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PI 3-kinase- and ERK-MAPK-dependent mechanisms underlie Glucagon Like Peptide-1-mediated activation of Sprague Dawley colonic myenteric neurons.

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Running title: GLP-1 in colonic myenteric neurons

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Abstract

Background: Glucagon-like peptide (GLP-1) can modify colonic function, with beneficial effects reported in the functional bowel disorder, Irritable Bowel Syndrome (IBS). IBS pathophysiology is characterized by hyper-activation of the hypothalamic-pituitary-adrenal stress axis and altered microbial profiles. This study aims to characterize the neuronal and functional effects of GLP-1 in healthy rat colons to aid understanding of its beneficial effects in moderating bowel dysfunction.

Methods: Immunofluorescent and calcium imaging of myenteric neurons prepared from Sprague Dawley rat colons was carried out to elucidate the neuromodulatory actions of the GLP-1 receptor agonist, exendin-4 (Ex-4). Colonic contractile activity was assessed using organ bath physiological recordings.

Key Results: Ex-4 induced an elevation of intracellular calcium arising from store release and influx via voltage-gated calcium channels. Ex-4 activated both ERK-MAPK and PI 3-kinase signaling cascades. Neuronal activation was found to underlie suppression of contractile activity in colonic circular muscle. Although the stress hormone, corticotropin-releasing factor (CRF) potentiated the neuronal response to Ex-4, the functional effects of Ex-4 on colonic circular muscle activity were not altered.

Conclusions & Inferences: Ex-4 evoked neurally-regulated suppression of rat colonic circular muscle activity. In myenteric neurons, the neurostimluatory effects of Ex-4 was dependent upon activation of PI 3-kinase and ERK-MAPK signaling cascades. No further change in circular muscle function was noted in the presence of CRF suggesting that stress does not impact on colonic function in health. Further studies in a model of IBS are needed to determine if mechanisms are modified in the context of bowel dysfunction.

Keywords: myenteric neurons; ERK-MAPK; GLP-1 receptors; voltage-gated calcium channels, circular smooth muscle.

Key points (max 80 words)

- GLP-1 receptor agonists have been shown to be beneficial in the treatment of functional bowel disorders, but the underlying cellular mechanisms are unclear.
- Activation of myenteric neurons by the GLP-1 receptor agonist induced activation of PI 3-kinase and ERK-MAPK signaling cascades.
- The GLP-1 receptor agonist modified colonic circular muscle contractile activity in healthy rats, an effect that was not further exacerbated by the stress hormone, corticotropin-releasing factor.

Chemosensory activation of enteroendocrine L-cells by ingested nutrients causes membrane depolarization, action potential firing and enhanced basolateral exocytosis of glucagon-like peptide-1 (GLP-1) [1]. Acting in its capacity as an incretin hormone, GLP-1 regulates glucose homeostasis. However, GLP-1 also exerts regulatory effects on the gastrointestinal (GI) tract, inhibiting motility, gastric emptying and the migrating motor complex; actions that have been reported both in both healthy controls and in patients with Irritable Bowel Syndrome (IBS) [2-5]. A common, functional GI disorder [6], IBS is characterized by abdominal pain, bloating, diarrhea and/or constipation. Administration of a GLP-1 mimetic to female IBS patients showed efficacy in reducing spasmodic and visceral pain symptoms [7]. Moreover, in constipation-predominant IBS (IBS-C) patients, decreased circulating GLP-1 and decreased mucosal expression of GLP-1Rs was correlated with the severity of abdominal pain [8]. Animal models of IBS suggest differential receptor expression levels in colons from animals with diarrhea or constipation [9].

The endocrine system has a recognized role in IBS symptom flares. Indeed, hormonal fluctuations associated with menstrual cycles, and activation of the Hypothalamic-Pituitary-Adrenal (HPA) stress axis, which is dysfunctional in IBS patients [10-13], have been linked to exacerbation of symptoms [14-16]. Interestingly, crosstalk between GLP-1 and corticotropin-releasing factor (CRF), a key stress hormone, has been demonstrated in central neurons. GLP-1 stimulates the HPA axis activation through CRF neurons [17]. Moreover, stress-induced defecation is attenuated by antagonists of both CRF [18] and GLP-1 [19], and GLP-1 accelerates stress-induced changes in colonic motility though vagal signaling [20]. Mechanistically, the modulatory effects of GLP-1 on GI function may be centrally orchestrated [21], with high levels of expression of GLP-1 receptors (GLP-1Rs) in the nucleus tractus solitarius, the ventrolateral medulla [22], the area postrema and hypothalamus [23]. Central administration of

GLP-1 increased colonic transit, also through vagal signaling [20], whereas peripherally applied GLP-1 has a mollifying effect on GI motility, which is likely to be mediated through local release of nitric oxide [24,25], regulated by myenteric neurons [8,26,27].

Somewhat counterintuitively, despite the reduced probability of nutrients being present at the more distal end of the GI tract, the abundance of GLP-1-secreting L-cells increases [28]. In fact, the chemosensory properties of GLP-1-secreting L-cells in the colon are likely to differ from those in more proximal regions, being activated by microbial products rather than nutrients [29,30]. Indeed, specific commensal bacteria increase intestinal and circulating GLP-1 [31,32]. In the context of IBS pathophysiology, altered microbial profiles have been reported [33], which could result in modified circulating GLP-1[8]. However, few studies have been carried out on the cellular mechanisms underlying the inhibitory effects of GLP-1 in the colon and how this may be impacted by other hormones in the context of bowel dysfunction. Thus, the aim of our study was to investigate the intracellular signaling mechanisms evoked by GLP-1 in myenteric neurons in rat colon and relate this to changes in colonic smooth muscle activity. We further investigated the potential exacerbation of gut function by the stress hormone, CRF.

Materials and Methods

Ethical approval

All experiments were in full accordance with the European Community Council Directive (86/609/EEC). Rats were euthanized by CO_2 overdose and cervical dislocation, which was approved by the local University College Cork animal ethical committee (ref. #2011/015). Rodents were euthanized at the same time of day (~9-10.30am) for all experiments.

Animals and Tissue collecting

Sprague Dawley (SD) rats approximately 10-12 weeks old (250-350g) were bred in the Biological Services Unit, University College Cork, Ireland. Rats were group-housed 3 per cage and maintained on a 12 / 12-hour dark-light cycle (08.00-20.00) with a room temperature of 22 ±1^{o}C. Food and water were available *ad libitum*.

A section of distal colon was excised from each rat and stored in ice-cold Krebs solution containing in mmolL^{-1}: 117 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4 and 11 D-glucose (pH 7.4). The colon was opened out along the mesenteric border and pinned out into a Sylgard-lined petri dish. To prepare a longitudinal muscle myenteric plexus (LMMP) tissue preparation for calcium and immunofluorescent imaging, the mucosal layer was removed and the circular muscle layer was peeled back to expose the myenteric plexus. For gut bath electrophysiology, distal colonic sections opened along the mesentery with mucosa removed, were suspended transversely to measure circular muscle contractility and longitudinally to measure longitudinal muscle contractile activity.

Calcium Imaging

For calcium imaging studies, LMMP tissue from the distal colon of Sprague Dawley rats was tightly pinned out in Sylgard (Sylgard 184 silicone elastomer kit, WPI, Sarasota FL, USA)-lined petri dishes and loaded with the ratiometic dye, Fura-2AM (7μM, 1 hr, Sigma Aldrich, UK). The calcium indicator was washed out and the tissue was continuously superfused with carbogen-bubbled Krebs saline solution containing nifedipine (1μM, Sigma Aldrich, UK) to inhibit smooth muscle contractions. Cytosolic changes in calcium were recorded from neuronal cell bodies using Cell R software (Olympus Soft imaging solutions, 1986-2009). Images were captured at 3Hz using a Xenon/Mercury arc burner (Olympus, Melville, NY, US), a charge-coupled device digital camera (F-view II, Soft imaging system, Munster, Germany) and a 40x water-immersion objective on a fixed stage upright microscope (Olympus BX51WI). The excitation or emission wavelengths of ratiometric Ca^{2+} indicators such as Fura-2 AM shift as concentrations of cytosolic Ca^{2+} change, and thereby allow dynamic measurements of intracellular Ca^{2+} in the selected cells. Excitation filters of 340/380nm and emission wavelengths of 510nm were used. Ganglionic neurons were identified based on morphology and responsivity to 75mM KCl, which was added at the end of each experiment. Neurons were considered to be responders if the Fura-2 AM signal increased by more than two standard deviations from baseline for each neuron. The baseline values were calculated as the average ratio during the 150 seconds preceding the stimulus. The tissue was incubated with pharmacological reagents for 20-30 mins under continuous perfusion.

Immunofluorescence and confocal microscopy

LMMP preparations were pinned out on Sylgard-lined petri dishes and fixed in 4% paraformaldehyde (4^{o}C, overnight), permeabilized with Triton X-100 (0.1%, 1 hour, room temperature) and blocked with 1% donkey serum (1 hour, room temperature). LMMP tissue

was incubated overnight at 4^{o}C with anti-GLP-1 receptor (GLP-1R) primary antibody (1:250, affinity purified rabbit polyclonal antibody; Abcam, Cambridge, UK) and a TRITC-conjugated secondary fluorophore (1:250, 2 hrs, 37^{o}C, Jackson Immunoresearch, PA, US). Tissues were dual-labelled with primary antibodies against cholinergic afferent and efferent neurons with calbindin (1:300, mouse, Swant, Bellinzona, Switzerland) and calretinin (1:300, goat, Swant), respectively. Neuronal nitric oxide synthase (nNOS, 1:300, goat, Abcam) was used to label nitrergic inhibitory efferent neurons. The glial cell marker, S100 (1:300, mouse; Sigma-Aldrich, St. Louis, MO), the presynaptic marker, synapsin I (1:200, Santa Cruz Biotechnology) and the post-synaptic marker, PSD-95 (1:200, Santa Cruz Biotechnology, TX, USA) were used to localize GLP-1 receptors in the neurons. These cell markers were identified using species-specific FITC-conjugated fluorophores (1:250, 2 hrs, 37^{o}C, Jackson Immunoresearch). Tissue was mounted on glass slides (VWR, Dublin 15, Ireland) using Dako-fluorescent mounting medium (Agilent Pathology Solutions, Santa Clara, California, USA) and a coverslip placed over the tissue. Antibody controls were performed by incubating LMMP tissue with primary antibodies in the absence of secondary fluorophores and fluorophores in the absence of the primary antibody. The GLP-1R antibody was incubated in the presence of excess GLP-1 protein and subsequently applied to the LMMP tissue. No non-specific staining was evident in any controls. At least three different tissue preparations from three different animals were used in each experiment. Images were captured using Olympus D71 upright fluorescent microscope and Cell F software (Soft Imaging Solutions) or FV1Oi-Olympus-confocal microscope with Fluoview software.

Gut Bath electrophysiology

Colonic tissue with the mucosa removed was suspended transversely or longitudinally from a tension transducer under 1g of tension in Krebs saline, in a water-jacketed tissue bath

maintained at 37.5ºC and allowed to equilibrate for up to an hour. Colonic sections were stimulated with the cholinergic agonist, carbachol (100μM, Sigma-Aldrich, 5 min) at the beginning and at the end of each protocol to stimulate a maximal contractile response and ensure no decline in muscle responsiveness was occurring over time. Baseline contractile activity was recorded for 10 minutes prior to addition of pharmacological reagents. Contractile changes in isolated muscle strips were recorded via a mechanical transducer and Powerlab system and LabChart7 (all AD instruments Inc, Colorado Springs, CO, USA). LabChart7 was used to measure the area under the curve (AUC) of colonic contractions. The area under the rectified trace was calculated by computing the integral of the raw data.

Materials

Pharmacological reagents used in these studies include carbachol (Sigma Aldrich), CRF (Abcam); Ex-4 (Abcam), exendin (9-39) (Santa Cruz Biotechnology), PD98059 (Tocris Bio-Techne, Abingdon, UK); tetrodotoxin (Tocris), thapsigargin (Sigma Aldrich); UO126 (Tocris); wortmannin (Sigma Aldrich); ω-agatoxin IVA (Tocris) and ω-conotoxin GVIA (Tocris).

Statistical Analyses

Data sets were normally distributed (assessed using the D'Agostino-Pearson normality test) and presented as box and whisker plots (5-95 percentile). Data was analyzed using GraphPad prism for windows (version 7) and compared using paired two-tailed t-tests or repeated measures ANOVA with Tukey post hoc test, where appropriate. P values of ≤ 0.05 were considered significant.

Results:

GLP-1 receptor agonist, Exendin-4 excites myenteric neurons

Myenteric ganglia in LMMP tissue preparations from the distal colon of Sprague Dawley rats expressed GLP-1Rs (figure 1A, red staining). Receptor expression appeared to be primarily localized to the plasma membrane of some, but not all neurons within the ganglia, with strong expression in nerve fibers. Immunofluorescent examination of the subtypes of myenteric neurons which express GLP-1Rs revealed that 25% (n=24/98) of calretinin-stained cholinergic efferent neurons expressed GLP-1Rs. 41% (n=12/29 neurons) of calbindin-stained cholinergic afferent neurons expressed GLP-1Rs and 16% (n=10/63 neurons) of nNOS-stained inhibitory neurons co-expressed GLP-1Rs. GLP-1R expression was not observed in S100-labelled glial cells in colonic myenteric ganglia (figure 1A). Given the punctate pattern of GLP-1R labelling, we investigated whether GLP-1Rs were clustered at synapses, where they may influence neuronal excitability. Dual-labelling of neurons with anti-GLP-1R and anti-synapsin I, indicated pre-synaptic expression of GLP-1Rs. Anti-PSD-95 labelling indicated GLP-1Rs also appeared to be expressed post-synaptically (indicated by arrows, n=9 ganglia, figure 1B).

Consistent with expression of GLP-1Rs on myenteric neurons, exposure of LMMP preparations to Ex-4 resulted in an increase in intracellular calcium ($[Ca^{2+}]_i$) in neuronal cell bodies. A concentration response protocol was carried out with 100nM, 1µM, 10µM and 100µM Ex-4 (3 min, figure 1C). All concentrations tested induced a calcium response but the peak amplitude was evoked by 10µM Ex-4 (0.06 ±0.015 (mean ±SD)), which was reproducible on second application (0.04 ±0.015 (mean ±SD), n=47 neurons from 3 rats). Thus, the remainder of experiments were carried at this concentration. Ex-4 (10µM, 3min) increased somatic calcium concentrations in 76% (47/62 neurons, n=3 rats) of ganglionic

myenteric neurons. The calcium response generated had a latency of 15±10 seconds and was abolished in the presence of exendin (9-39) (Ex(9-39), 1μM, 10 min), a GLP-1R antagonist, ($p<0.001$, n=25 neurons from 3 rats, figure 1D). The voltage-gated Na^+ channel blocker, tetrodotoxin (100nM, 30 min) similarly inhibited the evoked response ($p<0.01$, n=16 neurons from 3 rats, figure 2E).

Ex-4-evoked calcium release is mediated by activation of PI 3-kinase and ERK-MAPK

To investigate the role of intracellular signaling pathways in the GLP-1-evoked response, pharmacological inhibitors of key intracellular signaling molecules were used. The Phosphoinositide 3-kinase (PI 3-kinase) inhibitor, wortmannin (1μM, 20 mins), attenuated the Ex-4-evoked increase in $[Ca^{2+}]_i$ (n=13 neurons from 3 rats, $p<0.05$, figure 2A). Investigation of the extracellular-signal-regulated kinase /mitogen activated protein kinase (ERK/MAPK) pathway was carried out using two pharmacological inhibitors. PD98059 (10μM, 30 mins) inhibited the Ex-4-evoked calcium response (n=21 neurons from 3 rats, $p<0.01$, figure 2B). Using an altered protocol due to a two-hour incubation for UO126 (10μM), responses to Ex-4 in control tissue were compared to Ex-4 responses in tissue incubated with UO126. The responses evoked by Ex-4 were suppressed in the presence of UO126 (n=28 neurons, from 3 rats, $p<0.001$, figure 2C).

Ex-4 stimulates calcium influx into myenteric neurons via VGCCs

A pharmacological approach was used to investigate the source of intracellular calcium evoked by the GLP-1R agonist. Given the continuous presence of nifedipine (1μM) in all calcium imaging experiments to suppress smooth muscle activity, Ex-4-mediated responses appear to occur independently of L-type voltage gated calcium channels (VGCCs). However, the Ex-4 evoked calcium response was abolished by ω-agatoxin IVA (100nM, $p<0.001$, n=

20 neurons from 3 rats, figure 3A) and inhibited by the N-type calcium channel blocker, ω-conotoxin GVIA (100nM, $p<0.001$, n=14 neurons from 3 rats, figure 3B). When extracellular calcium was removed using a calcium free saline buffer, and Ex-4 was re-applied, the calcium response was suppressed (n=13 neurons, 3 rats, $p<0.001$), although a large amplitude peak was still evident (figure 3C). The peak may reflect release from intracellular stores. Indeed, incubation of the LMMP tissue with the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin (100nM, 30 minutes) in Ca^{2+}-containing saline, reduced but did not abolish the amplitude of the Ex-4-evoked activation of myenteric neurons (n=26 neurons, 3 rats, $p>0.001$, figure 3D).

Ex-4 modifies colonic circular and longitudinal smooth muscle activity

To assess the effects of Ex-4 on colonic smooth muscle activity, tissue sections were suspended in organ baths such that contractions of circular and longitudinal muscle could be recorded. Control maximal contractile responses were evoked by addition of the cholinergic agonist, carbachol (100μM, 5 min) at the beginning and end of the experiment. No differences in the amplitude of the maximal contractions in either circular or longitudinal muscle were noted ($p>0.05$, n=5). Basal circular muscle contractile activity in the distal colon of Sprague Dawley rats exhibited consistent tone with sustained contractions of regular amplitude and frequency. Addition of Ex-4 (10μM) caused modifications in the pattern of circular muscle contractile activity, resulting in a decrease in frequency ($p<0.05$) but no significant change in the mean amplitude of contractions ($p>0.05$, figure 4A). When the area under the curve (AUC), which was used to incorporate both the amplitude and frequency of contractions, was examined, Ex-4 decreased the AUC of circular muscle contractions in the majority of colonic tissue sections examined (n=8 out of 10, $p<0.05$). Incubation of the colonic tissue with the voltage-gated Na^{+} channel blocker, tetrodotoxin (TTX, 100nM), prior

to the addition of Ex-4 reversed the Ex-4-evoked decrease in AUC in circular muscle ($p=0.055$, n=4, figure 4C). TTX did not impact on the amplitude of contractions ($p>0.05$) but blocked the Ex-4-mediated decrease in frequency. When the colonic tissue was longitudinally orientated, the regular contractions observed at baseline were also modified by application of Ex-4 (figure 4B), although the response was more variable. All sections exhibited a change in tone which was transient in some tissue preparations and sustained in others. However, 7 of the 11 sections exhibited an increase in the AUC ($p<0.05$. n=7, figure 1B(i)), although no significant changes in amplitude ($p>0.05$) or contraction frequency ($p>0.05$) were detected. Four sections exhibited a decrease in AUC ($p<0.05$, n=4, figure 1B(ii)), with a significant decrease in amplitude of contractions ($p<0.01$) but no effect on contraction frequency ($p>0.05$). TTX did not impact on the variable response evoked by Ex-4 in longitudinal muscle when AUC ($p>0.05$, n=5, figure 4D), amplitude ($p>0.05$) or frequency of contractions ($p>0.05$) were compared.

CRF modifies Ex-4 evoked neuronal and functional responses.

CRF binds with highest affinity to CRF1 receptors (CRFR1), which we found to be expressed on colonic myenteric ganglia (figure 5A). To investigate potential crosstalk between CRF and GLP-1 in colonic myenteric neurons, ratiometic calcium imaging was carried out. Both Ex-4 (10µM) and CRF (100nM) evoked calcium responses in myenteric neurons, although the amplitude of the Ex-4-evoked response was larger (n=60 neurons, $p<0.001$). Co-application of both hormones had a modest additive effect on the calcium response in neurons ($p<0.001$, figure 5B). In organs baths, AUC of circular muscle contractile activity was not modified by co-application of Ex-4 and CRF (One-way ANOVA: $p>0.05$, n=4, figure 5C). The peak amplitude ($p>0.05$) and frequency ($p>0.05$) of contractions were unchanged when both hormones were co-applied.

Discussion

In order to understand normal physiological mechanisms that may be impaired in functional bowel disorders, such as IBS, this study has examined the neuromodulatory effects of GLP-1 in healthy Sprague Dawley rat colons. We have established that the GLP-1R agonist, Ex-4 induces an increase in $[Ca^{2+}]_i$ in some but not all myenteric neurons and this translates to changes in colonic contractile activity. Consistent with previous reports of suppression of colonic contractile activity by peripherally applied GLP-1 [25], we found that Ex-4 decreased contractile activity in *ex vivo* colonic circular muscle and this was inhibited by the neurotoxin, TTX, implicating neuronal regulation. In terms of neuronal cell biology, our studies found that activation of G-protein-coupled GLP-1 receptors on colonic myenteric neurons induced activation of both PI 3-kinase and ERK-MAPK intracellular signaling cascades. The increase in $[Ca^{2+}]_i$ was due to both an influx of extracellular Ca^{2+} through VGCCs and release from intracellular stores. We have previously reported that the stress hormone, CRF evokes stimulatory effects in colonic myenteric neurons, with associated changes in bowel function [34] however, at least in healthy tissue, co-application of Ex-4 and CRF did not further impact on circular muscle activity.

Functionally, myenteric neurons regulate smooth muscle contractile activity. We determined that exposure of colonic smooth muscle to Ex-4 resulted in disruption to the synchronicity of circular muscle contractile activity. Consistent with reports that GLP-1 inhibits colonic transit [35], Ex-4 suppressed circular muscle contractile activity with more frequent contractions of smaller amplitude but with little change in smooth muscle tone. Ex-4-mediated modulation of colonic function was attenuated by TTX, indicating neuronal regulation of this effect. In colonic longitudinal muscle, modulation of contractile activity by Ex-4 was more variable. Basal contractile activity in some colonic sections was suppressed as evidenced by a

reduction in tone and amplitude of contractions. Other tissue samples responded to Ex-4 with increased tone and more frequent, but smaller amplitude contractions. It is not clear why the responses are divergent although in mouse proximal colon, GLP-1 was effective in suppressing electrically stimulated contractions in circular muscle but did not modify longitudinal muscle function [24]. The insensitivity to TTX suggests that this unlikely to be due to differences in neuronal regulation.

We detected GLP-1R expression in subsets of neurons in Sprague Dawley rat colonic myenteric ganglia, which supports evidence from other studies in rodents [9,24], monkeys and humans [36]. Punctate GLP-1R expression was evident on neuronal cell bodies and nerve fibers but was absent from ganglionic glial cells. Just 16% of nNOS expressing neurons, a marker largely restricted to inhibitory motor neurons, were positive for GLP-1R expression, an observation consistent with reports in GLP-1R-cre mice with fluorescent reporters [23], and somewhat lower than that observed in the proximal colon [24]. In contrast to reports that GLP-1R mRNA is primarily found in nNOS positive enteric neurons [23,24], albeit more proximal in the GI tract, we found that in the distal colon a higher proportion of neurons labelled with markers for cholinergically-mediated efferent and afferent neurons, calretinin (25%) and calbindin (41%) co-expressed GLP-1Rs. This is broadly similar to findings in mice with fluorescently-tagged GLP-1Rs [23] and as such, supports the specificity of the anti-GLP-1R antibody used in our study. However, it may be at odds with the inhibitory effects of GLP-1 on GI transit, which is proposed to be mediated through release of NO [24,25]. That said, stimulation of myenteric neurons does not necessarily result in increased transit [37].

The punctate pattern of GLP-1R expression prompted us investigate if receptors were localized at synapses, where they could influence synaptic transmission. Similar to pre- [38] and

post-synaptic [39] effects of GLP-1 reported in central neurons, we found that myenteric neurons expressed GLP-1Rs at both pre- and post-synaptic neuronal sites and responded in a TTX-sensitive manner to GLP-1 with an increase in $[Ca^{2+}]_i$. This finding is consistent with increased action potential frequency in GLP-1 expressing cultured myenteric neurons exposed to GLP-1 [23]. In pancreatic β-cells, binding of GLP-1 to the seven transmembrane spanning G-protein coupled GLP-1R results in increased $[Ca^{2+}]_i$ [40] and activation of MAPK-ERK [41] and the PI 3-kinase downstream signaling cascades [42]. Calcium responses evoked by Ex-4 in myenteric neurons are similarly dependent upon MAPK-ERK and PI 3-kinase signaling pathways. Whilst GLP-1 stimulates activation of ERK through an influx of calcium through L-type VGCCs in pancreatic β cells [43], in myenteric neurons, we found that nifedipine had no impact on Ex-4-evoked calcium responses. However, complete inhibition of Ex-4-evoked calcium responses with ω-agatoxin IVA, a voltage-dependent blocker of P/Q calcium channels, and partial inhibition by ω-conotoxin GVIA, an N-type calcium channel blocker, suggests that influx of calcium through these VGCCs may be important in the activation of downstream signaling molecules in myenteric neurons. Activation of GLP-1Rs is also reported to induce release of sequestered calcium from intracellular stores in insulin secreting cell lines [44] and in myenteric neurons, we found that the calcium response evoked by Ex-4 was reduced but not eliminated in the absence of extracellular calcium and the SERCA Ca^{2+} ATPase inhibitor, thapsigargin, indicating the involvement of intracellular stores in addition to influx from extracellular sources.

Studies in the hypothalamus demonstrated GLP-1-IR terminals established synapses with CRF neurons, thus providing a neuromodulatory pathway for activation of the stress axis (28), which is a key feature of IBS symptom flares. However, we have previously shown that CRF can have direct neuromodulatory effects on enteric neurons [45,46], and stimulate circular

muscle contraction, an effect that was potentiated by the presence of interleukin-6 and interleukin-8 [45], pro-inflammatory cytokines that are elevated in IBS patient serum [13]. In circular muscle, where GLP-1 suppresses contractile activity using neutrally-regulated mechanisms, we found that the presence of CRF had no impact on this response, despite a modest increase in the amplitude of calcium responses in myenteric neurons evoked by combined exposure to CRF and Ex-4. Thus, in a healthy animal model, the functional effects of Ex-4, in terms of suppressing circular muscle contraction, are not altered in the presence of this stress hormone. Future research will determine if this relationship is maintained in animal models of IBS, where colonic expression of both CRF receptors [11] and GLP-1Rs [9] are modified.

Understanding the neuromodulatory and functional effects of GLP-1 in the distal colon is of particular interest in the context of functional bowel disorders such as IBS and other disorders where luminal microbial profiles are altered, and may thereby result in altered colonic GLP-1 secretion [33]. In a healthy model, we have determined that activation of GLP-1Rs on myenteric neurons results in increased $[Ca^{2+}]_i$ and activation of PI 3-kinase and MAPK signaling cascades. Functionally, Ex-4 modifies smooth muscle contractile activity, although only Ex-4-evoked suppression of circular muscle contractility was sensitive to the neurotoxin, TTX. The stress hormone, CRF, which mimics bowel symptoms of IBS [47], did not impact on the functional effects of Ex-4 in circular smooth muscle. However, repeating these studies in an animal model of IBS may find that this relationship is changed.

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Contributions:

RO'B: Performed and analyzed the research.

M.B.: Performed and analyzed the research.

A.K.: Performed the research.

D.O'M: Designed the research study, wrote the paper, sourced funding.

No competing interests declared.

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Figure Legends

Figure 1: Exendin-4 stimulates colonic myenteric neurons

A: The dual-labelled immunofluorescent images of myenteric ganglia from the distal colon of Sprague Dawley rats show GLP-1 receptor (GLP-1R) expression (red staining) in calbindin (n=12 ganglia, green staining), calretinin (n=17 ganglia, green staining) and nNOS-labelled neurons (n=13 ganglia, green staining). GLP-1Rs were not detected in S100 stained glial cells (n=16 ganglia, green staining). B: The punctate pattern of GLP-1R expression co-localized with the pre-synaptic marker, synapsin I and the post-synaptic marker, PSD-95 (indicated by arrows in the magnified regions). Scalebar: 20μm. C: The box and whiskers graph and representative trace illustrate the calcium responses in colonic myenteric neurons to increasing concentrations of the GLP-1 receptor agonist, exendin-4 (Ex-4, n=47) D: The box and whiskers graph and representative trace show that the Ex-4-evoked calcium responses is attenuated by a GLP-1R antagonist, exendin (9-39) (10μM, n=25) and E: tetrodotoxin (TTX, 100nM, n=16). ** and *** indicate $p<0.01$ and $p<0.001$, respectively.

Figure 2: Ex-4 stimulates intracellular signaling cascades.

A: The box and whisker plots and representative traces show that the PI 3-kinase inhibitor, wortmannin (1μM) inhibits the Ex-4-evoked calcium response. B: The ERK-MAPK inhibitors, PD98059 (10μM) and C: UO126 10μM) abolish the Ex-4-evoked calcium response. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Figure 3: Ex-4 evoked calcium responses are mediated by VGCCs.

A: The P and Q type voltage gated calcium channel inhibitor, ω-agatoxin IVA (100nM, n=20) and the B: N-type calcium channel blocker, ω-Conotoxin GVIA (100nM, n=14) inhibited the Ex-4-evoked calcium response in myenteric neurons as shown in the box and

whiskers charts and representative traces. C: The calcium response evoked by Ex-4 is attenuated but not abolished in the absence of extracellular calcium (n=13). D: Thapsigargin (1μM), the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor, reduced but did not abolish the Ex-4-evoked calcium response (n=26). *** indicates $p<0.001$.

Figure 4: Ex-4 modifies circular and longitudinal colonic contractile activity.

A: The box and whisker plot and representative trace illustrates the Exendin-4 (Ex-4, 10μM)-mediated change in colonic contractile activity in circular muscle from Sprague Dawley colons (n=8). B: A subset of colonic longitudinal muscle tissue responded to Ex-4 with (i) an increase in area under the curve (n=7) whereas the remainder (ii) responded with a decrease (n=4). C: The box and whisker plot shows the partial reversal of Ex-4-evoked suppression of colonic circular muscle activity in the presence of tetrodotoxin (TTX, n=4). D: TTX had no impact on the actions of Ex-4 on longitudinal muscle activity (n=5). * indicates $p<0.05$.

Figure 5: Corticotropin-releasing factor does not modify the functional effects of Ex-4.

A: The representative immunofluorescent image shows that CRF-1 receptor expression in myenteric ganglia. Scalebar: 20μm. B: The box and whisker plot with representative trace illustrate activation of colonic myenteric neurons by Ex-4 and CRF. When Ex-4 and CRF are co-applied it results in a larger response than when either are applied alone (n=60). C: The box and whisker plot and representative trace show that colonic contractile activity in circular smooth muscle was not significantly changed when CRF was co-applied with Ex-4 (n=4). *** indicates $p<0.001$.

Figure 1

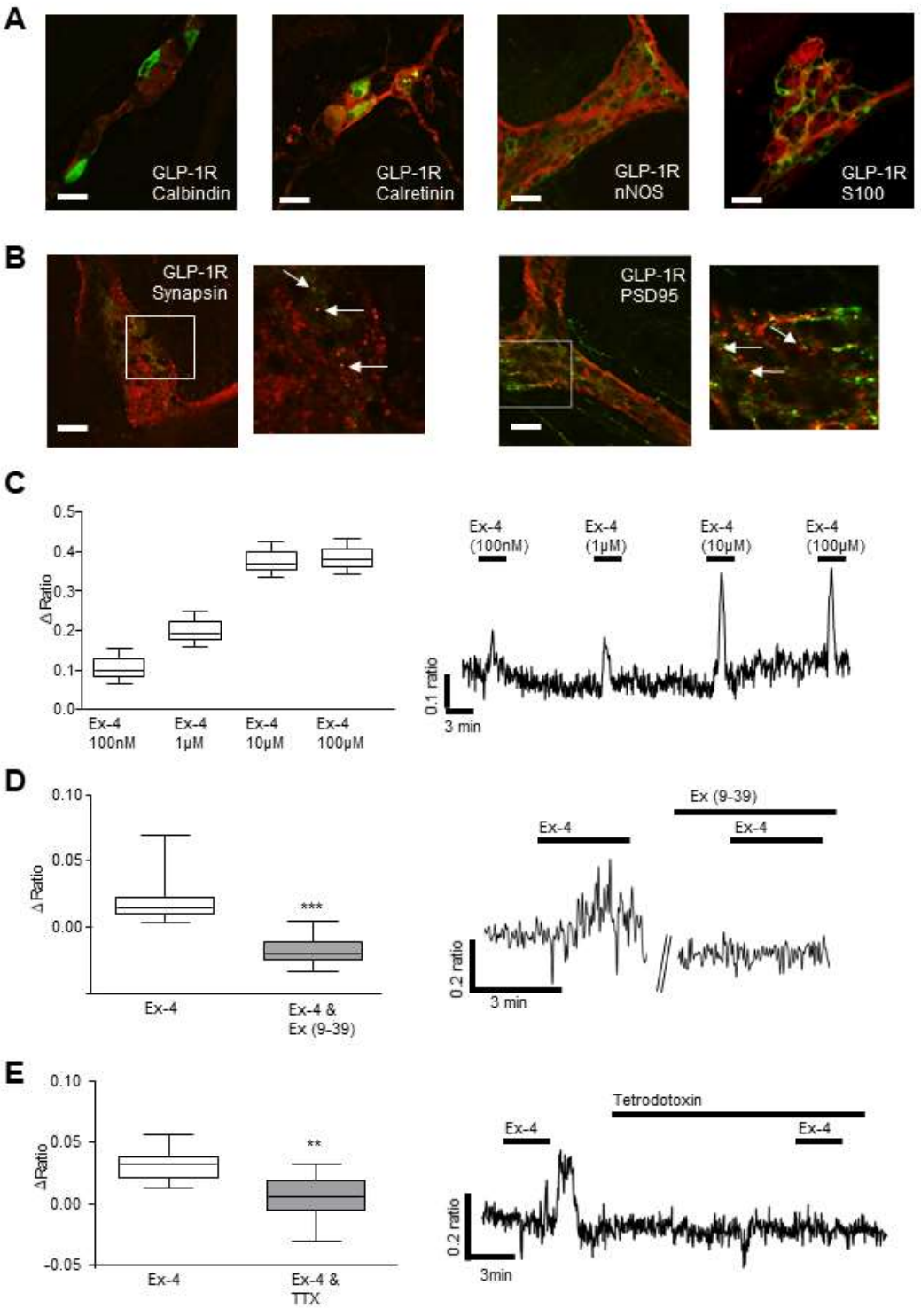

A
GLP-1R
Calbindin
GLP-1R
Calretinin
GLP-1R
nNOS
GLP-1R
S100
B
GLP-1R
Synapsin
GLP-1R
PSD95
C
Δ Ratio
0.5
0.4
0.3
0.2
0.1
0.0
Ex-4
100nM
Ex-4
1μM
Ex-4
10μM
Ex-4
100μM
Ex-4
(100nM)
Ex-4
(1μM)
Ex-4
(10μM)
Ex-4
(100μM)
0.1 ratio
3 min
D
Δ Ratio
0.10
0.05
0.00
***
Ex-4
Ex-4 &
Ex (9-39)
Ex (9-39)
Ex-4
Ex-4
0.2 ratio
3 min
E
ΔRatio
0.10
0.05
0.00
-0.05
**
Ex-4
Ex-4 &
TTX
Tetrodotoxin
Ex-4
Ex-4
0.2 ratio
3min

Figure 2

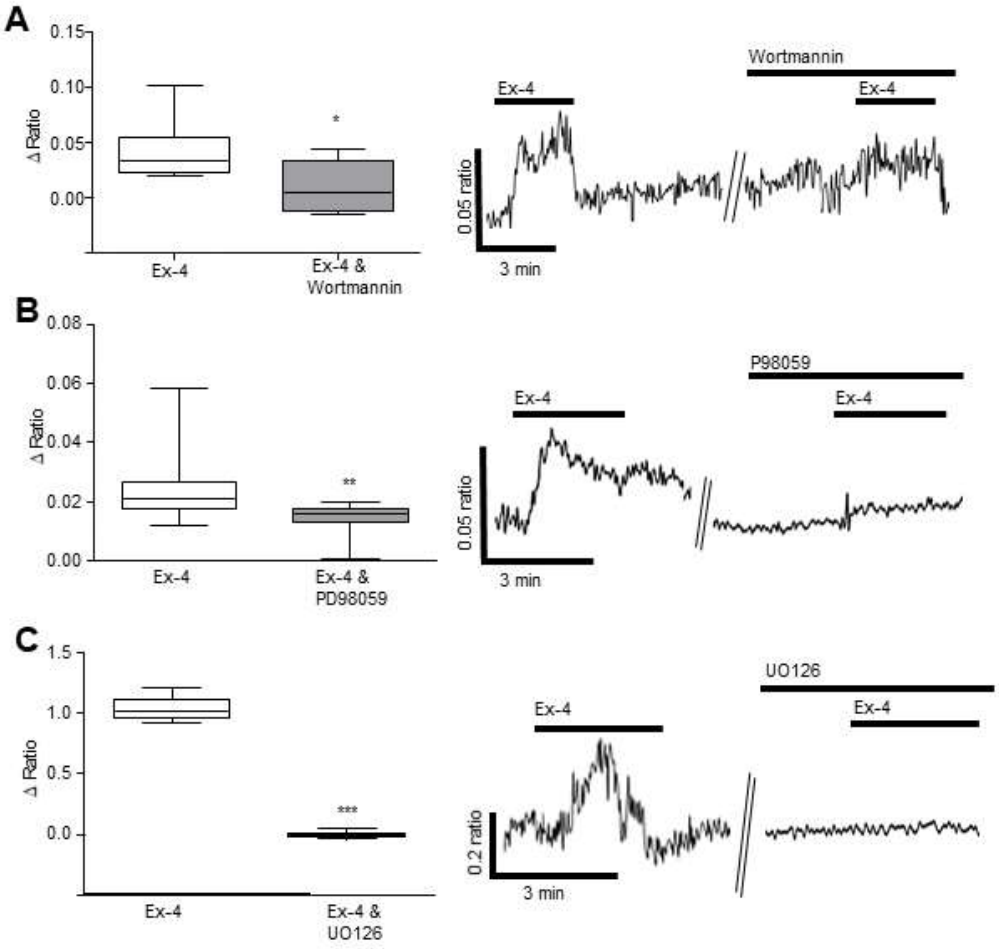

A
0.15
0.10
0.05
0.00
Δ Ratio
*
Ex-4
Ex-4 &
Wortmannin
Wortmannin
Ex-4
Ex-4
0.05 ratio
3 min
B
0.08
0.06
0.04
0.02
0.00
Δ Ratio
**
Ex-4
Ex-4 &
PD98059
P98059
Ex-4
Ex-4
0.05 ratio
3 min
C
1.5
1.0
0.5
0.0
Δ Ratio
***
Ex-4
Ex-4 &
UO126
UO126
Ex-4
Ex-4
0.2 ratio
3 min

Figure 3

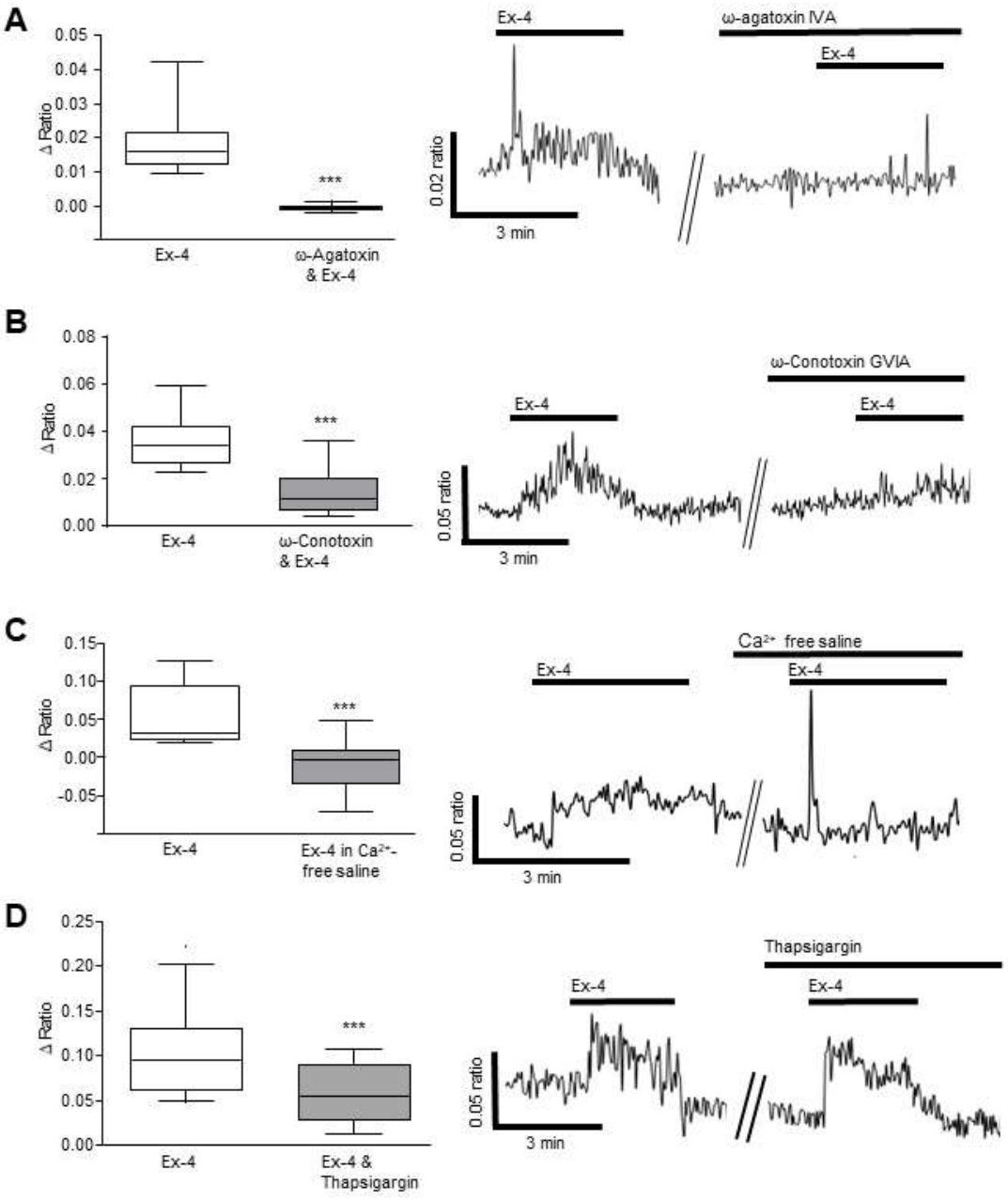

A
Δ Ratio
0.05
0.04
0.03
0.02
0.01
0.00
***
Ex-4
ω-Agatoxin & Ex-4
Ex-4
ω-agatoxin IVA
Ex-4
0.02 ratio
3 min
B
Δ Ratio
0.08
0.06
0.04
0.02
0.00
***
Ex-4
ω-Conotoxin & Ex-4
ω-Conotoxin GVIA
Ex-4
Ex-4
0.05 ratio
3 min
C
Δ Ratio
0.15
0.10
0.05
0.00
-0.05
***
Ex-4
Ex-4 in Ca2+-free saline
Ca2+ free saline
Ex-4
Ex-4
0.05 ratio
3 min
D
Δ Ratio
0.25
0.20
0.15
0.10
0.05
0.00
***
Ex-4
Ex-4 & Thapsigargin
Thapsigargin
Ex-4
Ex-4
0.05 ratio
3 min

Figure 4

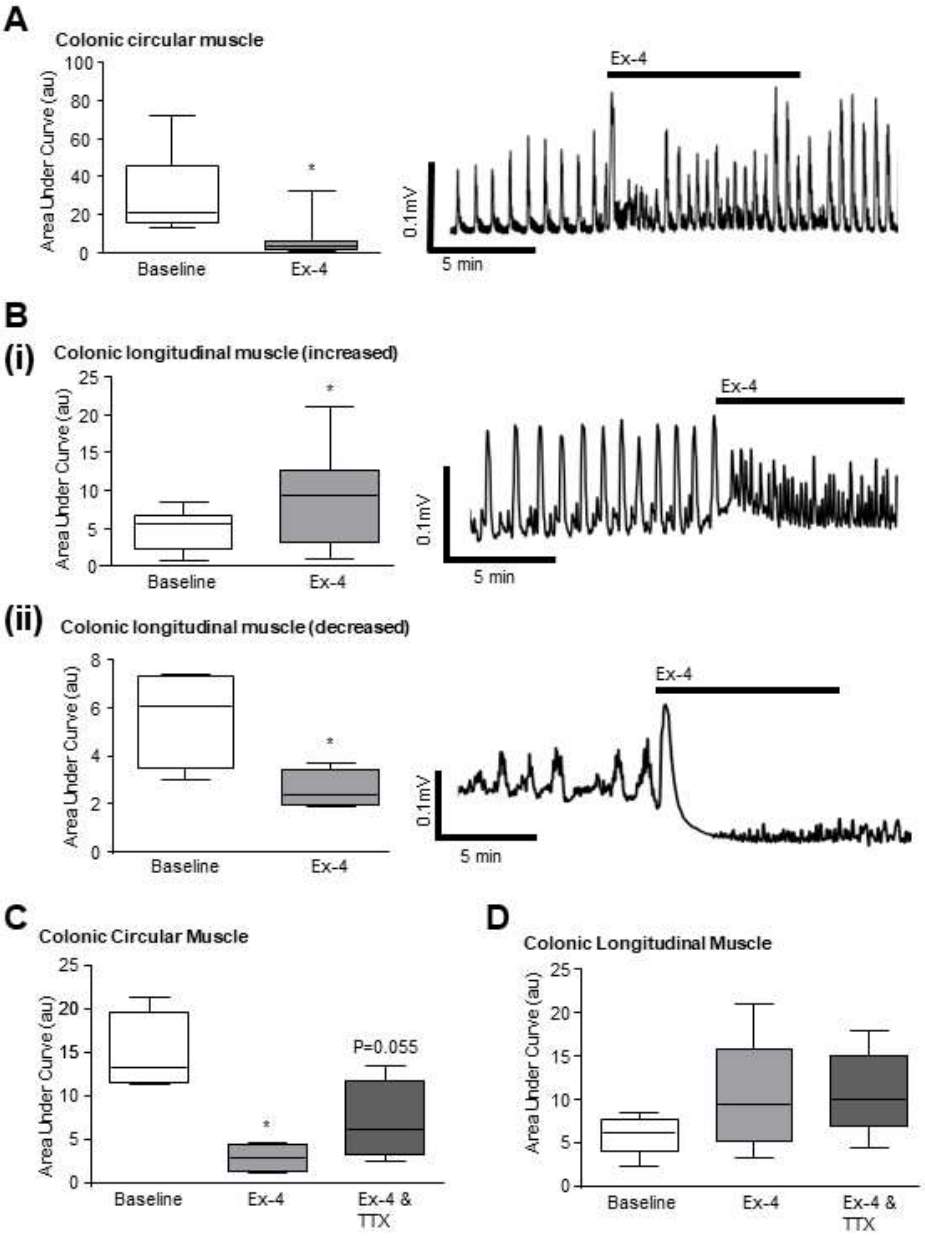
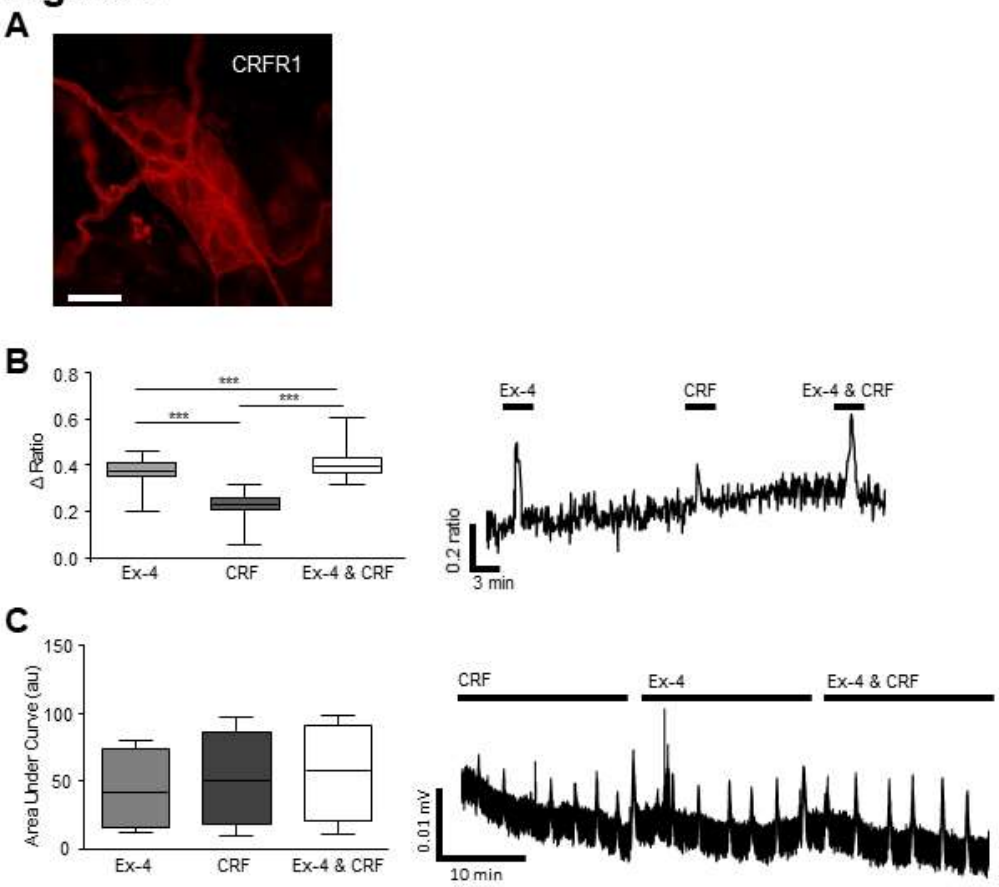

A
Colonic circular muscle
Area Under Curve (au)
100
80
60
40
20
0
*
Baseline
Ex-4
Ex-4
0.1mV
5 min
B
(i)
Colonic longitudinal muscle (increased)
Area Under Curve (au)
25
20
15
10
5
0
*
Baseline
Ex-4
Ex-4
0.1mV
5 min
(ii)
Colonic longitudinal muscle (decreased)
Area Under Curve (au)
8
6
4
2
0
*
Baseline
Ex-4
Ex-4
0.1mV
5 min
C
Colonic Circular Muscle
Area Under Curve (au)
25
20
15
10
5
0
*
P=0.055
Baseline
Ex-4
Ex-4 & TTX
D
Colonic Longitudinal Muscle
Area Under Curve (au)
25
20
15
10
5
0
Baseline
Ex-4
Ex-4 & TTX

Figure 5


A
CRFR1
B
Δ Ratio
0.8
0.6
0.4
0.2
0.0
***
***
***
Ex-4
CRF
Ex-4 & CRF
Ex-4
CRF
Ex-4 & CRF
0.2 ratio
3 min
C
Area Under Curve (au)
150
100
50
0
Ex-4
CRF
Ex-4 & CRF
CRF
Ex-4
Ex-4 & CRF
0.01 mV
10 min