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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

GHSR-1 agonist sensitizes rat colonic intrinsic and extrinsic neurons to exendin-4: a role in the manifestation of post-prandial gastrointestinal symptoms in Irritable Bowel Syndrome?

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Abstract

Background

Irritable Bowel Syndrome (IBS) patients may experience post-prandial symptom exacerbation. Nutrients stimulate intestinal release of glucagon-like peptide 1 (GLP-1), an incretin hormone with known gastrointestinal effects. However, prior to the post-prandial rise in GLP-1, levels of the hunger hormone, ghrelin, peak. The aims of this study were to determine if ghrelin sensitizes colonic intrinsic and extrinsic neurons to the stimulatory actions of a GLP-1 receptor agonist, and if this differs in a rat model of IBS.

Methods

Calcium imaging of enteric neurons were compared between Sprague Dawley and Wistar Kyoto rats. Colonic contractile activity and vagal nerve recordings were also compared between strains.

Key Results

Circulating GLP-1 concentrations differ between IBS subtypes. Mechanistically, we have provided evidence that calcium responses evoked by exendin-4, a GLP-1 receptor agonist are potentiated by a ghrelin receptor (GHSR-1) agonist, in both submucosal and myenteric neurons. Although basal patterns of colonic contractility varied between Sprague Dawley and Wister Kyoto rats, the capacity of exendin-4 to alter smooth muscle function was modified by a GHSR-1 agonist in both strains. Gut-brain signaling via GLP-1 mediated activation of vagal afferents was also potentiated by the GHSR-1 agonist.

Conclusions & Inferences

These findings support a temporal interaction between ghrelin and GLP-1, where the pre-prandial peak in ghrelin may temporarily sensitize colonic intrinsic and extrinsic neurons to the neurostimulatory actions of GLP-1. Whilst the sensitizing effects of the GHSR-1 agonist

was identified in both rat strains, in the rat model of IBS, underlying contractile activity was aberrant.

Key points:

- Many patients with Irritable Bowel Syndrome report exacerbation of symptoms following food intake, however the mechanisms underlying this phenomenon are unclear.
- Brief exposure to the ghrelin receptor agonist potentiated neuronal activation in colonic enteric neurons and vagal afferents by a GLP-1 mimetic. Colonic contractile activity was differentially modified in an animal model of Irritable Bowel Syndrome.
- In a rat model of gastrointestinal dysfunction, sensitization of colonic intrinsic and extrinsic neurons to the neurostimulatory actions of GLP-1 may potentiate IBS-like symptoms.

Keywords: colonic contractility, ghrelin, glucagon-like peptide 1, myenteric, vagal.

Ghrelin, a stomach-derived orexigenic hormone plays a key role in whole-body energy metabolism and is often termed the 'hunger hormone'. However, in addition to regulating this basic survival instinct, ghrelin also modifies gut motility and visceral pain sensitivity through its actions on intrinsic myenteric neurons and extrinsic vagal and pelvic nerves, respectively[1]. Interestingly, ghrelin-mediated modulation of myenteric neuronal activity is dependent on fasting state. Larger calcium responses are generated in the myenteric neurons of fasted guinea pigs as compared to re-fed animals, results which indicate the importance of neuro-endocrine regulation of satiety and satiation[2]. Glucagon-like peptide-1 (GLP-1) is a gut-derived hormone important in satiety. Released from intestinal L-cells in response to the arrival of nutrients in the small intestinal lumen[3], this incretin hormone enhances glucose-stimulated insulin biosynthesis and secretion. In the gastrointestinal (GI) tract the effects of GLP-1 vary with location. Gastric emptying, the migrating motor complex and small intestinal secretion are inhibited by GLP-1[4], in contrast to its effects in the colon, where a vagally-mediated increase in transit resulted from central administration of GLP-1[5]. GLP-1-mediated changes in motility have been observed in both healthy controls and in patients with Irritable Bowel Syndrome (IBS)[6-8].

IBS is a common[9], heterogeneous functional bowel disorder, characterized by abdominal pain and episodes of either diarrhea, constipation, or both[10]. Miscommunication in the brain-gut axis is thought to underlie the pathophysiology, with alterations in neuroendocrine signaling being implicated[11]. Clinical interventions in IBS patients demonstrated that a GLP-1 mimetic had anti-spasmodic and pain-relieving properties[12] and may have therapeutic efficacy in constipation-predominant IBS (IBS-C)[13]. A prevalent feature of the disorder is post-prandial exacerbation of symptoms[14-16], making hunger- and satiety-related hormones an area of interest in understanding IBS pathophysiology.

Ghrelin can enhance the post-prandial nutrient-evoked increase in GLP-1 secretion[17] and has been proposed as a potential incretin enhancer therapy[18]. This is consistent with the temporal profile of circulating ghrelin, which peaks with hunger prior to a meal[19], and GLP-1, which peaks 10-15 minutes following a meal[20]. However, GLP-1 receptor (GLP-1R)-expressing neurons are found in both neuronal plexi of the enteric nervous system throughout the GI tract[21], including the colon.

Vagal and spinal visceral afferents provide a constant stream of interoceptive information to the central nervous system. Unlike healthy controls, IBS patients appear to be conscious of these sensory inputs[22], which is interpreted as abdominal discomfort or pain. The vagus nerve has been linked to activation of the hypothalamic-pituitary stress axis, and modification of gut-originating pain signaling, which, in turn, leads to altered gut function[23]. The entire GI tract in rats is innervated by the vagus[24], and the dorsal motor nucleus of the vagus[25] and vagal afferents are sensitive to GLP-1[26]. We hypothesize that ghrelin may sensitize gut intrinsic and extrinsic neurons to the stimulatory effects of GLP-1 and thereby alter GI function. The aims of the study were to determine the importance of GLP-1 and ghrelin in the pathophysiology of bowel dysfunction using the stress-sensitive Wistar Kyoto (WKY) rat model.

Materials and Methods

Ethical approval

All experiments were in full accordance with the European Community Council Directive (86/609/EEC) and the local University College Cork animal ethical committee (#2011/015). Rats were sacrificed by CO_2 overdose and exsanguination.

The protocol for collecting biopsies from the transverse and distal colon with matched serum samples from IBS patients and healthy control volunteers was approved by the University College Cork Clinical Research Ethics Committee (ECM 4(r) 01/03/16) and was carried out in the Mater Private Hospital, Cork, Ireland. Informed consent was obtained from all participants.

Animals and Tissue collecting

Anxiety- and depression-like behaviors, including stress-induced defecation and time spent in the aversive, exposed inner zone of the open-field arena and behavior in the forced swim test are not reported to differ between male and female Sprague Dawley (SD) and Wistar Kyoto (WKY) rats[27]. Moreover, both male[28] and female[29] WKY rats exhibit lower pain threshold to CRD as compared to SD rats. Given the lack of sex difference and to avoid the cyclical hormonal changes, 8-12 week old male SD and WKY rats, purchased from Envigo, Derbyshire, UK, were selected for this study. Rodents were group-housed 5 per cage and maintained on a 12/12 hour light -dark cycle (08.00-20.00) with a room temperature of 22±1^{o}C. Animals were permitted at least a week to acclimatize to their new environment prior to experimentation. Food and water were available *ad libitum* and rats were not fasted prior to tissue collection. In comparison to SD rats, WKY rats exhibit increased sensitivity to stress[30-32]. In several studies where SD rats were used as comparators, WKY rats exhibited a

high anxiety phenotype, visceral hypersensitivity and increased stress-related defecation. This model of brain-gut axis dysfunction has been validated as a rodent model of IBS[32-35].

A section of distal colon was excised from each rat and stored in cold Krebs buffered saline 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). For calcium imaging and immunofluorescence studies, the mucosal layer was removed to expose the submucosal plexus (SMP). To expose myenteric neurons, the circular muscle layers were peeled away using forceps leaving a longitudinal muscle myenteric plexus (LMMP) preparation. Sections of distal colon stripped of the mucosa were used for gut bath studies. The dissection technique used for the colon-vagal nerve tissue preparation is described in detail below.

Human plasma and colon biopsy collection

Patients attending the General Surgery Clinic at the Mater Private Hospital, Cork, Ireland were recruited for the study. Patients aged between 18-65 years of age and able to provide written informed consent were enrolled. Inclusion criteria for IBS patients included confirmed clinical diagnosis of IBS that satisfies the Rome III criteria. Biopsies from age and weight-matched healthy controls were taken from patients undergoing routine colonoscopies that were good health and negative for bowel disease. Exclusion criteria for participation included acute or chronic co-existing illness, recent unexplained bleeding or prior GI surgery (apart from hernia repair and appendectomy), psychiatric disease, immunodeficiency, bleeding disorder, coagulopathy, a malignant disease or any concomitant end-stage organ disease. Subjects were also excluded if they were taking any experimental drugs or if the subject had taken part in an experimental trial less than 30 days prior to this study. Mucosal biopsies from the transverse and distal colons were taken from fasting patients at the same

time as obtaining a matched serum sample. Dipeptidyl peptidase-IV was added to the serum samples to inhibit GLP-1 degradation. Samples were assigned a study number, with the key held by the treating surgeon, so as to preserve patient confidentiality in accordance with the study protocol. The secretory products from biopsies incubated in Dubellco's Modified Eagle Medium (Sigma Aldrich, UK, overnight, 37^{o}C) were used to measure local tissue concentration of GLP-1 and ghrelin. Mucosal biopsies were subsequently fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose and stored at -80°C for later immunofluorescent staining.

Mesoscale Discovery Biomarker Assay

An immunoassay (MesoScale Discovery U-PLEX customized multiplex assay kit I, MesoScale Discovery, Gaithersburg, MD, USA) was carried out to determine the GLP-1 and ghrelin concentration in plasma samples and biopsy supernatants from 6 healthy, 6 IBS-C and 6 IBS-D patients. The assay was run in triplicate and an electrochemiluminescent detection method was used to measure protein levels in the samples. The plates were read using MesoScale Discovery plate-reader (MESO QuickPlex SQ 120). A calibration curve was generated using standards, and GLP-1 and ghrelin concentrations were determined from the curve.

Calcium Imaging

For calcium imaging studies, a LMMP or SMP tissue preparation was pinned out in Sylgard (Sylgard 184 silicone elastomer kit, WPI, Sarasota FL, USA)-lined petri dishes superfused with carbogen-bubbled Krebs saline solution with 1μM nifedipine to inhibit smooth muscle contractions. The tissue was loaded with either Fluo 4 (8μM, 1 hr, Thermo Fisher Scientific, Waltham, MA, USA) or Fura-2AM (Thermo Fisher Scientific 7μM, 1 hr) in the dark and

washed out prior to recording. Cytosolic changes in intracellular calcium ($[Ca^{2+}]_i$) were recorded from neuronal cell bodies loaded with Fluo-4 using WinFluor fluorescence image capture and analysis program (John Dempster, University of Strathclyde, Scotland) or from neurons loaded with Fura-2AM using Cell R software (Olympus Soft imaging solutions, 1986-2009). Images were captured at 2Hz using a Cairn optoscope 1200 (Cairn Research, Kent, UK) or a Xenon/Mercury arc burner (Olympus, Melville, NY, US), a charge-coupled device digital camera (Hamamatsu ORCA-ER, Hamamatsu Photonics, Hertfordshire, UK) and a 40x water-immersion objective on a fixed stage upright microscope (BX51WI, Olympus, South-End-on-Sea, UK).

Ganglionic neurons were identified based on morphology and responsivity to brief exposure to 75mM KCl, which was added at the end of each experiment. Neurons were considered to be responders if fluorescence increased (in 150 seconds from the point of the initiation of the calcium response) by more than two standard deviations from baseline noise values for each neuron. The baseline values were calculated as the average ratio during the 150 seconds preceding the stimulus and compared to the peak amplitude of response (150 seconds). A perfusion system continuously superfused the colonic tissue with carbogen-bubbled Krebs-buffered saline. The tissue was incubated with pharmacological reagents added to the superfusate.

Immunofluorescence and confocal microscopy

Human distal colonic biopsies, fixed in 4% paraformaldehyde (4^{o}C, overnight), were cryo-sectioned (10µm in thickness, Leica Biosystems, Wetzler, Germany) and mounted on glass slides (VWR, Dublin 15, Ireland). Mucosal biopsies were permeabilized with 0.1% Triton X-100 and blocked with 1% donkey serum (Sigma Aldrich, UK) and immunolabelled with

rabbit polyclonal anti-GLP-1 antibody[36] (1:250, overnight 4^{o}C, Abcam, Cambridge, UK). FITC-anti rabbit fluorophore (green staining, 1:250, 2 hrs, 37^{o}C, Jackson Immunoresearch, PA, US) was used to visualize GLP-1 expression. Tissue sections were mounted using Dako-fluorescent mounting medium containing DAPI (Agilent Pathology Solutions Santa Clara, California, USA) and a coverslip placed over all tissue. Images were captured using a FVl0i-Olympus-confocal microscope with Fluoview software (FV10i-SW, Olympus Europe, Hamburg, Germany). No non-specific fluorescence was detected in control experiments using a GLP-1 binding peptide prior to tissue exposure as compared to a positive control. Controls for the secondary fluorophore (tissues were incubated with primary antibody in the absence of secondary antibody or the secondary antibody was applied alone) similarly showed no non-specific immunofluorescent staining.

Extracellular recording from colonic vagal afferents

This dissection has been described previously in detail[37]. In brief, a vertical abdominal incision was made below the sternum to expose the intestine with intact peripheral nerves. The esophagus with intact posterior vagus and its branches were excised. An *ex vivo* preparation of the distal colon with intact inferior and superior mesenteric and coeliac ganglia and the vagus nerve was excised from the abdominal cavity of SD or WKY rats. Vagal nerve activity was recorded superior to the stomach near the attached portion of the esophagus. The nerve-gut recording rig consisted of two Sylgard-lined Perspex chambers. The colon chamber (2.5cm x 10cm) was separated from the adjacent nerve chamber (1.5cm x 10cm) by a 2mm barrier. Both chambers were superfused with 5% CO_2/95 % O_2 bubbled Krebs saline comprised of (in mmolL^{-1}): NaCl, 117; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2 and D-glucose maintained at 37^{o}C. The colonic tissue was opened at the mesenteric border and pinned out, mucosal side up. The serosal side of the tissue was

continuously irrigated with perforated plastic tubing embedded in the Sylgard. To expose myenteric neurons, the mucosal and submucosal layers were removed and the circular muscle fibers were peeled away using fine dissection forceps. To prevent damaging the nerves, glass rods were used to thread the nerves and ganglia through to the nerve-recording chamber, which was sealed with petroleum jelly. The vagal nerve bundles were placed on platinum bipolar recording electrodes (WPI, Sarasota, FL, USA) attached to a Power lab (AD Instruments, Oxford, UK). The nerve activity was viewed and recorded with Chart 7 (AD Instruments, Oxford, UK). High- and low-pass filters were set at 0.2 and 2kHz, respectively and the signal was filtered to exclude mains fluctuation. Data was analyzed in GraphPad Prism for Windows (version 5) and presented as area under the curve (AUC).

Gut Bath electrophysiology

Sections of distal colon were opened along the mesenteric border and the mucosa was removed. Tissue was either suspended transversely to measure circular muscle contractility or longitudinally to measure longitudinal muscle contractile activity. The colonic sections were suspended from a tension transducer under 1g of tension in a water-jacketed tissue bath, maintained at 37^{o}C in Krebs-buffered saline and allowed to equilibrate for up to an hour. Colonic sections were stimulated with the cholinergic agonist, carbachol (1μM, 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 reagents such as the GLP-1 mimetic, exendin-4 (10μM, Abcam Laboratories, Cambridge, UK) and the ghrelin receptor agonist, Ibutamoren mesylate (MK-667, 1μM, Sigma Aldrich, UK). 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), which was used to incorporate both the amplitude and frequency of colonic contractions. The rectified trace was calculated by computing the integral of the raw data.

Statistical Analyses

Data was analyzed using GraphPad prism for windows (version 7). Data were plotted as box and whisker plots with 95% confidence intervals. Data were compared using paired two-tailed Student's or paired t-tests, as appropriate or One-way or repeated measures ANOVA with Tukey post-hoc test. P values of <0.05 were considered significant.

Results

Circulating levels of GLP-1 differ between IBS patient subtypes.

Circulating levels of GLP-1 and ghrelin were compared between fasting healthy controls (HCs), IBS-C and IBS-D patients (n=6 samples per group). Plasma GLP-1 was similar in HCs and IBS-C patients (one-way ANOVA, $F(2,14)=1.51$, $p=0.25$) but depressed in IBS-D patients ($p=0.043$, figure 1A(i)). Plasma ghrelin did not differ between fasting HCs, IBS-D and IBS-C patients (one-way ANOVA, $F(2,14)=0.927$, $p=0.42$, figure 1A(ii)). Tissue secretion of GLP-1 from colonic biopsies was higher than plasma levels and, increased in IBS-C supernatants, as compared to HCs (one-way ANOVA, $F(2,11)=5.81$, $p=0.019$), with no change in secretion from IBS-D samples (figure 1A(iii), $p=0.8$). Ghrelin-secreting cells are not thought to be found in the colon, so it was unsurprising that ghrelin was undetectable in biopsy supernatants.

To determine if a change in the density of colonic L-cells accounted for altered colonic secretion of GLP-1, the numbers of L-cells as a fraction of total DAPI-labelled mucosal cells in a given field were compared. In HC biopsies, 3.8% of epithelial cells were identified as GLP-1-expressing L-cells, 4.8% of cells in IBS-C biopsies and 3% of cells in the IBS-D cohort expressed GLP-1. Although this pattern is consistent with increased secretion of GLP-1 in IBS-C biopsies (figure 1A(iii)), these differences were not significant (n= 6-10 sections per biopsy, n=4 biopsies per group, one-way ANOVA, $F(2,23)=1.76$, $p=0.195$, figure 1B).

GLP-1 and ghrelin contribute to the neurostimulatory action of IBS plasma on submucosal neurons.

Previous studies from our lab demonstrated that soluble mediators present in plasma from IBS patients but not healthy comparators evoked robust increases in $[Ca^{2+}]_i$ in enteric neurons

from SD distal colons[38,39]. Due to the innate variability of individual plasma samples, a standard preparation of pooled plasma samples from healthy (n = 6) and IBS (n=6 pooled samples from IBS-D, IBS-C and IBS-A) was used to standardize calcium responses and facilitate investigation of the potential contribution of circulating GLP-1 and/or ghrelin in the neurostimulatory effects of IBS plasma on SD and WKY submucosal neurons. IBS plasma was diluted 1:250 in Krebs saline and perfused onto the tissue for 3 minutes. Consistent with our previous studies[38,39], soluble mediators within the plasma stimulated a robust increase in $[Ca^{2+}]_i$ in submucosal neurons in both SD (n=41 neurons from 3 rats, figure 2A) and WKY (n=28 neurons from 4 rats, figure 2B) rats. Similar proportions of total SD (59%) and WKY (64%, Fisher's exact test, p=0.56) rat submucosal neurons responding to 75mM KCl were activated by IBS plasma, suggesting that neurons in both rat strains were equally sensitive to the stimulatory factors in IBS plasma.

Although we have already determined that stress and immune factors contribute to the stimulatory effect of IBS plasma[38,39], the GLP-1R antagonist, exendin (9-39) (Ex(9-39), 10μM, 10min) and the ghrelin receptor antagonist, YIL781 (10nM, 10min) were utilized to determine if either of these gut hormones contributed to the neurostimulatory effect. In SD SMP tissue, IBS plasma–evoked calcium responses were reduced by Ex(9-39) (n=20 neurons from 3 rats, one-way ANOVA, $F(2,75)=6.69$, $p=0.003$), whereas the GHSR-1 antagonist had no effect on IBS plasma-evoked responses (n=17 neurons from 3 rats, p=0.317, figure 2A). In WKY submucosal neurons, the IBS plasma-evoked calcium response was similarly attenuated by Ex(9-39) (one-way ANOVA, $F(2,39)=13.39$, $p<0.001$), but in contrast to its lack of effect in SD tissue, YIL781 attenuated the IBS plasma-induced calcium response in this tissue (n=14 neurons from 3 rats, $p<0.001$, figure 2B).

GLP-1 contributes to the neurostimulatory action of IBS plasma on myenteric neurons.

The neurostimulatory effects of IBS plasma were assessed in the myenteric neurons of SD and WKY distal colons. Pooled IBS plasma evoked a robust increase in $[Ca^{2+}]_i$ in both SD (n=32 neurons from 3 rats, figure 2C) and WKY (n=19 neurons from 3 rats, figure 2D) myenteric neurons. The percentage of myenteric neurons responding to IBS plasma (71% SD vs 69% WKY, Fisher's exact test: p=0.878) was similar between rat strains. To determine if GLP-1 and ghrelin may represent neurostimulatory soluble mediators present in IBS plasma, Ex(3-39) and YIL781 were applied to LMMP tissue prepared from each rat strain. In SD tissue, Ex(3-39) inhibited the response evoked by IBS plasma (n=14 neurons from 3 rats, one-way ANOVA, $F(2,61)=10.74$, $p<0.001$) whereas YIL781 had no effect (n=18 neurons from 3 rats, p=0.957, figure 2C). In WKY LMMP tissue, the GLP-1R antagonist similarly attenuated the IBS plasma-evoked response (n=18 neurons from 3 rats, one-way ANOVA, $F(2,51)=9.36$, $p<0.001$), whereas the ghrelin receptor antagonist had no effect (n=15 neurons from 3 rats, p=0.59, figure 2D).

MK-667 sensitizes colonic submucosal neurons to exendin-4.

We have previously noted differences between SD and WKY enteric neurons in their sensitivity to neuromodulatory molecules[40]. Calcium imaging of submucosal neurons demonstrated that the GLP-1R agonist, exendin-4 (Ex-4, 10µM, 3 min) evoked a small, short-lived increase in $[Ca^{2+}]_i$ in SD rats that was reproducible, with no difference between the first and second application (n=10 neurons from three rats, paired t-test: p=0.489, figure 3A). Ex-4 evoked a calcium response in both SD (n=18 neurons from 3 rats, figure 3B) and WKY rats (n=26 neurons from 3 rats, figure 3C). The ghrelin receptor agonist, MK-667 (1µM, 3 min) evoked an increase in $[Ca^{2+}]_i$, which was similarly short-lived in both strains. No strain difference was detected in the amplitude of response for Ex-4 (p=0.898) or MK-667

(p=0.08). However, re-application of Ex-4, 10 minutes after the brief exposure to MK-667, evoked an enhanced calcium response both in SD (repeated measures ANOVA, $F(2,44)=9.19$, $p=0.005$, figure 3B) and WKY (repeated measures ANOVA, $F(2, 25)=109.8$, $p<0.001$, figure 3C) rats. Sensitization of neurons by the GHSR-1 agonist appears to be a short-lived phenomenon as 30 minutes after exposure to MK-667, the calcium response evoked by Ex-4 in SD submucosal neurons (0.885 ±0.83) was no longer potentiated as compared to the control response (1.217 ± 0.86, n=27 neurons from 3 rats, paired t-test: p=0.167). The potentiated calcium response did not differ between SD and WKY strains (t-test: p=0.15).

Potentiation of an exendin-4 evoked calcium response in colonic myenteric neurons by MK-667 is larger in Wistar Kyoto rats.

To determine if the ghrelin agonist similarly sensitized myenteric neurons to the neurostimulatory actions of the GLP-1R agonist, Ex-4 (10μM, 3 min) was applied to colonic myenteric neurons. An increase in $[Ca^{2+}]_i$ was evoked in SD rats, which was reproducible upon re-application, with no significant difference between the first and second application (n=25, paired t-test: p=0.452, figure 4A). Both Ex-4 and MK-667 evoked calcium responses in SD (n=14 neurons from 3 rats, figure 4B) and WKY (n=37 neurons from 3 rats, figure 4C) rats. No strain difference in the amplitude of response was detected for Ex-4 (p=0.39). The amplitude of response to MK-667, however was larger in WKY rats (p=0.05). Ex-4 applied post-exposure to MK-667 resulted in an enhanced calcium response, such that the amplitude was significantly larger than the control Ex-4 response in SD (repeated measures ANOVA, $F(2,13)=8.9$, $p=0.0023$, figure 4B) and in WKY (repeated measures ANOVA, $F(2, 36)=39.13$, $p<0.001$, figure 4C) rats. Moreover, between strains, the potentiated Ex-4 response was significantly larger in WKY rats (t-test: p=0.0009).

MK-667 potentiates Ex-4-evoked smooth muscle colonic contractile activity.

To assess if the potentiation of Ex-4-evoked excitatory activity in myenteric neurons had a functional impact on colonic smooth muscle activity, colonic tissue was suspended in gut baths in the circular and longitudinal orientations. The cholinergic agonist, carbachol (1μM) was applied to the tissue at the beginning and the end of the experiment to evoke a maximal response. No run-down in response was detected in either circular (n=6, t-test, p=0.29) or longitudinal muscle (n=6, p=0.78). Baseline colonic circular smooth muscle contractions are regular in SD rats, with reasonably consistent amplitude and frequency (figure 5A). The pattern of circular muscle contractile activity in WKY rats appears more erratic (figure 5B) and the amplitude of contractions was reduced (p=0.01, t-test). Longitudinal muscle contractile activity in SD rats (figure 5C) is also less erratic than the pattern of activity in the WKY rat (figure 5D), which is also of lower amplitude (p=0.009, t-test). However, no differences in the frequency of contractions was detected between strains in either circular (p=0.427) or longitudinal muscle (p=0.467). As a combined measure of amplitude and duration of contractions above baseline, area under the curve (AUC) was compared between strains in remaining experiments.

In SD circular muscle, Ex-4 (10μM, 10min) modified the pattern of contraction (n=6, figure 5A), a response that was reproducible upon repeated application. The response was attenuated in the presence of the neurotoxin, tetrodotoxin (100nM, n=5, p=0.025), indicating that the action of Ex-4 is primarily neurally-regulated. However, the post-MK-667 (1μM, 20min) response evoked by Ex-4 was potentiated (repeated measures ANOVA, $F(2, 5)=5.576$, p=0.024, figure 5A) with evidence of increased amplitude and frequency of contractions, and a notable increase in colonic tone. In WKY circular muscle, Ex-4

suppressed the aberrant activity noted at baseline (n=6, figure 5B). The post-MK-667 application of Ex-4 stimulated increased colonic tone and circular contractile activity (repeated measures ANOVA, $F(2, 5)=5.2$, $p=0.028$). Despite differences in baseline contractile activity, the potentiated response did not differ between strains (t-test, $p=0.73$).

In SD colonic tissue suspended to record longitudinal muscle activity, the amplitude of colonic contractions was suppressed by Ex-4 (n=6, figure 5C) with a decrease in muscle tone. However, the effect of Ex-4 was not attenuated significantly by tetrodotoxin (n=5, $p=0.166$). After exposure to MK-667, Ex-4 evoked an increase in colonic tone and more contractile activity (repeated measures ANOVA, $F(2, 4)=5.27$, $p=0.035$, figure 5C), although the synchronicity of the response was lost. In WKY rat colons, Ex-4 suppressed tonic longitudinal muscle contractile activity, but the post-MK-667 response evoked by Ex-4 was potentiated (repeated measures ANOVA, $F(2, 5)=4.32$, $p=0.044$, figure 5D), with a large increase in tone and loss of synchronous contractions. The potentiated response in longitudinal muscle contractile activity did not differ between strains (n=5, t-test, $p=0.413$). While MK-667 appears to sensitize the colon of both rat strains to the actions of the GLP-1R agonist, the patterns of baseline contractile activity vary considerably between SD and WKY rats.

Potentiation of Exendin-4 evoked vagal nerve firing by MK-667.

To determine if the GHSR-1 agonist can sensitize afferent nerve signaling to Ex-4, extracellular recordings of colonic vagal nerve fibers were undertaken. In an isolated chamber, vagal nerve activity was recorded in response to stimulation of a colonic LMMP tissue preparation. Application of Ex-4 (10μM, 10 min) to SD distal colon LMMP stimulated a rapid but short-lived increase in vagal nerve firing (n=6 rats, figure 6A). Similarly, Ex-4-

evoked increase in compound vagal nerve activity in WKY rats (n=6 rats, figure 6B). Exposure to the GHSR-1 agonist, MK-667 also evoked a similar afferent nerve response in both strains, however, re-application of Ex-4 post-MK-667 resulted in a significantly larger increase in vagal firing than the control Ex-4. This potentiation was evident in both SD (repeated measures ANOVA, $F(2,5)=17.6$, $p=0.033$, figure 6A) and WKY (repeated measures ANOVA, $F(2,5)=13.05$, $p=0.027$, figure 6B) rats and did not significantly differ between strains (t-test: $p=0.073$). The duration of the response was over twice as long in WKY rats as compared to SD rats (SD: 1.46 ±0.61 min, WKY: 3.68 ±0.84 min) although it didn't quite reach statistical significance (t-test: $p=0.058$). Repeated application of Ex-4 in the absence of MK-667 did not result in an enhanced neural response (3.22 ±0.55 vs 3.61 ±0.74, n=3 t-test: $p=0.694$), indicating that exposure to MK-667 was the likely cause for the potentiated response.

Discussion

In many IBS patients, ingestion of a meal leads to exacerbation of symptoms, including abdominal bloating and pain, and diarrhea[14-16]. The cellular mechanisms underlying this acute exacerbation of symptoms is likely to involve both endocrine and afferent nerve signaling. In our study, we have used the stress-sensitive WKY rat, which exhibits visceral pain hypersensitivity[30,34] and increased stress-related defecation[32] to determine if endocrine-mediated modification of GI function differs in an animal model of bowel dysfunction. We investigated if ghrelin, which peaks prior to meal ingestion, sensitized colonic intrinsic and extrinsic neurons to the neurostimulatory actions of GLP-1. Our findings indicate that the ghrelin receptor agonist, MK-667, sensitizes colonic enteric neurons to the neurostimulatory effects of a GLP-1R agonist, Ex-4 in both SD and WKY rats. However, despite similar neuronal responses, differences between the strains became apparent when colonic function was examined. Under basal conditions, WKY rats exhibited aberrant colonic contractile activity. Ex-4 suppressed contractile activity in both SD and WKY strains, but this response was altered by exposure to MK-667. However, the underlying patterns of contractile activity were maintained in WKY rats. In separate experiments, we found that vagal nerve activity, which provide the CNS with interoceptive information from the viscera, were stimulated by application of Ex-4 to myenteric neurons of the distal colon. Exposure to the GHSR-1 agonist, potentiated this response. The finding that circulating GLP-1 was altered in human IBS-D patients suggested that our mechanistic findings in the rat model may be translatable to the human functional bowel disorder.

Ghrelin is primarily secreted by gastric epithelial cells thus it was unsurprising that no ghrelin was detected in secretions from colonic biopsies. Nonetheless, acetylated ghrelin, the endogenous ligand for GHSR-1, has a reasonably long half-life of ~10 minutes[41] and can

activate colonic enteric neurons[42]. Indeed, MK-667 stimulated a short-lived increase in $[Ca^{2+}]_i$ in submucosal and myenteric neurons prepared from rat colons. We previously demonstrated that IBS plasma, which contains neurostimulatory soluble mediators, such as corticotrophin-releasing factor and interleukin-6, which are not present in plasma from healthy controls, evokes larger calcium responses in enteric neurons[38,39]. However, blocking GHSR-1 did not alter calcium responses evoked by IBS plasma in SD submucosal or myenteric neurons. Indeed, ghrelin levels were not different between healthy controls and IBS patients, which is consistent with a previous study[43]. Interestingly, the amplitude of the response evoked by pooled IBS plasma in WKY submucosal, but not myenteric neurons, was reduced in the presence of a GHSR-1 antagonist. Submucosal neurons regulate GI absorpto-secretory function, which is altered in WKY rats[33,44]. These findings may indicate a possible role for ghrelin in absorpto-secretory dysfunction in the WKY model of IBS. However, what is perhaps more interesting, is the capacity of MK-667 to modify the sensitivity of enteric neurons to the GLP-1R agonist, Ex-4.

Some[17,45], but not all studies[46,47] show that ghrelin can stimulate L-cells to secrete GLP-1, or ghrelin may prime intestinal L-cells for nutrient-induced GLP-1 secretion[17], fitting with the temporal peak in ghrelin concentrations prior to ingestion of a meal and subsequent GLP-1 release[18]. In our experiments, only supra-physiological concentrations of Ex-4 evoked small, short-lived increases in intracellular calcium in both submucosal and myenteric neurons. This may reflect a mechanism where local L-cell activation facilitates the accumulation of higher concentrations of GLP-1 to activate enteric neurons through paracrine mechanisms. However, research demonstrating that feeding status tunes the sensitivity of myenteric neurons to orexigenic or anorexigenic molecules[2,48], and our finding that GHSR-1 activation sensitizes enteric neurons to Ex-4, could explain how colonic neurons gain sensitivity to post-prandial

physiological concentrations of GLP-1. This increase in sensitivity is time-limited however as it is not apparent 30 minutes after exposure to MK-667. GLP-1 may also contribute to the neurostimulatory actions of IBS plasma in both neuronal plexi of both rat strains as the GLP-1R antagonist resulted in a decrease in the amplitude of response. However, it is likely that GLP-1 is just one contributory factor in the altered modulation of enteric neurons, as we have previously noted the importance of immune factors and circulatory hormones in this effect[38,39,49]. In our cohort we found that fasting GLP-1 was lower in IBS-D with no change in IBS-C patients, which contrasts with a report that indicated plasma GLP-1 was decreased in IBS-C patient samples[50].

Others have examined the functional effects of GLP-1 on mucosal secretory physiology and postulated that activation of submucosal GLP-1Rs leads to a decrease in neurally evoked chloride secretion, a pathway mediated through suppression of neural acetylcholine[4]. Thus, we focused on colonic contractile activity. Striking differences in basal smooth muscle contractile activity were noted in SD and WKY rats, which is similar to differences in colonic contractility reported between WKY and SHR rat comparators[51]. Sustained colonic tone and regular circular muscle contractions in SD rats contrasted with an erratic baseline tone and contractile frequency observed in WKY colons. Basal activity in longitudinal smooth muscle also differed between strains and combined, this is likely to underlie dysfunctional GI transit in WKY rats[32]. In SD circular muscle, Ex-4 evoked a small increase in tone and contractions that were more frequent but of smaller amplitude. In WKY rats, the large amplitude erratic contractions, characteristic of basal contractile activity, was suppressed by Ex-4. In longitudinal muscle, Ex-4 caused suppression of colonic contractions and tone in SD rats, although regular contractile activity returned following washout. In WKY rats the inhibitory effects of Ex-4 on longitudinal muscle contractile function was reversed upon washout,

although the amplitude of contractions remained suppressed. The suppressive effects of Ex-4 in WKY rat colons are consistent with reports that GLP-1 mimetics suppress intestinal motility in both humans[6] and rats[52], and this may underpin the therapeutic benefits of GLP-1 mimetics in IBS.

Prior exposure to the GHSR-1 agonist, which aimed to replicate the peak in ghrelin prior to food ingestion, modified the effect of Ex-4 in circular and longitudinal muscle in both rat strains, although the basal pattern of contraction in each strain influenced the outcome. While Ex-4 applied to naïve tissue made contractile responses more quiescent and caused a decrease in colonic tone, re-application of Ex-4 after MK-667 exposure caused an increase in colonic tone and larger, more frequent contractions. In health, increased contractile activity following food ingestion may be important in stimulating fecal transit in preparation for the arrival of more luminal content. However, in pathophysiological conditions, such as IBS this contractile activity may contribute to abdominal pain associated with contraction of hollow viscera, which are the most frequently reported symptoms exacerbated by food intake in IBS[15]. Consistent with the potentiating effects of MK-677 on Ex-4-evoked activation of myenteric neurons, tetrodotoxin inhibited Ex-4-evoked suppression of basal contractile activity in circular muscle but not longitudinal muscle in SD rats. Given that the sensitivity of myenteric neurons to Ex-4 is similar between SD and WKY rats, we would expect to see a similar outcome in the pre-clinical IBS model, however this does need to be experimentally verified.

The physiological mechanisms by which ROSE-010, a GLP-1 analogue, inhibits acute abdominal pain in IBS[12] and relieves constipation in IBS patients[53], remain unclear. It may be via an endocrine pathway, however, given the short half-life of GLP-1 in plasma, direct

neural signaling is also likely to be important. Spinal afferents are important in pain signaling, and a recent paper demonstrated expression of GLP-1Rs in DRG neurons[54]. However, GLP-1 did not directly stimulate calcium responses in these neurons. Moreover, the nociceptive receptor, TRPV1 was not affected by acute application of GLP-1, leading the authors to conclude that pain signaling is perhaps not modulated via this pathway. The vagus has been implicated in the pathophysiology of IBS, as it can stimulate the stress hypothalamic-pituitary-adrenal axis, modulate the neuro-immune axis and pain signaling originating in the gut, contributing to bowel dysfunction[23]. The dorsal motor nucleus of the vagus is sensitive to GLP-1[25] and intravenous injection of GLP can stimulate vagal firing[26]. GLP-1Rs are expressed in the nodose ganglion[55], where vagal afferent neurons innervating the abdominal viscera are found[56]. Thus, central regulation of visceral pain sensitivity could be influenced by GLP-1. Ex-4 increased the compound neural activity in vagal nerves when it was applied to myenteric neurons in the distal colon. Moreover, Ex-4-evoked vagal nerve activation was enhanced by prior exposure to the GHSR-1 agonist.

The reported exaggeration of the sensory component of the gastro-colonic response[57] and increased colo-rectal sensitivity to balloon distension[58] following food ingestion in IBS could be due to modulation of peripheral neurons by orexigenic and anorexigenic hormones. This study has provided evidence to support a role for ghrelin, which is primarily described as a driver of feeding, in priming both colonic enteric neurons and vagal afferents originating in the colon for the neurostimulatory actions of GLP-1. GLP-1 is secreted upon arrival of food in the small intestine but also in response to luminal molecules such as microbial factors, bile acids and short-chain fatty acids, which are more abundant in the distal intestine. Our data suggest that ghrelin receptor activation sensitizes intrinsic and extrinsic gut neurons to GLP-1 as part of the normal physiological response to the arrival of food in the gut. However,

underlying dysfunction of contractile activity or gut-brain signaling, as evident in the WKY rat model of IBS, may dictate whether the outcome is physiological or pathophysiological. Thus, the temporal regulation of ghrelin and GLP-1 may contribute to the post-prandial exacerbation of IBS symptoms.

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MMB and RO'B performed the research and analyzed the data. JMB contributed human samples. DO'M designed the research study and wrote the paper.

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Figure Legends

Figure 1: Circulating GLP-1 is depressed in IBS-D samples.

A: The box and whisker plots illustrate circulating plasma levels of (i) GLP-1 and (ii) ghrelin and (iii) supernatant concentration of GLP-1 in healthy controls (HCs), constipation- (IBS-C) or diarrhea (IBS-D) – predominant irritable bowel syndrome patient samples. B: The box and whisker plot and representative immunofluorescent images of GLP-1-expressing L-cells compare L-cell numbers in HC, IBS-C and IBS-D human colonic biopsies. Scalebar: 50μm. * indicates $p<0.05$.

Figure 2: GLP-1 contributes to the neurostimulatory effects of IBS plasma.

The box and whisker plots and representative calcium imaging traces illustrate the stimulatory effect of IBS plasma on A: Sprague Dawley (SD) and B: Wistar Kyoto (WKY) colonic submucosal (SMP) neurons. IBS plasma also stimulates myenteric (LMMP) neurons in C: SD and D: WKY rats. The calcium response in SMP and LMMP neurons is inhibited by the GLP-1 receptor antagonist, exendin (9-39) (Ex(9-39)) in both strains. The ghrelin receptor (GHSR-1) antagonist, YIL781 has no effect on SD SMP neurons or LMMP neurons in both strains, but does attenuate the response in WKY submucosal neurons. ** and *** indicate $p<0.01$ and $p<0.001$, respectively.

Figure 3: MK-667 sensitizes submucosal neurons to Ex-4 in both Sprague Dawley and Wistar Kyoto colons.

A: The box and whisker plots and sample calcium imaging traces illustrate the reproducibility of the calcium response evoked by Ex-4 in SD submucosal neurons. The change in fluorescence evoked by Ex-4 (10μM) was potentiated by exposure to MK-667 in both B:

Sprague Dawley and C: Wistar Kyoto rats. ** and *** indicate p<0.01 and p<0.001, respectively.

Figure 4: MK-667 potentiates the Ex-4-evoked response in Sprague Dawley and Wistar Kyoto myenteric neurons.

A: The box and whisker plots and sample calcium imaging traces illustrate the reproducible calcium response evoked by Ex-4 (10μM) in Sprague Dawley myenteric neurons. B: The change in fluorescence in myenteric neurons evoked by Ex-4 before and after exposure to the ghrelin receptor agonist, MK-667 (1μM) is shown in Sprague Dawley and C: Wistar Kyoto rats. ** and *** indicate p<0.01 and p<0.001, respectively.

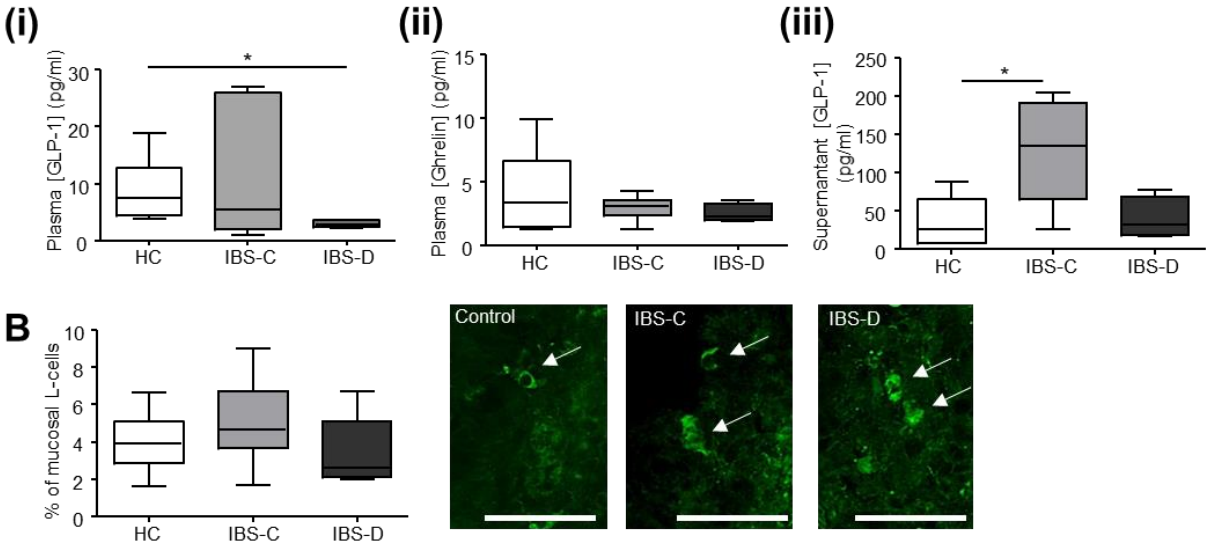
Figure 5: MK-667 potentiates Ex-4 evoked contractile activity in Sprague Dawley and Wistar Kyoto colons.

The box and whisker plots and representative colonic contractile activity recordings illustrate how Ex-4 differentially modifies circular muscle contractility in A: Sprague Dawley and B: Wistar Kyoto rats. Following exposure to MK-667 the response to Ex-4 is potentiated. The box and whisker plots and colonic contractile activity traces illustrate the effect of Ex-4 on baseline contractile activity in longitudinal muscle in both C: Sprague Dawley and D: Wistar Kyoto rats. Application of Ex-4 following exposure to MK-667 resulted in a dramatic change in colonic tone in both Sprague Dawley and Wistar Kyoto rats and a potentiation of the contractile response. * indicates P<0.05.

Figure 6: Vagal nerve activity evoked by Ex-4 application to myenteric neurons is enhanced by exposure to MK-667.

The box and whisker plots and representative un-filtered and filtered extracellular recordings from vagal nerves, recorded superior to the stomach, show that application of Ex-4 to colonic myenteric neurons stimulates afferent nerve firing in both A: Sprague Dawley and B: Wistar Kyoto rats. While MK-667 itself evokes a moderate stimulatory response, the capacity of Ex-4 to evoke vagal nerve firing is enhanced following exposure to MK-667 in both strains of rat, although this effect is potentiated in the Wistar Kyoto rat. * indicates $p<0.05$.

Figure 1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(i)
Plasma [GLP-1] (pg/ml)
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HC
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(ii)
Plasma [Ghrelin] (pg/ml)
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10
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HC
IBS-C
IBS-D
(iii)
Supernantant [GLP-1] (pg/ml)
250
200
150
100
50
0
*
HC
IBS-C
IBS-D
B
% of mucosal L-cells
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8
6
4
2
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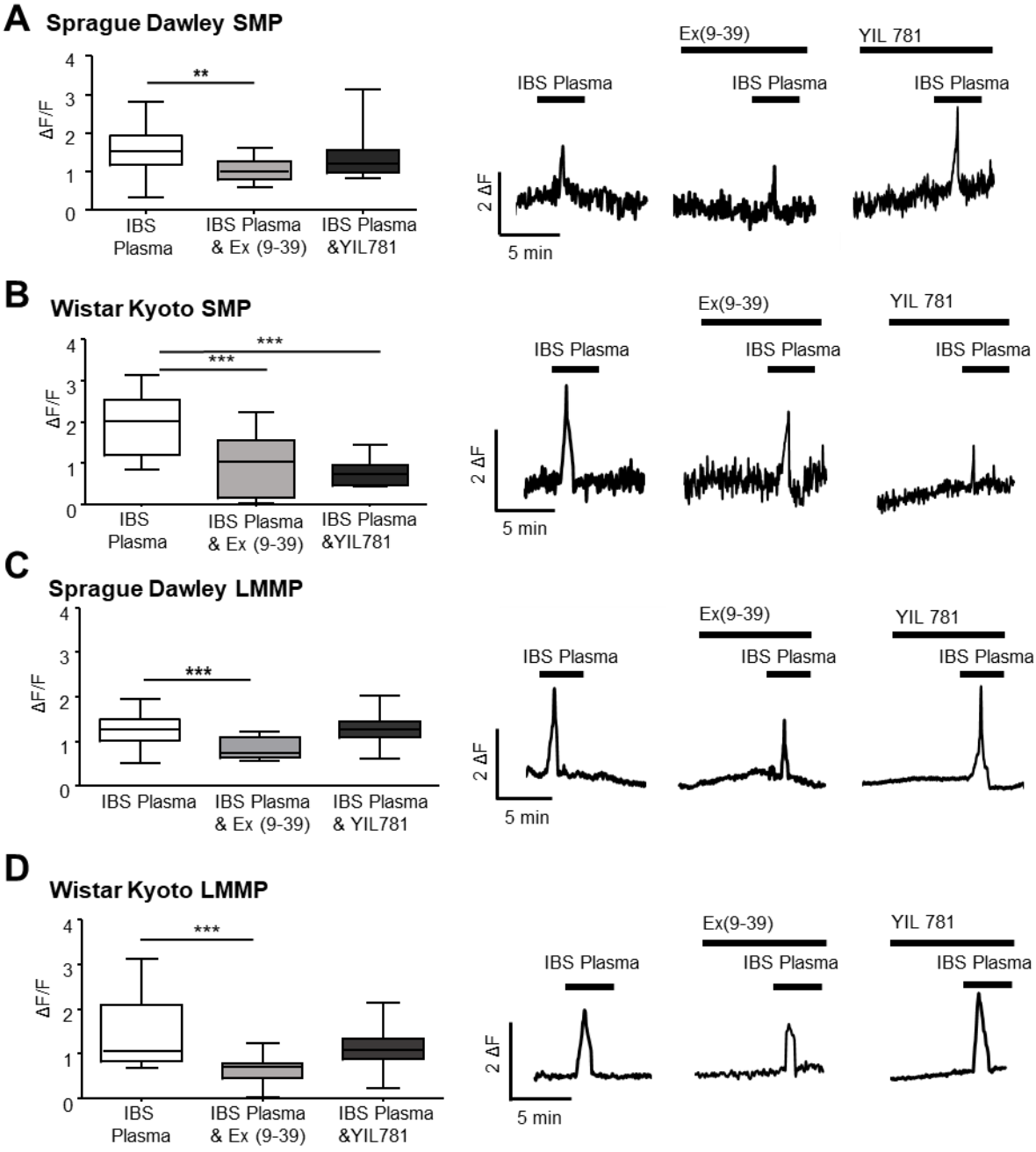
HC
IBS-C
IBS-D
Control
IBS-C
IBS-D

Figure 2


A
Sprague Dawley SMP
ΔF/F
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IBS Plasma
IBS Plasma & Ex (9-39)
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Ex(9-39)
YIL 781
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IBS Plasma
IBS Plasma
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Wistar Kyoto SMP
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ΔF/F
IBS Plasma
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Sprague Dawley LMMP
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2 ΔF
5 min
D
Wistar Kyoto LMMP
***
ΔF/F
IBS Plasma
IBS Plasma & Ex (9-39)
IBS Plasma &YIL781
Ex(9-39)
YIL 781
IBS Plasma
IBS Plasma
IBS Plasma
2 ΔF
5 min

Figure 3

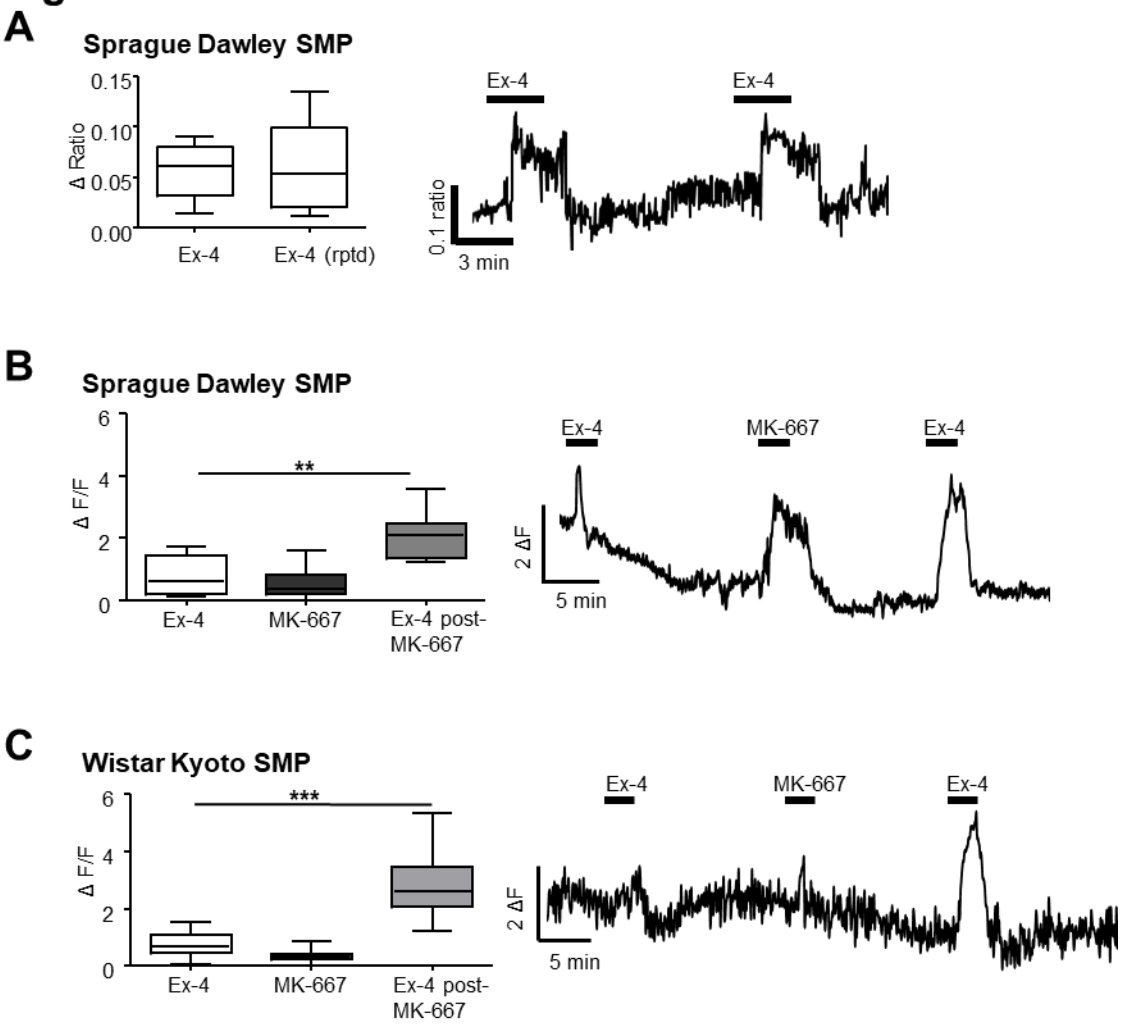

A
Sprague Dawley SMP
0.15
0.10
0.05
0.00
Δ Ratio
Ex-4
Ex-4 (rptd)
Ex-4
Ex-4
0.1 ratio
3 min
B
Sprague Dawley SMP
6
4
2
0
Δ F/F
**
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
2 ΔF
5 min
C
Wistar Kyoto SMP
6
4
2
0
Δ F/F
***
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
2 ΔF
5 min

Figure 4

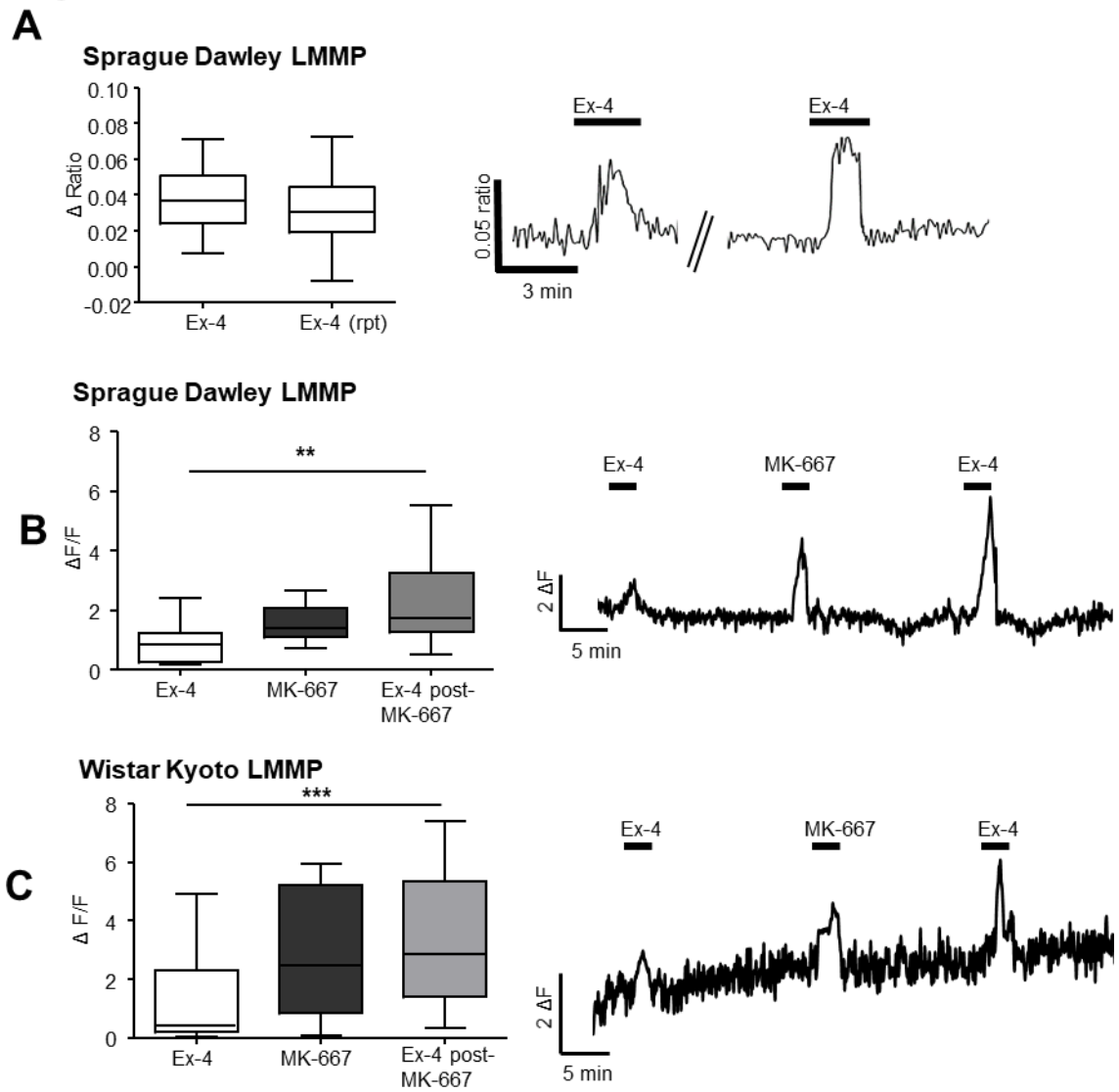

A
Sprague Dawley LMMP
Δ Ratio
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
Ex-4
Ex-4 (rpt)
Ex-4
Ex-4
0.05 ratio
3 min
B
Sprague Dawley LMMP
ΔF/F
8
6
4
2
0
**
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
2 ΔF
5 min
C
Wistar Kyoto LMMP
Δ F/F
8
6
4
2
0
***
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
2 ΔF
5 min

Figure 5

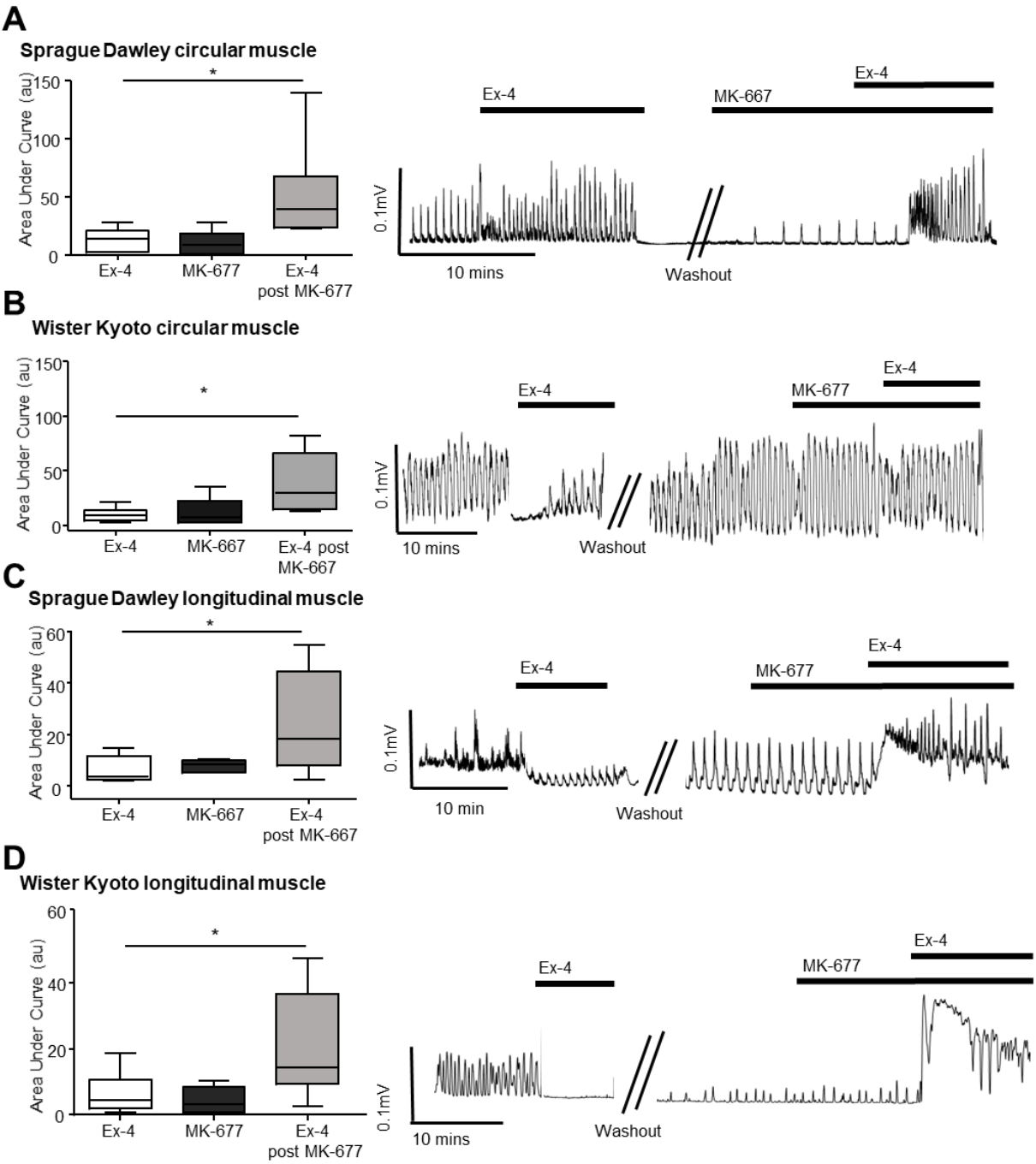
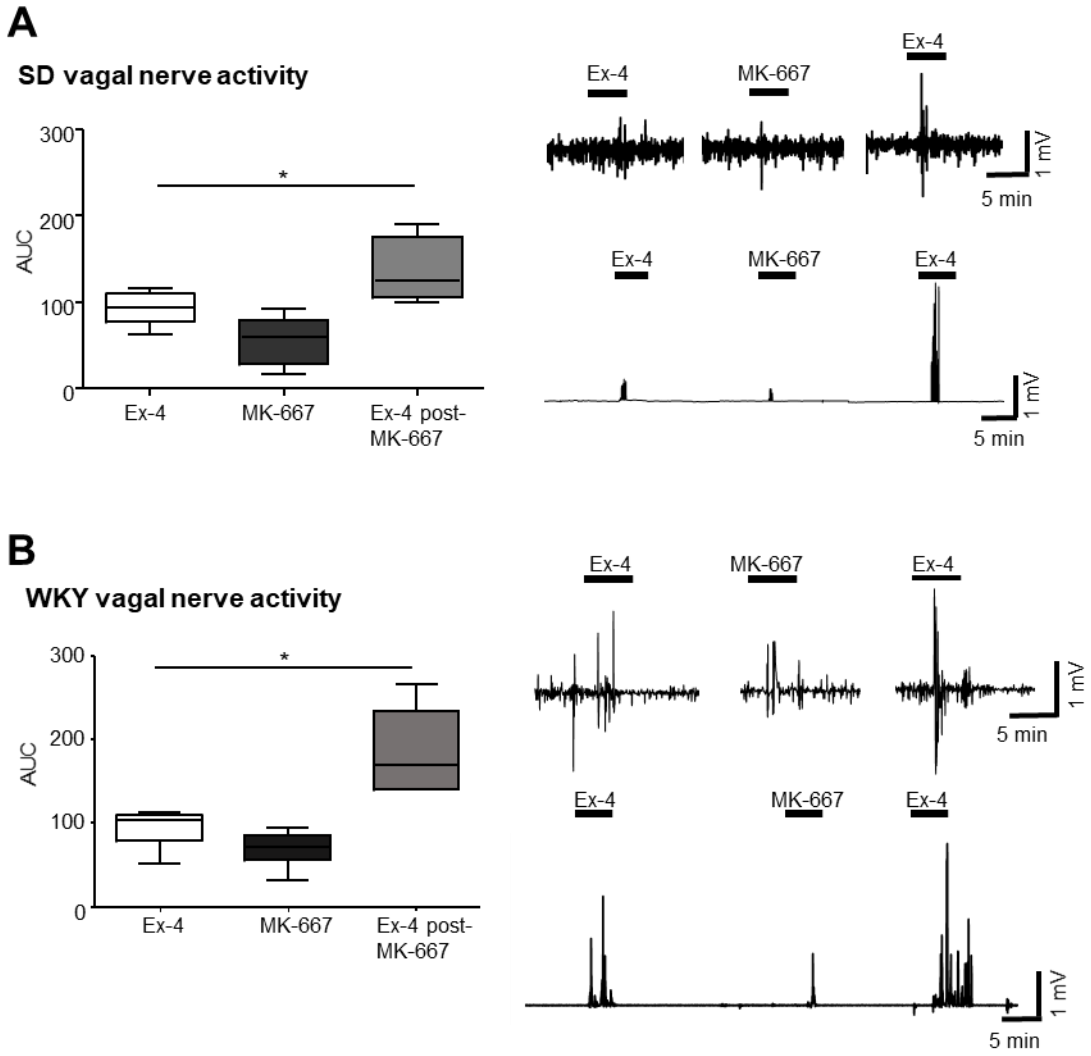

A
Sprague Dawley circular muscle
Area Under Curve (au)
150
100
50
0
*
Ex-4
MK-677
Ex-4 post MK-677
Ex-4
MK-667
Ex-4
0.1mV
10 mins
Washout
B
Wister Kyoto circular muscle
Area Under Curve (au)
150
100
50
0
*
Ex-4
MK-667
Ex-4 post MK-667
Ex-4
MK-677
Ex-4
0.1mV
10 mins
Washout
C
Sprague Dawley longitudinal muscle
Area Under Curve (au)
60
40
20
0
*
Ex-4
MK-667
Ex-4 post MK-667
Ex-4
MK-677
Ex-4
0.1mV
10 min
Washout
D
Wister Kyoto longitudinal muscle
Area Under Curve (au)
60
40
20
0
*
Ex-4
MK-677
Ex-4 post MK-677
Ex-4
MK-677
Ex-4
0.1mV
10 mins
Washout

Figure 6


A
SD vagal nerve activity
300
200
100
0
AUC
*
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
1 mV
5 min
Ex-4
MK-667
Ex-4
1 mV
5 min
B
WKY vagal nerve activity
300
200
100
0
AUC
*
Ex-4
MK-667
Ex-4 post-MK-667
Ex-4
MK-667
Ex-4
1 mV
5 min
Ex-4
MK-667
Ex-4
1 mV
5 min