodies via a TGR5-dependent mechanism (1), suggesting a potential role for bile acid signaling in visceral pain. However, *in vivo* studies examining pain signaling via bile acids have revealed conflicting results. For example, DCA instillation into the rat colon for 3 consecutive days induced mild inflammation and persistent visceral hyperalgesia (42). In another study using *in vivo* afferent recordings of pelvic nerves, a mixture of sodium cholate and DCA increased baseline firing rate but mechanosensitivity remained unaltered (40). Conversely, while intraplantar injection of bile acids in mice caused inflammation, it also resulted in analgesia to mechanical stimulation independent of inflammation (1). Discrepancies in these *in vivo* studies highlight the need for *in vitro* studies at the level of primary sensory nerve terminals within the intact colon to examine mechanisms of neural signaling by bile acids in the distal GI tract. To achieve this, we studied two regions of the colon, the proximal colon, innervated by a combination of vagal and spinal afferent nerves, and the distal colon

125 that is predominantly spinal innervation (6). This allowed us to discriminate between
126 bile acid modulation of spinal and vagal afferent pathways.
127
128

Material and methods

Animals and ethical approval

All experiments were approved by Queen's University Animal Care Committee, in accordance with the guideline of the Canadian Council for Animal Care. Male C57BL/6 mice (body weight 22-25 gm) were purchased from Charles River Laboratories. They were housed individually under a standard light-dark cycle (lights on: 7 am, lights off: 7 pm) with free access to food and water. Mice were euthanized by isoflurane inhalation followed by cervical dislocation.

Human bile was obtained from 3 patients undergoing endoscopic retrograde cholangiopancreatography for removal of choledocholithiasis at Kingston Health Sciences Centre with informed consent. Experimental procedures were approved by Queen's University Human Ethics Committee.

Extracellular afferent nerve recording

The proximal colon was defined as the first 3 cm segment of colon immediately after the cecum. The proximal colon was also distinguished from the distal colon by the presence of distinct mucosal folds that are easily seen through the wall of the proximal colon. After identification of the superior mesenteric artery that enters the proximal end of the colon, we isolated the nerve associated with this artery. The distal colon was defined as a 3 cm segment immediately proximal to the pelvic brim; this segment is supplied by the inferior mesenteric artery that branches from the abdominal aorta. The nerve associated with this artery was isolated proximal to the inferior mesenteric ganglion. This classification is similar to a previous study (13). Nerve activity of

153 colonic afferents was recorded as previously described (9). Segments of proximal or
154 distal colon were placed in an organ bath continuously superfused with gassed (5% CO_2
155 and 95% O_2) Krebs buffer (composition, in mM: NaCl, 118.4; $NaHCO_3$, 24.9; $MgSO_4$,
156 1.2; KH_2PO_4, 1.2; glucose, 11.7; $CaCl_2$, 1.9) at 34°C. Preparations were cannulated at
157 both ends with one end connected to an infusion pump to allow continuous perfusion of
158 Krebs solution (0.2 mL/min) while the other end was connected to a pressure transducer
159 (NL108, Digitimer, Welwyn Garden City, UK). Ramp distention was applied by closing
160 the outflow drain of the preparation until the pressure reached 60 mmHg. Nerve bundles
161 were identified in the mesentery and drawn into a glass suction electrode attached to a
162 Neurolog headstage (NL100, Digitimer). Afferent nerve signals were amplified
163 (NL104), filtered (NL125 band pass filter) and recorded on a computer via a Micro
164 1401 interface and Spike 2 software (Version 7, Cambridge Electronic Design,
165 Cambridge, UK). Krebs contained the L-type calcium channel blocker nifedipine (3 μM)
166 and the muscarinic acetylcholine receptor antagonist atropine (5 μM) to suppress
167 smooth muscle activity, as well as the cyclooxygenase inhibitor indomethacin (3 μM) to
168 suppress potential inhibitory actions of endogenous prostaglandins (33).

169

170 DCA was applied either intraluminally (0.2 ml/min) or into the bath (10 ml/min), and
171 granisetron (a selective 5-HT_3 antagonist, 1μM) (37) was applied into both the bath and
172 lumen 15 minutes prior to DCA. Baseline afferent nerve firing frequency was
173 determined during a 120-second period just prior to application of DCA. The effect of
174 intraluminal DCA on baseline firing was calculated as a ratio of increased baseline
175 firing frequency 15 minutes after DCA administration compared to control baseline.
176 Response to bath-applied DCA was analysed similarly but a 30-second period at the

peak was used for analysis. The afferent nerve response to ramp distention was assessed as the increase in firing rate with increased intraluminal pressure using a custom-made script in Spike2. To compare distention response within the same preparations, firing frequency was normalized to the peak firing rate of the control distention. Single unit analysis was performed offline using the spike sorting function of Spike2 to discriminate the afferent nerve activity of individual units. Based on their sensitivity to ramp distention, afferent units were classified into two subpopulations, low threshold (LT) and high threshold (HT), with a cut-off threshold at 15 mmHg. This cut-off threshold is in keeping with previous studies in the small intestine and colon (9, 11, 28). A unit was considered as responding to DCA if the afferent firing frequency increased or decreased by 20% from baseline.

5-HT release assay

A 1cm segment of proximal and distal colon from each mouse were placed in ice-cold Krebs solution. Segments were cut open and pinned flat with mucosa up in Sylgard-coated wells. Tissue was incubated with the serotonin reuptake inhibitor fluoxetine (1μM) in Krebs solution (1 mL) at 37 ˚C for 10 minutes and supernatants were then collected. Following a brief rinse, the same tissue was incubated in 1mM DCA plus 1μM fluoxetine (1 mL) at 37 ˚C for 10 minutes. Supernatants were then collected. The wet weight of the tissue was recorded. The concentration of 5-HT in the supernatants was measured using an immunoassay kit (Beckman Coulter, IM1749, Indianapolis, IN, US) in accordance with the manufacturer's instructions. The concentration of 5-HT was normalized to the tissue weight.

Immunohistochemistry

Segments of proximal and distal colon were fixed overnight at 4°C in 4% paraformaldehyde dissolved in 0.1 M phosphate-buffered saline (PBS), followed by 3 times wash with PBS. Fixed specimens were cryo-protected in 30% sucrose/PBS overnight, embedded in optimal cutting temperature (OCT) compound (Wolf Labs, York, UK), and sectioned at 10 μm in a cryostat (Bright Instrument, OTF5000, Huntingdon, UK). Slides with sections were incubated with 5% goat serum/ PBS for 20 minutes to block non-specific binding, and then incubated overnight with a rabbit anti-serotonin antibody previously validated in mice (24, 36) (1:50; AbD Serotec, AHP522, Kidlington, UK) at 4°C, followed by PBS rinse and 2-hour incubation with a goat anti-rabbit secondary antibody conjugated to Cy3 (1:400; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature. Slides were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Peterborough, UK). A negative control was performed by omitting the primary antibody; this abolished immunofluorescence. When acquiring images, sections were oriented by aligning the muscularis mucosae to the bottom. Ten random images from ten sections of each specimen were acquired under 20× objective lens using an Olympus ColourView II digital camera for offline quantification. The number of enterochromaffin (EC) cells was counted in a blinded fashion. Since the transverse mucosal folds were much longer in the proximal colon, EC cell density was expressed as cells per unit area of mucosa (measured using ImageJ 1.43u; National Institutes of Health, Bethesda, MD, USA).

Perforated patch clamp recording

Dorsal root ganglion (DRG)(T9-T13) and nodose ganglion (NG) neurons were isolated as previously described (9, 38). Following overnight culture, coverslips containing isolated neurons were placed in a recording chamber on an inverted microscope and superfused with external solution containing (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10 and glucose 10, pH 7.4 with NaOH. While we did not perform retrograde labelling to specifically identify colon projecting DRG neurons, only small-diameter DRG neurons (≤30 pF) were selected, as they are putative nociceptors. Patch electrodes were pulled from Premium Custom 8520 Patch Glass (Warner Instruments) and filled with an internal solution containing (in mM): K-gluconate 110, KCl 30, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, pH 7.25 with KOH. Amphotericin B (240 μg/ml) was added to the pipette solution. Neuronal excitability was assessed by determining rheobase, the minimum amount of current required to elicit an action potential. Input resistance was determined by the hyperpolarizing response to current step from 0 to -10 pA. These parameters were measured again after 10-min superfusion of vehicle or DCA on the same neurons. Junction potential was calculated as 12 mV and the resting membrane potential was adjusted accordingly.

Drugs and compounds

Sodium deoxycholate (D6750) and fluoxetine hydrochloride (F132) were purchased from Sigma-Aldrich, and granisetron (21239) was obtained from Cayman Chemical. Sodium deoxycholate was made fresh in distilled water and diluted to their final concentration in Krebs buffer (with a final pH at 7.5) immediately prior to application in afferent recordings. DCA stock was diluted in the external solution in patch clamp

recordings. Fluoxetine and granisetron were prepared as stock solution, kept frozen at -20°C and diluted to their final concentration prior to application.

Data analysis and statistics

All data are expressed as means ± SD unless otherwise stated. Significant difference was determined by Student's t-test (two-tailed), one or two-way ANOVA with Bonferroni test as appropriate using GraphPad Prism 6. N refers to number of animals, and n indicates number of cells or afferent units. $P<0.05$ was considered significant. Significance indicator was defined as: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

DCA increased baseline afferent firing in mouse proximal and distal colon

DCA is a secondary bile acid that encompasses a significant proportion of the colonic bile acid pool (39). Therefore, we examined the effect of DCA on colonic afferent nerve firing. In the proximal colon, increase in baseline firing in response to DCA of LT afferents was small and not statistically significant (Fig. 1A, P=0.176, one-way ANOVA with Bonferroni test, N=4, 5 and 10 for 100, 300 μM and 1 mM), whereas DCA augmented HT afferent firing in a concentration-dependent manner with a significant change observed with 1 mM (Fig. 1C, $P<0.01$). It is generally accepted that HT afferents are almost exclusively spinal afferents as opposed to vagal afferents (6), and so we examined the response of afferent nerves in the distal colon as it is innervated predominantly by spinal afferents. Although the increase in LT afferent firing rate was not statistically significant (Fig. 1B, P=0.091, N=6 for both 100 μM and 1 mM), a significant change was observed for 1mM DCA in HT afferents in distal colon (Fig. 1D, $P<0.05$). In the proximal colon, 46% of units (n=45) were LT afferents and of these 62% showed increased baseline firing frequency in response to 1 mM DCA, while 47% units were HT afferents with 81% responsive to DCA (Fig.1E). In the distal colon, 44% of afferents (n=27) were LT units and 58% of these responded to DCA, while 41% units were HT afferents and 46% were DCA responders (Fig. 1F). The proportion of mechanically insensitive (MI) units was very low and thus were not included in the analysis of DCA response. Intraluminally-applied human bile (1:10 diluted in Krebs) in mouse proximal colon significantly increased baseline afferent firing rate with a similar response profile to 1 mM DCA (Supplementary Fig. 1).

Intraluminal administration of DCA activated afferent nerves indirectly via 5-HT release

Bath-applied DCA (1mM) elicited an immediate increase in baseline afferent nerve firing frequency, while intraluminal administration evoked a slow and smaller increase in baseline firing (Fig. 2A). Given the short time for bath perfusion (1 min), this is most likely due to direct actions of DCA on the afferent endings. Considering the delayed response to intraluminal application and evidence that bile acid induces release of 5-HT (2, 25, 35), we hypothesized that intraluminal application of DCA increased afferent discharge indirectly via mucosal 5-HT release. In agreement with this, pre-treatment with granisetron (1 μM), a 5-HT_3 antagonist, did not change the afferent response to bath-applied DCA (1mM) in both proximal (Fig. 2B left, P=0.075, paired t-test, N=5) and distal colon (Fig. 2C, P=0.405, N=5). Since nerve activity did not return to baseline after bath application of DCA in most proximal colon recordings, we reversed the order of treatments to confirm that granisetron did not inhibit afferent responses to bath-applied DCA (Fig. 2B right, P=0.092, paired t-test, N=5). However, granisetron significantly inhibited the afferent response to intraluminal application of DCA in the proximal colon (Fig. 2C, P<0.01, paired t-test, N=5), and reversal of the order of treatments confirmed the inhibitory effect of granisetron (Fig. 2D, P<0.05, N=5). However, in the distal colon, while the response to intraluminal application of DCA was reduced by granisetron, this was not significant (Fig. 2E, P=0.266, paired t-test, N=8), although 5 out of 8 preparations showed a smaller response in the presence of granisetron. The percent of inhibition on afferent response to DCA by granisetron was significantly lower in the distal colon compared to proximal (Fig. 2F, P<0.05, unpaired

306 t-test). Since distention itself can evoke 5-HT release from the mucosa (5) that may
307 impact the availability of mucosal 5-HT to be released upon repeated applications of
308 DCA, we did not perform distention during these experiments and thus were unable to
309 define the proportion of LT and HT units attenuated by granisetron.
310
311 **DCA stimulated 5-HT release in the proximal and distal colon**

312 Given the effect of granisetron on afferent response to DCA, we examined the effect of
313 DCA on 5-HT release. Compared to basal release, 1 mM DCA increased 5-HT release
314 in both proximal (Fig. 3A, $P<0.05$, paired t-test, N=5) and distal colon (Fig. 3B, $P<0.01$,
315 paired t-test, N=5). Basal 5-HT release in the proximal colon was higher compared to
316 the distal colon (Fig. 3C, $P<0.01$, unpaired t-test). Absolute 5-HT release upon DCA
317 stimulation was greater in the proximal colon than the distal colon (Fig. 3D, $P<0.05$,
318 unpaired t-test), although the net increase (subtracted by basal release) was not
319 significantly different (0.5±0.1 vs. 0.5±0.1 nM/mg, P=0.897).
320
321 We next examined the density of EC cells in the proximal and distal colon with an anti-
322 serotonin antibody. 5-HT immunoreactive EC cells were identified in the epithelium
323 lining in both proximal and distal colon (Fig. 3E). Similar to the greater 5-HT release
324 observed in the proximal colon, EC cell density was greater in the proximal colon
325 compared to the distal colon (Fig. 3F, $P<0.01$, unpaired t-test, N=5 for proximal colon
326 and 6 for distal colon).
327
328 **Continual exposure to DCA potentiated mechanosensitivity of HT spinal afferents**
329 **independent of 5-HT**

Our next series of experiments examined the effect of DCA on afferent nerve mechanosensitivity. A representative trace in Fig. 4A illustrated a time-dependent effect of intraluminal administration of DCA (1 mM) on spinal afferent response to distention in mouse distal colon. DCA did not change the overall afferent nerve response to distention after 10 minutes of perfusion (Fig. 4B, $P=0.225$, two-way ANOVA with Bonferroni test, N=7, although $P<0.05$ at 10 and 20 mmHg), but significantly potentiated the distention response after 30 minutes of perfusion ($P<0.05$ for overall response, $P<0.001$ for all pressure points). This potentiation was selective for HT afferents ($P<0.05$, two-way ANOVA with Bonferroni test, n=9, $P<0.05$ at 20 and 40 mmHg, $P<0.01$ at 30, 50 and 60 mmHg), whereas there was no effect on LT afferents ($P=0.372$, n=12). In a few recordings we recorded as long as 50 minutes and this potentiation appeared to persist. Interestingly, following DCA application 6 out of 11 HT units were now activated at pressures <15 mmHg (i.e. behaved like a LT unit). This increased mechanosensitivity after 30 minutes DCA perfusion was not affected by pre-treatment with granisetron (Fig. 4C, $P<0.05$, two-way ANOVA with Bonferroni test, N=7, $P<0.01$ at 20 mmHg, $P<0.05$ at 40 mmHg, $P<0.001$ at 50 and 60 mmHg). A lower concentration of DCA at 100 μM was not able to potentiate mechanosensitivity (Fig. 4D, $P=0.909$, N=6). However, bath application of this lower dose (100 μM) recapitulated the sensitizing effect of intraluminal 1 mM DCA after only 10 minutes of perfusion (Fig. 4E, $P<0.05$ for overall response, N=5, $P<0.001$ at 60 mmHg). Changes in compliance, the ability of a hollow organ to distend and increase volume with increasing pressure, may influence afferent nerve sensitivity to distention (30). By comparing pressure-volume curves, compliance was slightly increased after 30-minute intraluminal perfusion of 100 μM DCA ($P<0.01$, two-way ANOVA) whereas no change was

observed in the other 3 groups, suggesting that the change in mechanosensitivity is independent of compliance.

DCA increased the excitability of DRG neurons but decreased the excitability of NG neurons

Differences observed in the role of 5-HT in afferent nerve signalling by DCA in the proximal and distal colon may reflect differences in the innervation of these regions by spinal and vagal afferents. Therefore, in perforated patch clamp recordings we compared the effect of DCA on dissociated DRG and NG neurons. A brief superfusion (10 min) of DCA at 100 μM decreased the rheobase (i.e. increased the excitability) of DRG neurons (Fig. 5A, $P<0.01$, paired t-test, n=11). Conversely, DCA increased the rheobase (i.e. decreased the excitability) of NG neurons (Fig. 5B, $P<0.01$, paired t-test, n=12). DCA also had opposing effects on the resting membrane potential; the resting membrane potential of DRG neurons was depolarized after DCA application (Fig. 5C, $P<0.05$, paired t-test, n=11), whereas the resting membrane potential of NG neurons became hyperpolarized (Fig. 5D, $P<0.01$, paired t-test, n=12). However, input resistance was not significantly changed in both DRG (1509±244 vs. 2091±447 MΩ, P=0.142, paired t-test, n=11) and NG neurons (746±92 vs. 708±101 MΩ, P=0.796, paired t-test, n=11). Vehicle superfusion (external solution, 10 min) had no effect on any of the parameters measured above.

Discussion

Bile acids are increasingly recognized as important signalling molecules in the GI tract. While excess bile acids within the colon are known to increase secretion and transit, there has been little study of their impact on extrinsic sensory nerves innervating this region of the gut, which could have important implications for nociceptive signalling. The current study revealed different mechanisms underlying activation of vagal and spinal afferents innervating mouse colon by the major colonic bile acid, deoxycholic acid, at the level of nerve terminals compared to their neuronal cell bodies. DCA excited spinal afferents directly, and to a lesser extent, via 5-HT release. In contrast, the activation of vagal afferent pathways appears to depend on mucosal 5-HT release, as direct administration of DCA to nodose ganglion neurons inhibited their excitability. Interestingly, a longer exposure to DCA potentiated the mechanosensitivity of high threshold spinal afferents, which are thought to be nociceptors (6), implying that bile acids have the potential to evoke visceral hypersensitivity.

We identified that DCA activates extrinsic afferent nerves by both direct and indirect mechanisms with the predominant mechanism differing between regions of the colon. Bath application of DCA increased baseline firing frequency; this effect was unaltered in the presence of 5-HT_3 receptor antagonist. This, combined with the almost immediate increase in firing frequency upon DCA application, strongly suggests that it's the result of direct activation of nerve terminals by DCA. Our patch clamp recording experiments confirmed direct activation of DRG neurons by DCA. Conversely, granisetron reduced the response to DCA applied through the colonic lumen suggesting an indirect activation of 5-HT_3 receptors on nerve terminals by 5-HT, which may be released from

399 enterochromaffin cells via a TGR5 dependent mechanism (2). However, the effect of
400 granisetron was much greater in the proximal colon where it significantly blocked the
401 afferent response to intraluminal DCA. This difference may be explained in part by our
402 observation of lower EC cell density and lower absolute 5-HT release in the distal colon
403 upon DCA stimulation compared to the proximal colon. Moreover, while spinal
404 afferents are activated by 5-HT via 5-HT_3 receptors (20), previous work has shown
405 greater 5-HT_{3a} receptor subunit transcript expression on microarray in NG neurons
406 compared to DRG neurons (32) and thus there may be more 5-HT_3 receptor expression
407 in the proximal colon as it is innervated by both vagal and spinal afferent nerves
408 compared to the distal colon, which has predominant spinal afferent nerve innervation.
409 Thus, the smaller inhibition by granisetron on excitation by DCA on distal colon
410 afferent nerves may result from both less 5-HT release from the epithelium and lower 5-
411 HT_3 expression on afferent nerves in this region. Bile acids may also promote release
412 of other mediators from enteroendocrine cells such as glucagon-like peptide-1 (GLP-1)
413 (8, 23, 41). While GLP-1 is able to activate vagal afferents (14), previous studies did not
414 find a direct activation on DRG neurons (3) and clinical data suggests it may reduce
415 pain (19). Thus, a role for bile acid induced release and modulation of colonic afferents
416 for other enteroendocrine mediators requires further study. Bile acids may also promote
417 peristalsis either via 5-HT release to activate 5-HT_4 receptors on intrinsic afferents or by
418 directly activating enteric neurons (2, 7). Although peristalsis activates muscular
419 afferents (16), this would have little role in our experiments as they were performed in
420 the presence of the L-type calcium channel blocker nifedipine and muscarinic receptor
421 antagonist atropine to suppress smooth muscle activity. Although a variety of 5-HT
422 receptor subtypes are expressed in the gut, in the context of sensory signalling, most

423 attention has focused on 5-HT_3 and $5HT_4$ receptors (15). Unlike the high abundance of
424 mRNA for 5-HT_3 receptor, 5-HT_4 receptor expression was low in both NG and DRG
425 neurons. A 5-HT_4 receptor agonist had no effect on the gastric vagal afferent activity
426 (43). In the colon, a 5-HT_4 agonist inhibited visceral hypersensitivity, although it was
427 not clear whether this was due to a direct action on nociceptive nerves (21). Conversely,
428 5-HT_3 receptor agonists directly excite distal colonic afferent nerves (20). Thus, while
429 we cannot exclude a role of other 5-HT receptor subtypes, we focused on 5-HT_3
430 receptors in the current study. Taken together, our results suggest that DCA activates
431 colonic afferents predominantly via release of 5-HT in the proximal colon whereas it
432 has both a 5-HT mediated activation and a direct activation of the extrinsic afferents in
433 the distal colon.
434
435 Since the distal colon is predominantly innervated by spinal afferents (6), some of
436 which are putative nociceptors, effect of DCA on mechanosensitivity was examined in
437 the distal colon. A brief intraluminal exposure to DCA did not alter mechanosensitivity
438 whereas a longer exposure (30 min) selectively sensitized high threshold spinal
439 afferents to mechanical stimulation. This was only observed at a concentration of 1mM
440 intraluminally, whereas bath application of a lower concentration quickly increased the
441 distention response. This suggests that DCA directly increases the mechanosensitivity
442 of spinal afferents but higher concentrations within the lumen are required. This effect
443 of the higher concentration within the lumen might be analogous to increased bile acid
444 delivery in the colon in a subpopulation of IBS patients (4, 34, 39). It has been
445 estimated that DCA 100 μM is within the physiological range in the colon whereas
446 1mM may be pathophysiological as has been observed in disorders such as IBS (2, 17,

447 39). This is consistent with an *in vivo* study that DCA (4 mM) instillation into the rat
448 colon daily for 3 days induces increased visceromotor response to noxious colorectal
449 distention (42). Additionally, a recent study using an *in vitro* mouse colorectal
450 preparation revealed that 67% of mechanical insensitive afferents acquire
451 mechanosensitivity after a 5-min exposure to 0.5% bile salts (12). Our study showed
452 that the sensitizing effect of DCA on mechanosensitivity was not affected by
453 granisetron, suggesting a mechanism independent of mucosal 5-HT release.
454 Additionally, our patch clamp results revealed increased excitability of cultured DRG
455 neurons following exposure to DCA, strengthening the contention that direct
456 sensitization of DRG neurons by DCA is at least one of the underlying mechanisms of
457 the observed increased mechanosensitivity. The cellular mechanisms of this increased
458 mechanosensitivity such as the involvement of the transient receptor potential ankyrin 1
459 (TRPA1) channel that may be sensitized via $G_{\beta\gamma}$, protein kinase C and Ca^{2+} following
460 TGR5 activation (27) require further study.
461
462 A definitive distinction between vagal versus spinal afferent units within a given
463 recording in the proximal colon is not possible with our extracellular recordings, and
464 thus we performed patch clamp recordings to examine the direct effect of DCA on these
465 two neural populations. While we did not perform retrograde labelling to specifically
466 identify colon-projecting neurons, previous studies have shown sensory neurons,
467 including small diameter DRG neurons recorded in this study, express TGR5 (1). Our
468 finding that the excitability of unlabelled NG and DRG neurons is affected by DCA
469 suggests that TGR5 expression is not limited to gut-projecting afferent neurons.
470 Interestingly, in contrast with DRG neurons DCA inhibited the excitability of NG

neurons. This difference in the effect of DCA on the excitability is in keeping with distinct gene expression profiles (e.g. ion channels) between DRG and NG neurons (32), although specific channels and mechanisms involved require future study. The inhibition of NG neurons by DCA is consistent with an *in vivo* study showing that glycocholic acid decreases gastric vagal afferent response to distention at neutral pH (22). Since a major function of vagal afferents in the GI tract is transmission of meal-related satiety signals to the brain, the effect of DCA on vagal afferent excitability, together with its effects on stimulating GLP-1 and 5-HT release, satiety mediators that can activate vagal afferents (45), may suggest implications for satiety regulation.

FXR and TGR5 are the two most studied bile acid receptors. FXR is highly expressed in the liver and ileum (10), with no evidence of its presence in the primary sensory neurons. Since FXR is a nuclear receptor that fulfils bile acids' regulative roles at a transcriptional level, it is unlikely to be involved in the current study, given the relative short-term treatment of DCA. TGR5 is widely expressed, including in DRG neurons (1) and enterochromaffin cells (2), and a key mediator of many rapid physiological and pathophysiological effects of bile acids (7). Furthermore, FXR is activated mainly by primary bile acids, whereas the most potent activators for TGR5 are secondary bile acids (44). As such, the effects of DCA on colonic primary afferent signalling observed in this study are likely mediated via TGR5. However, due to lack of specific pharmacological tools, the present study did not directly address the involvement of TGR5.

494 In conclusion, this study has elucidated different mechanisms underlying activation of
495 spinal and vagal afferents innervating mouse colon by the major secondary bile acid
496 DCA, and provided evidence that it can induce visceral hypersensitivity. The findings
497 of this study have important implications for studying mechanisms related to pain
498 signalling in GI disorders such as IBS.
499

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Author contribution:

- DR obtained funding and supervised the project.
- DR, YY, EV and AL designed the study.
- YY, EV, SP, CB, CK and DG acquired, analysed and interpreted the data.
- YY, EV, AL and DR drafted and revised the manuscript.

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649

650

651 # Figure legends

652 **Figure 1. DCA increased afferent firing frequency in mouse proximal and distal**
653 **colon.**

654 (A&C) DCA (100, 300 μM and 1 mM) induced increased baseline firing frequency on
655 LT (A) and HT (C) afferents innervating proximal colon, one-way ANOVA with
656 Bonferroni test. (B&D) DCA (100 μM and 1 mM) increased firing rate of LT (B) and
657 HT (D) afferents innervating distal colon. (E&F) The number of LT and HT afferents
658 responding to DCA (1mM) in the proximal and distal colon. N=10 for proximal colon;
659 N=6 for distal colon. LT, low threshold; HT, high threshold.

660

661 **Figure 2. Intraluminal administration of DCA activated afferent nerves indirectly**
662 **via 5-HT release.**

663 (A) Recording showing the response of an afferent nerve to bath and intraluminal
664 application of 1 mM DCA in a proximal colon preparation. (B) Pre-treatment with
665 granisetron (1 μM) did not change afferent response to bath-applied DCA in the
666 proximal colon (paired t-test, N=5), regardless the order of treatments. (C) Granisetron
667 did not change afferent response to bath-applied DCA in the distal colon (N=5). (D)
668 Granisetron decreased afferent response to intraluminal application of DCA in the
669 proximal colon, regardless of the order of treatments ($P<0.01$, paired t-test, N=5). (E)
670 Granisetron did not significantly change afferent response to intraluminal administration
671 of DCA in the distal colon (paired t-test, N=8). (F) The percent inhibition on the
672 response to DCA by granisetron was lower in the distal colon compared to proximal
673 colon ($P<0.05$, unpaired t-test).

674

675 **Figure 3. DCA stimulated greater 5-HT release in the proximal colon.**
676 (A&B) 5-HT release during DCA incubation (1 mM, 10 min) was greater than basal
677 release (incubated in Krebs for 10 min) in both proximal ($P<0.05$, paired t-test, N=5)
678 and distal colon ($P<0.05$, N=5). (C) Basal 5-HT release was greater in the proximal
679 colon compared to the distal colon ($P<0.05$, unpaired t-test). (D) DCA stimulated 5-HT
680 release was also higher in the proximal colon ($P<0.05$). Fluoxetine (1μM) was present
681 in both basal and DCA conditions. The concentration of 5-HT was normalized to the
682 tissue weight. (E) Representative images showing 5-HT immunoreactivity in the
683 proximal and distal colon. Scale bar =50 μm. (F) EC cell density was greater in the
684 proximal colon compared to the distal colon ($P<0.01$, unpaired t-test, N=5 for proximal
685 colon and 6 for distal colon).
686
687
688

689 **Figure 4. Continual exposure to DCA potentiated mechanosensitivity of HT spinal**
690 **afferents independent of 5-HT.**

691 (A) Representative trace showing afferent response to distention in the presence of DCA
692 (1 mM, intraluminal perfusion for 30 minutes) in mouse distal colon. (B) DCA did not

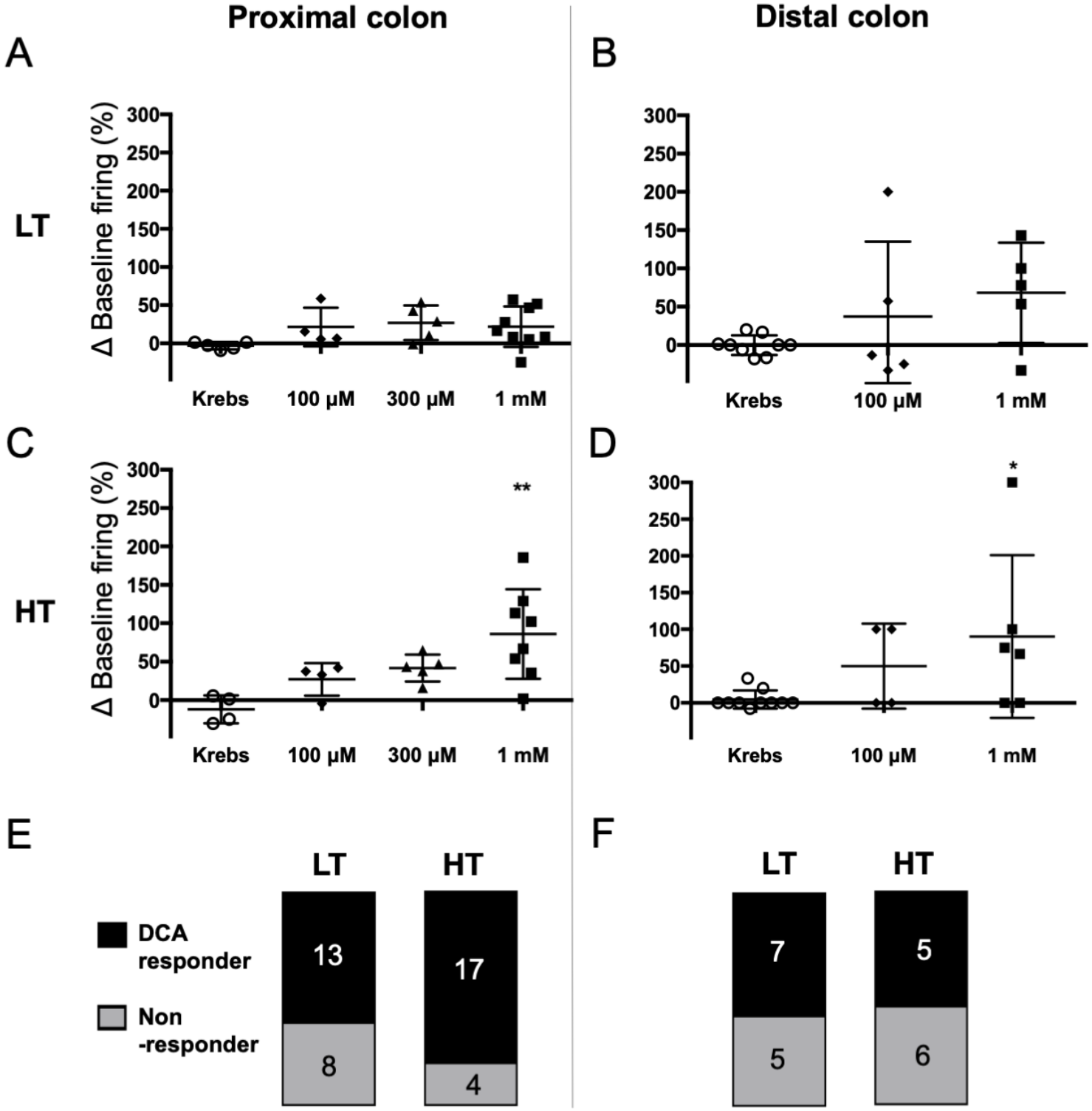
potentiate afferent response to distention until 30 minutes after perfusion, P<0.05, two-way ANOVA, N=7. P=0.225 for 10 min. Single unit analysis revealed that the main effect was on HT afferents, P<0.05. (C) This potentiation was not blocked by granisetron (1 μM), P<0.05. (D) A lower concentration (100 μM) did not cause any potentiation to distension. (E) Bath application of 100 μM DCA for 10 minutes increased afferent response to distension, P<0.05.

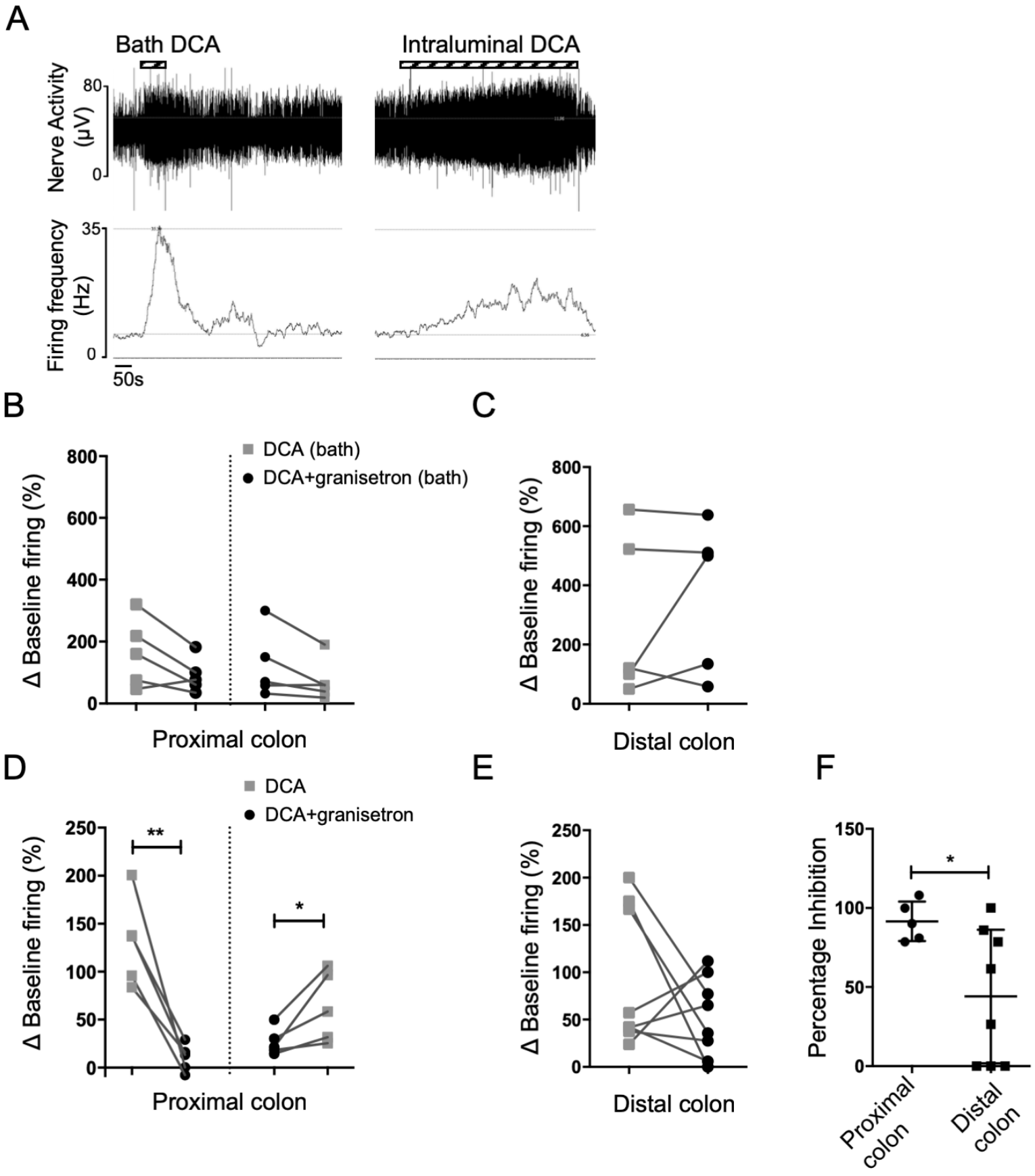
Figure 5. DCA increased excitability of DRG neurons whereas decreased excitability of NG neurons.

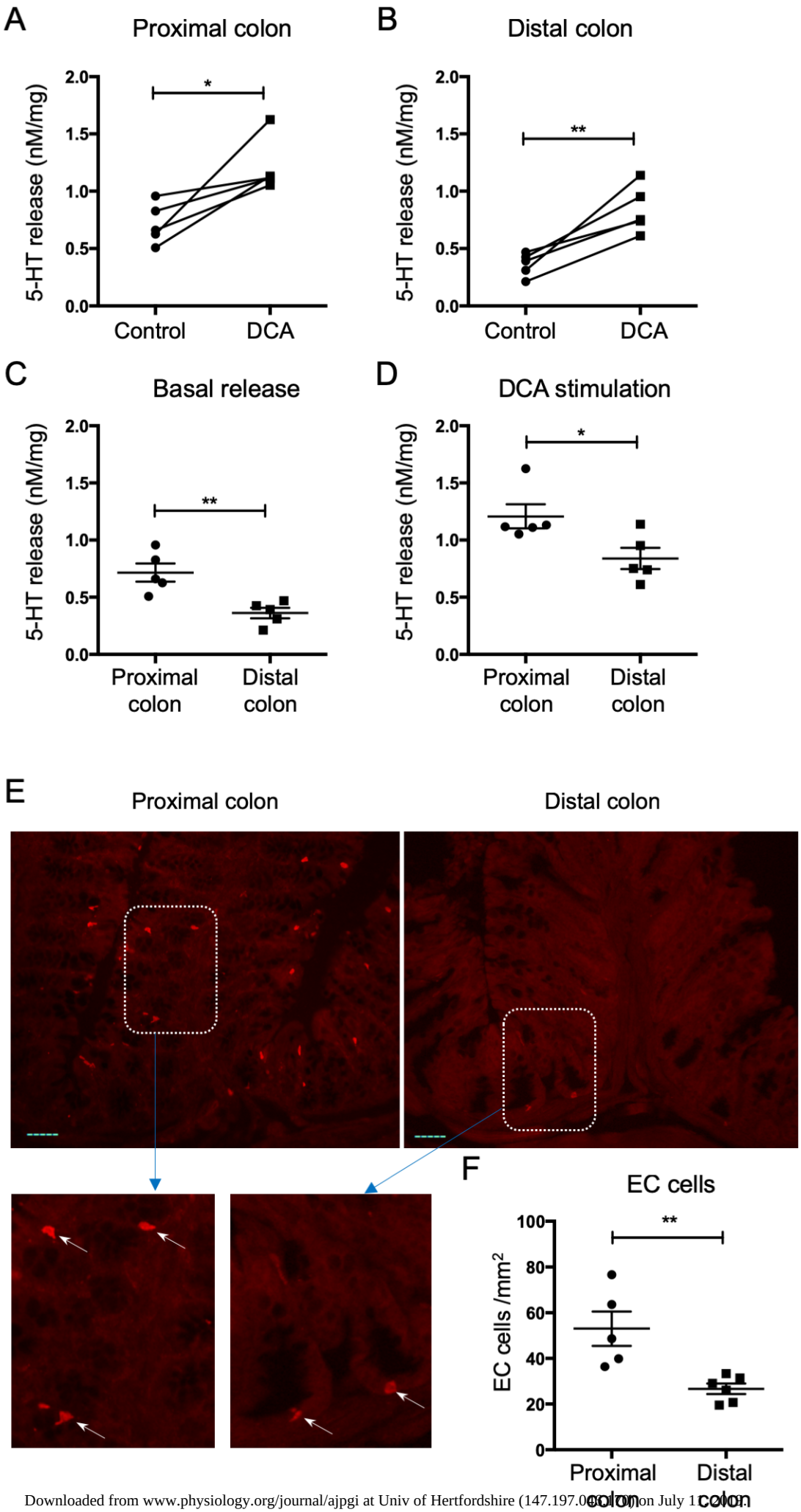
DCA superfusion (100 μM for 10 minutes) decreased the rheobase in DRG neurons (A, P<0.01, paired t-test, n=11) but increased the rheobase in NG neurons (B, P<0.01, n=12). DCA depolarized resting membrane potential in DRG neurons (C, paired t-test, P<0.05) but hyperpolarized that in NG neurons (D, P<0.01). Vehicle had no effect on rheobase and resting membrane potential. DRG, dorsal root ganglion; NG, nodose ganglion.

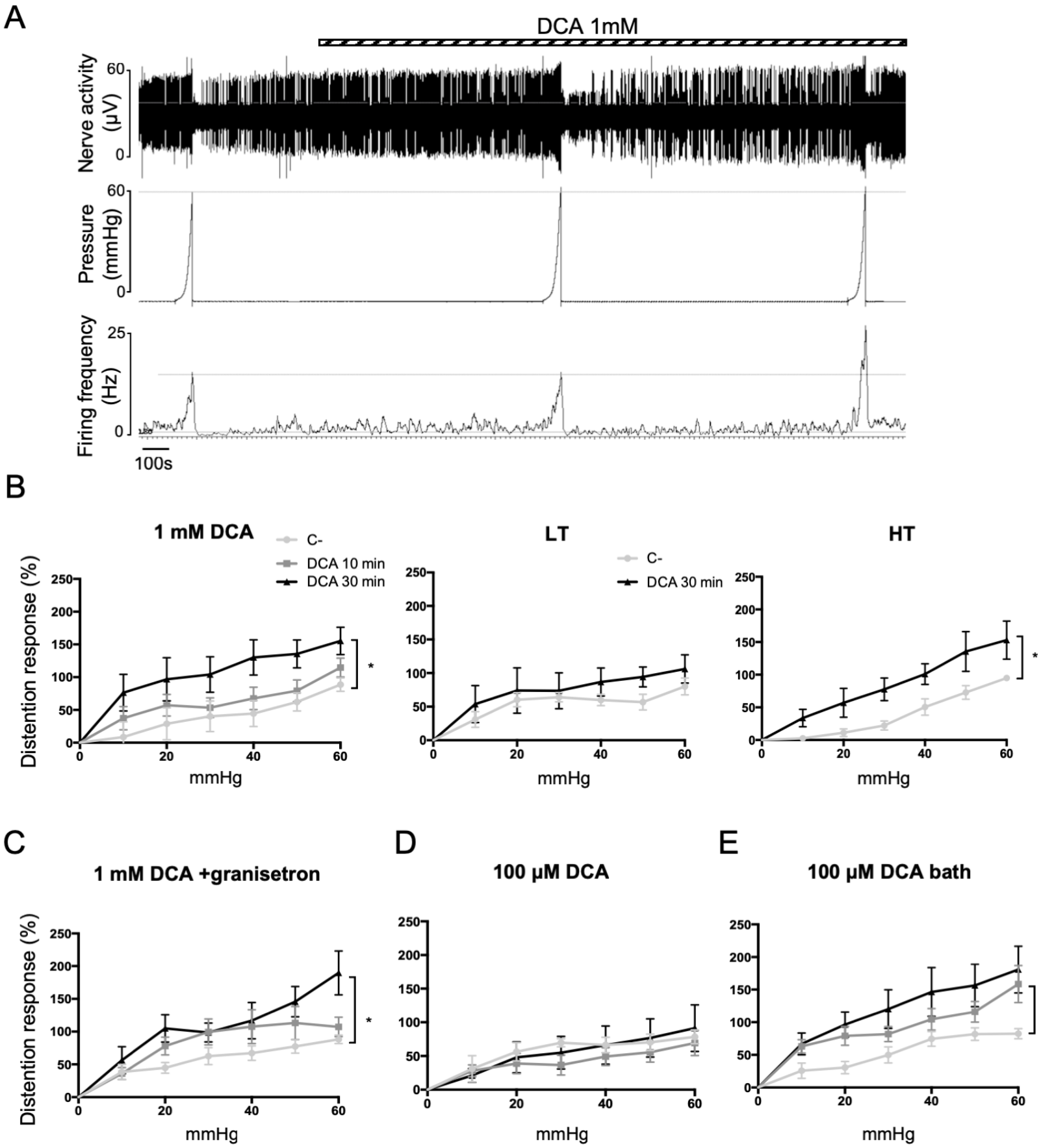
Supplemental Material

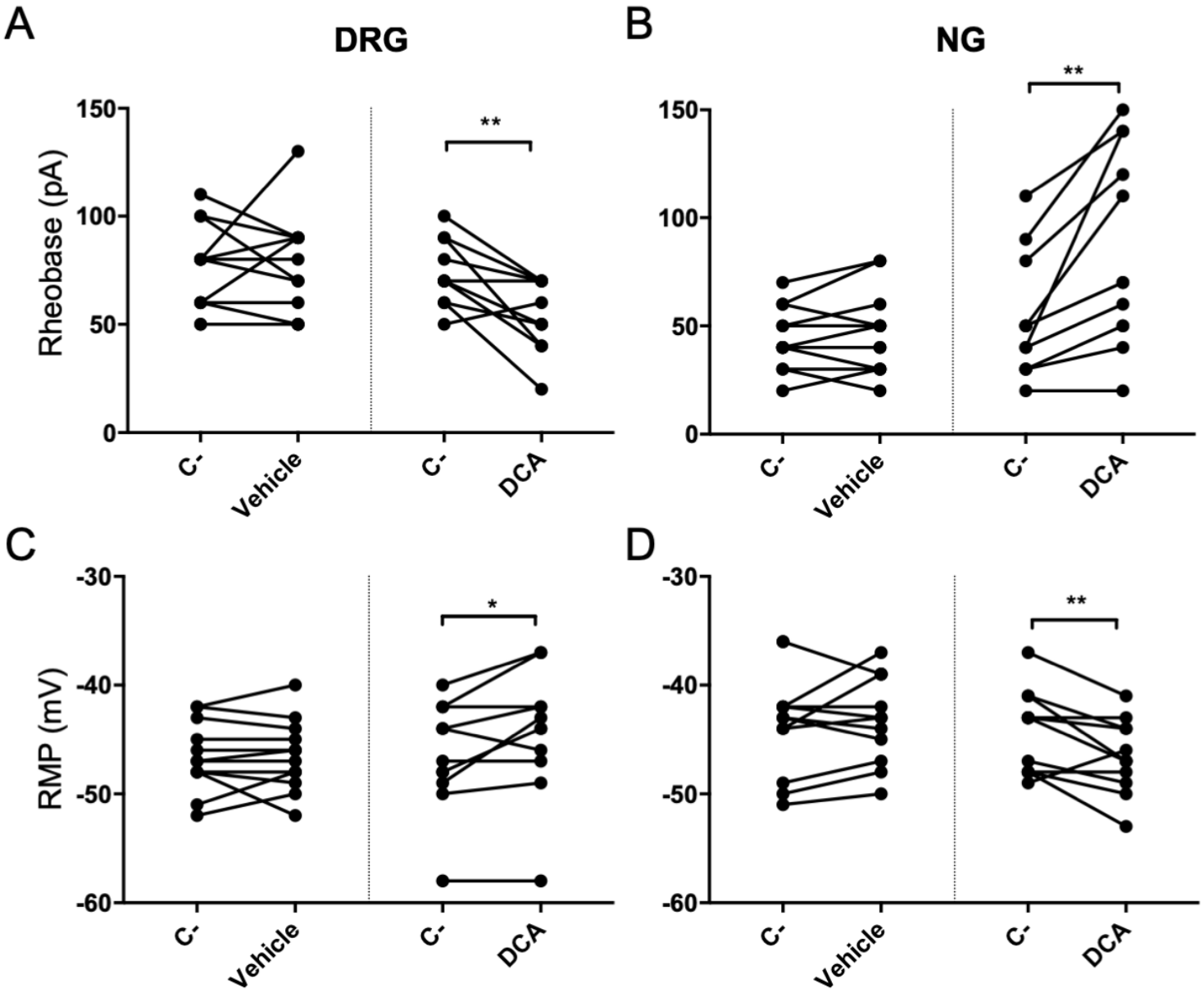
https://figshare.com/articles/supp_figure_DCA_AJP_pptx/8243252/1

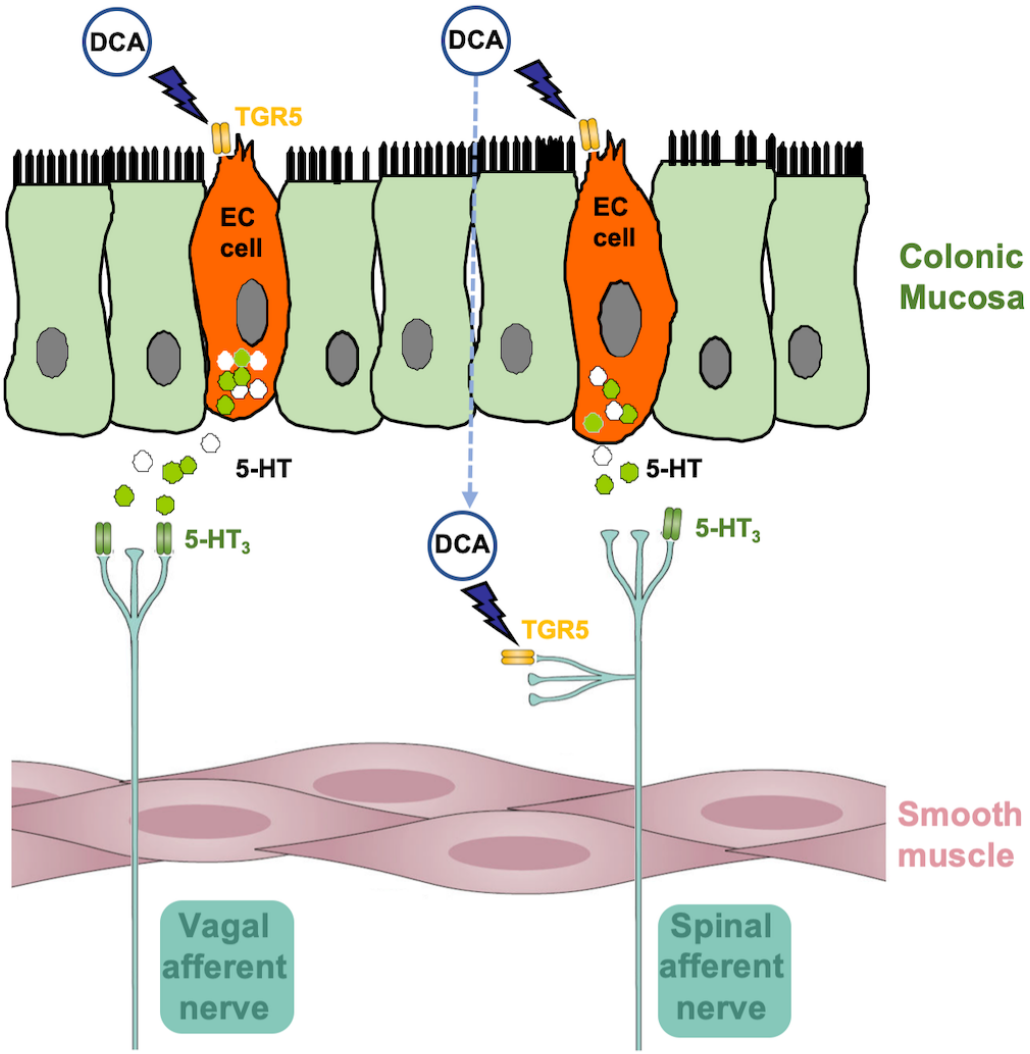












DCA (deoxycholic acid)
EC (enterochromaffin)
TGR5 (G protein-coupled bile acid receptor)