Leptin modifies the pro-secretory and pro-kinetic effects of the inflammatory cytokine, interleukin-6 on colonic function in Sprague Dawley rats.

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New Findings:

- **What is the central question of this study?**

  Does crosstalk exist between leptin and interleukin-6 in colonic enteric neurons and is this a contributory factor in gastrointestinal dysfunction associated with irritable bowel syndrome (IBS)?

- **What is the main finding and its importance?**

  Leptin ameliorates the pro-secretory and pro-kinetic effects of the pro-inflammatory cytokine, interleukin-6 on rat colons. Leptin also suppresses the neurostimulatory effects of IBS plasma, which has elevated levels of interleukin-6, on enteric neurons. This may indicate a regulatory role for leptin in immune-mediated bowel dysfunction.

Abstract:

In addition to its role in regulating energy homeostasis, the adipokine, leptin modifies gastrointestinal (GI) function. Indeed, leptin-resistant obese humans and leptin-deficient obese mice exhibit altered GI motility. In the functional GI disorder, irritable bowel syndrome (IBS), circulating leptin levels are reported to differ from healthy controls. Additionally, IBS patients display altered cytokine profiles, including elevated circulating levels of the pro-inflammatory cytokine, interleukin (IL)-6, which bears structural homology and similarities in intracellular signalling to leptin. This study aims to investigate interactions between leptin and IL-6 in colonic neurons and their possible contribution to IBS pathophysiology. The functional effects of leptin and IL-6 on colonic contractility and absorpto-secretory function were assessed in organ baths and Ussing chambers in Sprague Dawley rat colons. Calcium imaging and immunohistochemical techniques were employed to investigate the neural regulation of GI function by these signalling molecules. Our findings provide a neuromodulatory role for leptin in submucosal neurons, where it inhibited the stimulatory effects of IL-6. Functionally, this translated to suppression of IL-6 evoked potentiation of veratridine-induced secretory currents. Leptin also attenuated IL-6-induced colonic contractions, although it had little direct effect on myenteric neurons. Calcium responses evoked by IBS plasma in both myenteric and submucosal neurons were also suppressed by leptin, possibly through interactions with IL-6, which is elevated in IBS plasma. As leptin has the capacity to ameliorate the neurostimulatory effects of soluble mediators in IBS plasma and modulated IL-6-evoked changes in bowel function, leptin may have a role in immune-mediated bowel dysfunction in IBS patients.

Abbreviations

AUC, area under the curve; χ^2, Chi squared; CRF, corticotropin-releasing factor; ERK-MAPK, extracellular signal regulated kinase-mitogen activated protein kinase; FITC, Fluorescein isothiocyanate; GI, gastrointestinal; HC, healthy control; IL, interleukin; IL-6R, interleukin-6 receptors; IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-A, alternating IBS; I_{SC}, short circuit current; LMMP, longitudinal muscle myenteric plexus; ObR, leptin receptor; PI-3kinase, phosphoinositide 3-kinase; SMP, sub-mucosal plexus; STAT, signal transducer and activator of transcription, TER, transepithelial resistance; TRITC, Tetramethylrhodamine isothiocyanate; TTX, tetrodotoxin; xleptin, monoclonal leptin antibodies.

Introduction

Irritable bowel syndrome (IBS) is a highly prevalent functional gastrointestinal (GI) disorder, affecting approximately 15-20% of the worldwide population (Lovell & Ford, 2012). Characterized by episodes of abdominal pain, bloating and altered bowel habit (Longstreth *et al.*, 2006), IBS severely impinges on the quality of life of sufferers and confers a heavy economic and societal burden (Nellesen *et al.*, 2013). Symptoms vary amongst patients with constipation-predominant (IBS-C), diarrhoea-predominant (IBS-D) or alternating (IBS-A) subtypes (Yale *et al.*, 2008). Such heterogeneity in symptom phenotype, in addition to the absence of reproducible biomarkers and a lack of clarity on the underlying pathophysiology all hamper the search for effective treatments.

A number of factors are thought to contribute to the initiation, persistence and severity of IBS symptoms, including stress (Folks, 2004) and immune activation (Quigley, 2006; Elsenbruch, 2011; O'Malley *et al.*, 2011b), with evidence of elevated levels of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 (Dinan *et al.*, 2006; Liebregts *et al.*, 2007; Dinan *et al.*, 2008; McKernan *et al.*, 2011) and an altered IL-10/IL-12 ratio (O'Mahony *et al.*, 2005). Such changes in cytokine profile indicate a pro-inflammatory state in IBS patients, although not all studies have detected these immune changes in the circulation or mucosa (Chang *et al.*, 2012). Observations that soluble mediators, both secreted by IBS mucosal biopsies (Buhner *et al.*, 2009) and in IBS plasma (O'Malley *et al.*, 2011a; Buckley *et al.*, 2014) have functional effects in the gut suggest that cytokines, hormones and enzymes have a role in the pathophysiology of IBS. Supernatants containing histamine, serotonin and proteases from both IBS-C and IBS-D patients activate human enteric neurons (Buhner *et al.*, 2009) and IL-6 and corticotropin-releasing factor

(CRF) in IBS plasma modulate enteric neurons resulting in altered bowel contractile and absorpto-secretory function.

Another circulating factor with neuromodulatory actions is leptin, an adipokine secreted by white fat cells and gastric secretory cells. Leptin has important effects on satiety and energy homeostasis but also plays a role in GI function, as obese humans (Gallagher *et al.*, 2007) and leptin-deficient obese mice (Kiely *et al.*, 2005) exhibit altered gut motility. However, the effects of leptin may be dependent on the source. If leptin is secreted from gastric secretory cells, it may engage with leptin receptors (ObRs) on the luminal border of intestinal cells. Luminally-secreted leptin can modify nutrient uptake in the small intestine but has also been characterized as a pro-inflammatory cytokine, contributing to barrier breakdown and inflammation (Sitaraman *et al.*, 2004) and causing upregulation of pro-inflammatory cytokines such as IL-6 (Padidar *et al.*, 2011). If it originates from adipocytes, leptin may bind to ObRs on the basolateral surface of epithelial cells or on enteric neurons (Liu *et al.*, 1999). Indeed, leptin can activate both submucosal and myenteric neurons (Reichardt *et al.*, 2011), which regulate absorpto-secretory function and motility, respectively. A recent study demonstrated a neuroprotective effect of diet-induced obesity on the enteric nervous system that is likely to involve leptin (Baudry *et al.*, 2012). Serum leptin has been reported to be reduced in IBS patients, independent of factors such as IBS sub-type, body mass index or stress levels (Semnani *et al.*, 2009), although others have found that it was elevated in IBS-D plasma (Russo *et al.*, 2013). This study will examine the cellular and functional effects of interactions between leptin and IL-6 in the context of the functional bowel disorder, IBS.

Materials and Methods

Ethical Approval

The study protocol (APC020, 2009), for collecting blood samples form IBS patients and healthy volunteers was approved by the University College Cork Clinical Research Ethics committee of the Cork University Hospital in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. All animal experiments were in full accordance with the European Community Council Directive (86/609/EEC) and the local University College Cork Animal Experimentation Ethics Committee.

Animals and Tissue collecting

Male Sprague Dawley rats (8-12 weeks old) purchased from Envigo, Derbyshire, UK, were group-housed 6 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. Animals were permitted at least one week to acclimatize to their new environment prior to experimentation. Food and water were available *ad libitum*. Rats were sacrificed using CO_2 and decapitation. A section of the colon was excised from each rat, cut 8cm proximal to the anus. The excised distal colon was stored in ice-cold Krebs saline (pH7.4), 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.

As previously reported (McKernan *et al.*, 2011), human plasma was collected from healthy controls and IBS patients. IBS patients were aged 18–65 years and satisfied Rome II criteria for the diagnosis of IBS, with recurrent abdominal pain or discomfort for at least 3 days per month in last 3 months. Additionally, patients recruited from gastroenterology clinics at Cork

University Hospital, Cork, Ireland had two or more of the following symptoms: improvement with defecation, onset associated with a change in frequency of stool or onset associated with a change in form of stool. According to Rome II sub-classifications bowel habit was defined as constipation-predominant (IBS-C), diarrhoea-predominant (IBS-D) or alternating (IBS-A) subtypes. Healthy controls were recruited from hospital staff or the research institute (UCC). Individuals with a history of psychiatric illness, inflammatory bowel disease, coeliac disease, lactose intolerance, immunodeficiency or abdominal surgery were excluded. No patient was categorized as having post-infectious IBS. Alternative diagnoses were ruled out based on clinical history, physical examination, laboratory tests, imaging and endoscopy, as appropriate. Each individual was evaluated with a full review of their family history, details of current and recent medications, a physical examination and documentation of body mass index. All patients had active IBS, however disease activity was not used as a discriminatory factor, only IBS subtype. None of the controls were taking any medications (contraception excluded). Most IBS patients (17/18) were being treated with laxatives, antispasmodics, anti-peristaltic drugs, antidepressants, contraceptives or hormone replacement therapy.

20ml of venous blood was donated between 11:00 and 13:00 h to avoid diurnal variations. Whole blood (15 ml) was added to an equal volume of Histopaque 1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400g (30 min, room temperature). Plasma was collected and stored at -80^{o}C. A subset of plasma samples (n = 6 samples from each group) from both healthy volunteers and IBS patients (2 IBS-D, 2 IBS-C and 2 IBS-A) were randomly selected from a larger previously published study (McKernan *et al.*, 2011).

Calcium Imaging

For calcium imaging studies, whole mount preparations of myenteric and submucosal plexus were prepared from the distal colon of healthy Sprague Dawley rats. The colon was kept whole and mounted on a glass rod, where the outer serosal layer was scored lightly with a blade along the mesenteric border. Using a damp cotton swab, the outer serosa was removed. The colonic tissue (~2cm x 2cm) was subsequently pinned out in Sylgard-lined dishes and the mucosa removed. For myenteric preparations, the mucosa was removed, the tissue was turned over and the circular muscle was removed using forceps to leave longitudinal muscle and myenteric neurons. Enteric neuronal preparations were loaded with Fura-2AM (7μM, 1 hr) in Krebs saline solution prior to imaging. Cell R software (Olympus Soft imaging solutions, 1986-2009) was used to record excitation changes in intracellular calcium (O'Malley *et al.*, 2011a). Images were acquired 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). Ganglionic neurons were retrospectively identified based on morphology and responsivity to brief application of 75mM KCl at the end of the recording protocol. Responding neurons were defined as those with increases in intracellular calcium $[Ca^{2+}]_i$ greater than two standard deviations from baseline (calculated as the average ratio during the 150 seconds preceding stimulus application). Reagents were applied in random order to avoid run-down of calcium stores being a confounding factor. A perfusion system continuously superfused the colonic tissue with carbogen-bubbled Krebs-buffered saline at room temperature. Nifedipine (1μM), which is a potent inhibitor of calcium channels in smooth muscle, was continuously present in the perfusate to inhibit smooth muscle contractions.

Immunofluorescence of whole mount preparations

Whole-mount preparations were fixed in Zamboni's fixative (4^{o}C, overnight), permeabilised with 0.1% Triton X-100 and blocked with 1% donkey serum. Sub-mucosal plexus (SMP) and longitudinal muscle myenteric plexus (LMMP) tissue preparations were incubated with antibodies against leptin or IL-6 (1:100 @ 4^{o}C overnight), leptin receptors (ObR, 1:300 @ 4^{o}C overnight), interleukin-6 receptors (IL-6R, 1:300 @ 4^{o}C overnight) or cFos (1:100 @ 4^{o}C overnight, all procured from Santa Cruz Biotechnology, Dallas, TX, USA) and the associated FITC (Fluorescein isothiocyanate) - or TRITC (Tetramethylrhodamine isothiocyanate) - conjugated secondary antibodies (1:250, incubated at room temperature for 2 hours, Jackson Immunoresearch, Westgrove, PA, USA). Images were captured using Olympus D71 upright fluorescent microscope and Cell F software (Soft Imaging Solutions) or a FVlOi-Olympus-confocal microscope with Fluoview software. Control experiments detected no non-specific fluorescence when the FITC - and TRITC - conjugated secondary antibodies were incubated in the absence of primary antibodies. Similarly, no fluorescence was detected on neurons when anti-ObR and anti-IL-6R antibodies were applied to the neurons in the absence of secondary fluorophores.

Organ baths

The distal colon was excised from Sprague Dawley rats, the mucosa removed and the tissue was orientated transversely or longitudinally to measure circular or longitudinal muscle contractility, respectively. The colonic tissue was suspended from a tension transducer in a tissue bath of carbogen-bubbled Krebs saline under 1g of tension and allowed to equilibrate (20 – 30 minutes)

before reagents were added to the bath. Changes in tension were recorded and analysed using Powerlab and LabChart7 (AD instruments Inc, Colorado Springs, CO, US). Responses are reported as a percentage of the maximal response evoked by the cholinergic agonist, carbachol (100μM), in each experiment. The tissue was exposed to carbachol at the start and end of the experiment and no deterioration in the contractile response was noted. Reagents were added to bath at the final concentration. Before the addition of the next reagent, the bath was flushed twice with Krebs saline and the no further reagents were added until the baseline was restored (~20 minutes).

Ussing chambers

Mucosa–submucosa preparations were mounted in Ussing chambers (exposed area of 0.12 cm^2) with 5 ml of Krebs solution (95% O_2/5% CO_2, 37^oC) in both the basolateral and luminal reservoirs. Tissues were voltage-clamped at 0 mV using an automatic voltage clamp (EVC 4000, World Precision Instruments, Sarasota, Florida, US) and the short-circuit current (I_{sc}) required to maintain the potential at 0 mV was monitored as a reflection of the net active ion transport across the epithelium. Resistance was calculated using Ohms law. Experiments were carried out simultaneously in chambers connected to a PC equipped with DataTrax II software (World Precision Instruments). Following mounting, tissue was allowed to equilibrate (~ 1 hour) until a stable baseline was achieved. Reagents were added to the basolateral chamber.

Statistical Analyses

Data was analysed using GraphPad prism for windows (version 7). The data are represented as mean values ± the standard error of the mean. Assuming a null hypothesis, Students' or paired t-

tests were used when comparing two groups and repeated measures ANOVA with Bonferroni's multiple comparison post-hoc test were used when comparing more than two groups. Chi squared (χ^2) tests were used to compare percentages. $P \leq 0.05$ was considered significant.

Results:

Leptin has neurostimulatory effects in submucosal but not myenteric neurons.

Locally secreted leptin could have neurostimulatory effects on colonic neurons. Thus, submucosal and myenteric neurons were investigated to determine if leptin could induce signalling in this region. Fixed preparations of submucosal ganglia from healthy rat colons displayed abundant, cytosolic expression of both leptin protein and ObRs (figures 1A and 1B). Dual-labelling of SMP tissue preparations for leptin and IL-6 displayed significant co-localization. Expression of both proteins was punctate and concentrated in the neuronal cytosol and at the cell membrane. Quantification of neurons determined that 79% of neurons expressing IL-6 also expressed leptin (n=39 neurons from n=9 ganglia from 3 rats, figure 1A). Expression of IL-6Rs and ObRs was also prevalent in submucosal neurons, however only 14% of neurons were found to co-express both receptors (n=32 neurons from 9 ganglia from 3 rats, figure 1B). Calcium imaging studies demonstrated that exposure of submucosal neurons to leptin resulted in an increase in intracellular calcium. A dose-response curve for leptin in SMP neurons revealed robust but short-lived increases in intracellular calcium which increased in amplitude in a concentration dependent manner from 10pM to 1nM (each applied for 3 minutes). No further increase in amplitude was noted by application of 10nM leptin so 1nM recombinant leptin was used for the remainder of the calcium imaging experiments (figure 1C). At this concentration,

leptin activated 17% of submucosal neurons responsive to high K^+ whereas we previously observed that ~43% of neurons are excited by IL-6 (O'Malley *et al.*, 2011a). Sequential addition of 1nM leptin evoked reproducible calcium responses (0.04 ±0.004 vs 0.03 ±0.004 vs 0.04 ±0.01, n=24 neurons, $p>0.05$, repeated-measures ANOVA). With evidence of IL-6R and ObR co-localization in submucosal neurons, calcium imaging was used to confirm that the same submucosal neurons responded to both leptin and IL-6 and to assess potential crosstalk between the adipokine and cytokine. Recombinant leptin (1nM, 3 min) evoked a calcium response of equivalent amplitude to the calcium influx stimulated by IL-6 (1nM, 3 min, $p>0.05$, n=24 neurons from 3 rats). However, it was interesting to note that the application of both IL-6 and leptin together induced a response of smaller amplitude than leptin alone ($p<0.05$, figure 1D), indicating potential interactions between these neuromodulatory reagents.

Leptin activates JAK-STAT signalling cascades in submucosal neurons.

Given neurostimulatory effects of leptin in submucosal neurons, further calcium imaging studies were carried out in the SMP to determine the intracellular mechanisms underlying this action. The neurotoxin, tetrodotoxin (TTX, 100nM, 20min) resulted in just a small decrease in amplitude of the leptin-induced response (n=34, $p<0.001$), revealing a considerable TTX-insensitive component (figure 2A). In terms of intracellular signalling molecules, a signal transducer and activator of transcription (STAT) 3-specific pharmacological inhibitor, WP1006 (10µM, 20min), which had no effect on calcium levels *per se*, attenuated the leptin-evoked response (n=31, $p<0.001$). The smaller neuronal response to leptin and IL-6 together ($p<0.001$), was unchanged in the presence of WP1006 ($p>0.05$, figure 2B). Interestingly, inhibition of the PI 3-kinse signalling pathway with wortmannin (10µM, 20min) which had no effect on $[Ca^{2+}]_i$,

potentiated the leptin-evoked response in submucosal neurons (n=25, $p<0.001$). However, the smaller amplitude response to leptin and IL-6 together ($p<0.05$) was not further changed by wortmannin (n=27, $p>0.05$, figure 2C). The ERK-MAPK inhibitor, PD98059 (10μM, 30min) had no effect on $[Ca^{2+}]_i$ nor the leptin-evoked calcium response (n=22, $p>0.05$). The suppressed calcium response of leptin in the presence of IL-6 was also unaffected by PD98059 (n=32, $p>0.05$, figure 2D).

Leptin does not stimulate colonic absorpto-secretory function

As submucosal neurons are important in the regulation of colonic absorption and secretion, Ussing chambers were used to determine if leptin had an impact on absorptive or secretory function in healthy rat colons. Given that levels of IL-6 are elevated in the plasma of IBS patients (Dinan *et al.*, 2006; Liebregts *et al.*, 2007; Dinan *et al.*, 2008; McKernan *et al.*, 2011), blood vessels feeding the basolateral side of the colon are the likely site of action for this cytokine. Leptin secreted locally by adipocytes are also likely to interact with this side of the mucosal barrier. Hence, both exogenous leptin and IL-6 and other pharmacological reagents were added to the basolateral Ussing chamber. Short circuit current (I_{SC}) measurements were used as an indicator of ionic movement across colonic tissue and transepithelial resistance (TER) as an indicator of gut permeability. Similar to previous findings (O'Malley *et al.*, 2013) the relative change in TER over the course of the experiment increased in tissues stimulated with IL-6 (22.8 ±2.9 Ω/cm^2, $p<0.01$ compared to the start of the experiment), whereas saline-treated control preparations showed no change (n = 8, $p>0.05$). Leptin didn't evoke any change in TER over the course of the experiment (n=8, $p>0.05$). However, tissue incubated with both IL-6 & leptin showed an increase in TER (n=8, 17.9±2.4 Ωcm^{-2}, $p<0.01$, figure 3A) that was not different to

the change in resistance evoked by IL-6 alone ($p>0.05$, ANOVA comparing TER at the end of the experiment).

Fifteen minutes after the addition of leptin (1nM) a small increase in I_{SC} was recorded but this didn't reach significance ($p>0.05$, $n=9$). As previously reported (O'Malley *et al.*, 2011a), IL-6 stimulated a small increase in basal current ($p<0.05$, $n=8$) and this was unaffected by the presence of leptin ($n=8$, $p>0.05$, figure 3B). We have earlier demonstrated that IL-6-evoked calcium responses in submucosal neuron are inhibited by TTX. Moreover IL-6 potentiates currents induced by the Na^+ channel activator, veratridine (O'Malley *et al.*, 2011a). We reconfirmed the importance of neural regulation in the IL-6-evoked modulation of absorpto-secretory function ($n=8$, $p<0.01$) but found no such effect with leptin, which didn't potentiate veratridine- (10µM, 15 minutes) evoked secretory currents ($n=8$, $p>0.05$). However, interestingly, leptin did suppress the stimulatory actions of IL-6 on the veratridine-evoked current ($n=8$, $p<0.05$, figure 3C).

To assess the role of the parasympathetic neurotransmitter acetylcholine, in colonic secretion, the I_{SC} evoked by the cholinergic agonist, carbachol (10µM, 15 min) was compared under basal conditions and in the presence of leptin, IL-6 and IL-6 & leptin. We previously observed IL-6-mediated enhancement of a current evoked by bethanechol, another cholinergic agonist (O'Malley *et al.*, 2011a), and in these studies IL-6 similarly potentiated the carbachol-evoked cholinergic current ($n=8$, $p<0.05$) whereas leptin had no effect on cholinergic secretion *per se* ($n=8$, $p>0.05$), nor did it affect the capacity of IL-6 to potentiate secretion ($n=8$, $p<0.01$, figure 3D). Finally, the effects of leptin on capsaicin-evoked colonic currents were assessed. This

sensory nerve stimulant (1μM, 15 minutes) evokes a biphasic change in I_{SC} with a small secretory response followed by a larger and more sustained anti-secretory current. Consistent with previous findings (O'Malley *et al.*, 2011a), IL-6 did not significantly alter this current in either of the phases (n=8, p>0.05). Exposure of the basolateral colonic surface to leptin had no impact on the current evoked by capsaicin, which was similar to the control response (n=8, p>0.05). Similarly, the capsaicin-evoked current in the presence of leptin and IL-6 was not significantly different to the control (n=8, p>0.05, figure 3E).

Leptin modulates IL-6 mediated increases in colonic contractility

We have previously determined that IL-6-evoked colonic contractions are mediated via direct effects on smooth muscle and also via activation of enteric neurons (Buckley *et al.*, 2014). Immunofluorescent staining of myenteric ganglia displayed weak expression of ObRs in comparison to strong expression of IL-6Rs in the same neurons (n=4, figure 4A). Consistent with these expression levels, IL-6 (1nM, 3 min) evoked robust calcium responses in myenteric neurons whereas the change in intracellular calcium evoked by leptin was negligible. Co-application of leptin and IL-6 resulted in a calcium response similar to that evoked by IL-6 alone (p>0.05, figure 4B).

In organ baths, addition of carbachol (100μM) at the beginning and end of the experiment demonstrated that evoked contractions in the distal colon were not diminished over time (p>0.05, n=5, data not shown). When colonic samples were suspended to measure circular muscle activity, we found that both the amplitude (0.3 ±0.01 vs 0.8 ±0.2 milli Newtons (mN), p<0.05) and frequency (0.009 ±0.001 vs 0.2 ±0.03 contractions/minute, p<0.05) of contractions was enhanced when leptin (1nM) was added to the organ bath. The area under the curve (AUC) was

also measured as an assessment of the total response (n=5, figure 4C). The stimulatory effects of leptin on circular muscle contractile activity was smaller than contractions evoked by IL-6 both in amplitude (4.0 ± 1.0 mN, $p<0.05$) and frequency (1.39 ±0.45 contractions per minute, n=5, $p<0.05$). However, application of IL-6 to the bath caused a notable decrease in colonic tone (decrease of 2.0 ±0.2 mN, noted in 4/5 colonic strips) in circular muscle tissue preparations. Interestingly, addition of leptin and IL-6 together, resulted in some recovery of colonic tone (increase of 3.0 ±1.0 mN, n=5, $p<0.01$), but suppressed the generation of distinctive colonic contractions as compared to control IL-6 effects (n=5, $p<0.01$ figure 4C).

Overall, contractions evoked in longitudinal muscle were of smaller amplitude than those observed for circular muscle, but continuous contractions were frequently seen in unstimulated tissue. Leptin evoked contractions of increased amplitude (0.15 ±0.04 mN) and frequency (0.48 ±0.09 contractions/min, n=5) as compared to baseline values of 0.09 ±0.01 mN and 0.11 ±0.03 contractions/min ($p<0.05$) in longitudinal muscle. However, the contractile activity stimulated by IL-6 was also of larger amplitude (1.0 ±0.3 mN), frequency (1.32 ±0.45 contractions per minute) and AUC than that evoked by leptin ($p<0.05$), although no change in tone was observed. The IL-6-evoked response was suppressed when leptin was also added to the bath ($p<0.05$, figure 4D) similar to the effect seen in circular muscle.

Tetrodotoxin (TTX, 100nM, 20min) had little effect on the stimulatory actions of leptin on either circular (n=3, $p<0.05$, figure 4E) or longitudinal (n=3, $p<0.05$, figure 4F) muscle contractile activity indicating that the leptin-mediated excitation of colonic contractility is not likely to be neurally-mediated and is probably due to direct effects on smooth muscle. This is consistent with the observation that leptin had minimal neuroexcitatory effects on myenteric neurons, which are

the primary regulators of colonic motility with no further change in calcium levels noted in the presence of TTX (change in ratio: 0.003 ±0.002 vs 0.01 ±0.01, n=23, $p>0.05$).

Leptin attenuates calcium responses evoked by soluble mediators in IBS plasma.

Although leptin levels are undetermined in these samples, a previously published report using these samples found no difference in body mass index between healthy controls and IBS patients, which were both in the healthy weight range (McKernan *et al.*, 2011). To minimize variability in responses, a pooled sample of plasma from 6 IBS patients representing each of the IBS subtypes, was used to investigate the importance of leptin in the neuromodulatory effects of IBS plasma. In immunofluorescent studies, exposure of submucosal neurons to IBS plasma (1:250 dilution, 15 minutes) resulted in nuclear expression of the cFos early transgene in 51% of leptin immunopositive submucosal neurons. By comparison only 37% of submucosal neurons exposed to plasma from healthy controls (HCs, 1:250 dilution, 15 minutes) expressed nuclear cFos staining in leptin-expressing neurons ($p<0.01$, $\chi^2 = 8.4$, figure 5A).

We have previously found that IBS plasma evokes robust increases in calcium in both enteric neuronal plexi whereas healthy plasma is not neurostimulatory, effects mediated in part by interleukins and CRF (Buckley *et al.*, 2014; O'Malley *et al.*, 2015). In the current studies, IBS plasma similarly evoked robust calcium responses in 38% of myenteric and 27% of submucosal neurons tested. To mimic elevated leptin levels, which would be characteristic of obesity and may have neuro-protective effects in the enteric nervous system (Baudry *et al.*, 2012), recombinant leptin (1nM) was added to the sample of pooled IBS plasma prior to application. A smaller calcium response was evoked in both submucosal ($p<0.05$, figure 5B) and even

myenteric ($p<0.001$, figure 5C) neurons when leptin levels were elevated, as compared to control IBS plasma responses.

In the submucosal plexus, 27% (n=26/98) of neurons were activated by IBS plasma. When IBS plasma was incubated with leptin, only 14% (n=14/98) of neurons were activated. Of the 26 neurons activated by IBS plasma, 17 (65%) were inhibited and 9 (35%) were insensitive to the effects of leptin. Similarly, other submucosal neurons, not initially activated by IBS plasma, were activated when leptin was co-applied. While 38% (n=45/118) of myenteric neurons were stimulated by IBS plasma, only 18% (n=21/118) of neurons responded to IBS plasma when leptin was co-applied. Thus, the decrease in mean amplitude of the calcium responses was due to two contrasting effects of leptin. Of the 45 myenteric neurons activated by IBS plasma, 30 (67%) were inhibited by leptin whereas 15 (33%) were not sensitive to the inhibitory effects of leptin. Another subgroup of neurons not initially activated by IBS plasma, were activated by the addition of leptin.

Complimentary studies found that prior incubation of the IBS plasma sample with antibodies against leptin (xleptin), to bind and inactivate any endogenous leptin present in the plasma, potentiated the response evoked by IBS plasma in both myenteric (n=46, $p<0.001$) and submucosal (n=26, $p<0.05$, figure 5D) neurons.

Discussion

Previous studies demonstrated that leptin, an adipokine with modulatory effects on bowel function (El Homsi *et al.*, 2007; Reichardt *et al.*, 2011), increases neuronal firing in both submucosal and myenteric neurons in the guinea-pig colon, an effect that was more pronounced in the submucosal plexus (Reichardt *et al.*, 2011). On the whole, our data concur with these findings, with leptin stimulating increased neuro-excitability in submucosal neurons from rat colons with little or no responses generated in myenteric neurons. Similar to a report that failed to see functional effects of leptin in contractility or secretion (Reichardt *et al.*, 2011), our studies did not find that leptin directly stimulated colonic secretion or contractile activity. However, Reichardt *et al* proposed that leptin exerts subtle effects in the colon, that are likely to alter function only in concert with other mediators (Reichardt *et al.*, 2011) and indeed, we have shown that leptin can modify the pro-secretory and pro-kinetic effects of the pro-inflammatory cytokine, IL-6 on colonic function. Intriguingly, leptin suppressed the neuro-stimulatory effects of IBS plasma, which has elevated levels of IL-6, in both submucosal and myenteric neurons, indicating a possible role for leptin in ameliorating the unfavourable effects of pro-inflammatory cytokines in GI dysfunction associated with IBS.

An Iranian study with 80 IBS patients and 80 controls found that leptin levels are lower in IBS plasma (Semnani *et al.*, 2009), suggesting a possible role for leptin in the pathophysiology of the disorder. That said, another group comparing diarrhoea-predominant IBS patients with healthy controls and those with coeliac disease noted elevations in both leptin and IL-6 in the IBS-D group (Russo *et al.*, 2013). In our studies, soluble mediators present in IBS plasma stimulated more cFos activation in leptin-containing submucosal neurons that healthy control plasma.

Although leptin levels are unknown in these samples, the patients had comparable body mass indices (McKernan *et al.,* 2011). As we have previously shown that IBS secretory products, which include cytokines and CRF, potentiate neuronal excitability in enteric neurons (O'Malley *et al.*, 2011a; O'Malley *et al.*, 2012; Buckley *et al.*, 2014), it was interesting to note in the current studies that artificially increasing leptin levels in IBS plasma ameliorated the neuro-excitatory effects in both myenteric and submucosal neurons. The regulatory role of leptin was further confirmed by neutralizing leptin present in IBS plasma using an antibody against the leptin protein, which resulted in potentiation of the calcium responses in both myenteric and submucosal neurons. Although these results are consistent with a study that demonstrated that elevated levels of circulating leptin is neuroprotective in the enteric nervous system (Baudry *et al.*, 2012), the mechanism by which leptin can modulate the amplitude of calcium response in these neuronal plexi requires further investigation. This is particularly true in the myenteric neurons, which showed minimal expression of ObRs and responsivity to recombinant leptin. The effects of leptin on gut function are complex, as confirmed by a study in rats which found that two groups of vagal afferent nerve endings innervated the stomach, one group is leptin-sensitive and stimulates motility, whereas a second group is leptin-insensitive and inhibits motility (Wang *et al.*, 1997). We similarly noted two groups of neurons that were either sensitive or insensitive to leptin. In both myenteric and submucosal neurons, approximately two thirds of neurons excited by IBS plasma became quiescent when leptin was co-applied, with the remainder being insensitive to leptin.

The source of leptin may be important (Hanani *et al.*, 2007), but whether leptin stimulates the gut from the luminal side, acting in a manner similar to pro-inflammatory cytokines (Sitaraman *et*

al., 2004) or from the adipocytes, where diet-induced obesity is related to increased colonic transit with concurrent increases in both leptin and IL-6 levels (Reichardt *et al.*, 2013), it is apparent that a relationship exists between leptin and IL-6. Indeed, leptin induces upregulation of pro-inflammatory cytokines, such as IL-6, in colonic cells (Padidar *et al.*, 2011) and ObR and IL-6R signalling cascades exhibit significant homology. Both activate signalling cascades involving JAK-STAT (Bjorbaek & Kahn, 2004), PI 3-kinase (Shanley *et al.*, 2002) and MAPK (Cui *et al.*, 2006). Our studies found that the JAK-STAT nuclear transcription activating pathway was key to the neurostimulatory effects of leptin in submucosal neurons. Interestingly, PI 3-kinase, which is an important neuronal signalling molecule that regulates cellular differentiation, growth, survival and intracellular trafficking, appeared to be a negative regulator of leptin-evoked neuronal stimulation as wortmannin potentiated leptin-evoked stimulation of submucosal neurons. ERK-MAPK signalling did not appear to mediate the neuro-excitatory effects of leptin in either the absence or presence of IL-6. These observations suggest that activation of leptin receptors in submucosal neurons evokes calcium responses mediated by JAK-STAT signalling. The suppression of the leptin response in the presence of IL-6 was unchanged by the STAT3 inhibitor but this may reflect a floor effect. PI-3 kinase appears to negatively regulate the leptin evoked response but didn't affect the calcium response evoked by combined application of IL-6 and leptin. Potential crosstalk between leptin and IL-6 was indicated by suppression of the leptin-evoked calcium response when both leptin and IL-6 were applied to the neurons at the same time. However, it is not clear at which point in the cellular signalling cascades this crosstalk occurs as the calcium response to leptin and IL-6 together was unaffected by any of the inhibitors. Though the leptin-evoked calcium response in submucosal neurons was mediated in part by TTX-sensitive Na^+ channels, a significant component of the rise in neuronal calcium

levels was due to mechanisms which did not involve voltage gated Na^+ channels, or perhaps utilized TTX-insensitive Na^+ channels (Yoshida, 1994).

To determine if the neuro-stimulatory effects of leptin on submucosal neurons resulted in functional changes in gut barrier permeability and ionic movement across the mucosa, changes in TER were measured. Consistent with previous studies (Wang *et al.*, 2007; O'Malley *et al.*, 2012), IL-6 evoked an increase in TER, indicating that acute exposure to this pro-inflammatory cytokine helps to maintain the integrity of the gut barrier. Leptin has previously been observed to contribute to barrier breakdown and inflammation (Sitaraman *et al.*, 2004); however, these effects are thought to be mediated from the luminal side. Application of leptin to the basolateral side of the colonic tissue had no effect on TER over the observed period, nor did leptin influence the protective effects of IL-6. Indeed, leptin-deficient ob/ob mice do not display changes in gut permeability (Stenman *et al.*, 2013). Similar to previous observations (O'Malley *et al.*, 2012) and consistent with the neurostimulatory effects of IL-6 on submucosal neurons, a small secretory current was evoked by IL-6. However, despite the neuroexcitatory effects of leptin in submucosal neurons, no changes in I_{SC} were noted, which is consistent with previous reports (Reichardt *et al.*, 2011), nor did leptin alter the current evoked by IL-6. Moreover, while IL-6 potentiated the neurally-mediated secretory current evoked by the Na^+ channel agonist, veratridine and the cholinergically-mediated current evoked by carbachol, leptin had no effect on these currents nor the biphasic current evoked by capsaicin, a TRPV1 agonist which activates visceral afferent neurons to stimulate mucosal electrolyte transport and fluid secretion (Vanner & MacNaughton, 1995). When the tissue was exposed to IL-6 in the presence of leptin, no change was noted in the capacity of IL-6 to directly evoke a secretory current. However, leptin did

attenuate the IL-6-mediated enhancement of the veratridine-evoked current, although it had no effect of the carbachol-evoked current. When considering this result in the context of the calcium responses in submucosal neurons, it may be that leptin-sensitive and leptin-insensitive neurons underlie changes in secretion. Given the lack of effect of leptin on the IL-6-evoked potentiation of the carbachol current, it may indicate that cholinergic neurons in the submucosal ganglia are insensitive to leptin, although further investigation is required to confirm this.

In terms of colonic contractility, we have previously reported the stimulatory actions of IL-6 on both circular and longitudinal colonic muscle (Buckley *et al.*, 2014), an effect that was mediated, at least in part, by myenteric neurons. Leptin also stimulated increased contractile activity, although this was smaller in amplitude than the IL-6 effect. Interestingly, when both leptin and IL-6 were added together, leptin suppressed the IL-6-evoked contractile activity. This was noted in both circular and longitudinal muscle layers and indicates likely cellular crosstalk between the factors. While myenteric neurons contributed to the IL-6-evoked colonic contractility, this is not the case for leptin. Myenteric neurons exhibited strong expression of IL-6Rs but little ObR expression. Consistent with this finding, leptin did not evoke calcium responses in myenteric neurons. Furthermore, TTX had no effect on leptin-evoked contractile activity in either the circular or longitudinal colonic muscle strips, suggesting that the capacity of leptin to suppress IL-6-evoked activity may be mediated through its actions on smooth muscle rather than via myenteric neurons.

These studies demonstrate that leptin has neuromodulatory effects in submucosal neurons, stimulating increases in intracellular calcium via the JAK-STAT signalling pathway. Although

there was little direct effect of leptin on absorpto-secretory function, it did modify the neuronally-mediated stimulatory effects of IL-6. Furthermore, a potentially protective role has been identified for leptin in colonic dysmotility, a key symptom of IBS. IL-6, which is elevated in IBS, evoked an increase of colonic contractile activity and leptin suppressed this activity. The mechanism of this crosstalk between leptin and IL-6 remains to be elucidated but it is likely to be through indirect effects, possibly on smooth muscle rather than in the myenteric neurons. These studies have demonstrated that leptin can modify the pro-secretory and pro-kinetic effects of IL-6 on colonic function. Intriguingly, leptin suppressed the neuro-stimulatory effects of IBS plasma, which has elevated levels of IL-6, in both submucosal and myenteric neurons, indicating a possible role for leptin in ameliorating IBS-associated GI dysfunction.

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DO'M: conception or design of the work; acquisition, analysis, or interpretation of data for the work AND drafting the work or revising it critically for important intellectual content.

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Competing Interests

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Figure 1: Leptin stimulates submucosal neurons

A: The representative immunofluorescent images illustrate strong staining of rat submucosal neurons with antibodies against IL-6 (red staining) and leptin (green staining) with evidence of co-localization (yellow staining) in the merged image. **B:** Submucosal neurons also express IL-6 receptors (IL-6R, green staining) and leptin receptors (ObR, red staining) with evidence of co-expression (yellow staining, n=3 colons) in the merged image. Scale bar: 200µm **C:** The dot plot and trace illustrate the calcium response to increasing concentrations of leptin and a control response to high K^+. **D:** The dot plot and representative trace illustrates activation of the same submucosal neurons by both IL-6 and leptin and suppression of the IL-6-evoked response by leptin (n=24 neurons). * and *** indicate $p<0.05$ and $p<0.001$, respectively.

Figure 2: Leptin-evoked calcium responses in submucosal neurons activate JAK-STAT signalling

A: The dot plot and representative trace demonstrate a large calcium response evoked by leptin (1nM, 3min) in submucosal neurons which was reduced by TTX (100nM, 20min, n=34 neurons). **B:** The leptin-evoked calcium response is diminished by the STAT3 inhibitor, WP1006 (WP, 10 µM, 20min, n=31) although the inhibitory effect of IL-6 on leptin is not further changed by WP (n=13). **C:** The leptin-evoked calcium response is potentiated by the PI 3-kinase inhibitor, wortmannin (wort, 10µM, 20 min, n=25). However, potentiation of the leptin-evoked response by wort was suppressed by the presence of IL-6. **D:** The ERK-MAPK inhibitor, PD98059 (PD, 10µM, 30 min, n=22) has no effect on the leptin-evoked calcium response. The presence of IL-6 (1nM) suppresses the leptin-evoked response but PD has no additional effect (n=32). * and *** indicates $p<0.05$ and $p<0.001$, respectively.

Figure 3: Colonic absorpto-secretory function is modified by IL-6 but not leptin.

A: The graph shows the changes in transepithelial resistance (TER) in colonic tissue exposed to saline (control), leptin (1nM), IL-6 (1nM) or leptin & IL-6 over the course of an experiment (1.8 ±0.3 hours). TER in the IL-6 and leptin & IL-6 groups is significantly increased when the end of the experiment is compared to the start. **B:** The histogram and representative traces show changes in colonic short circuit current (I_{SC}). Basal, unstimulated conditions are labelled control. A secretory current was generated by IL-6 (1nM, 15 min) in the absence or presence of leptin (1nM, 15 min). Leptin did not stimulate colonic secretion (n=9). **C:** Veratridine (10µM, 15 min) stimulates a large Na^+ channel-mediated secretory current which is further enhanced by IL-6 but not leptin. The IL-6-evoked potentiation of this current is inhibited by leptin (n=9). **D:** Carbachol (10µM, 15 min) - evoked secretion is enhanced by IL-6 but not leptin. Leptin does not affect the IL-6-evoked potentiation of carbachol secretion (n=9). **E:** The biphasic response to capsaicin (1µM, 15 min) wasn't significantly altered by IL-6, leptin or IL-6 & leptin in these experiments (n=9). * and ** indicate $p<0.05$ and $P<0.01$, respectively.

Figure 4: Leptin attenuates IL-6-evoked contractile activity.

A: The representative immunofluorescent images illustrate strong staining of myenteric neurons with antibodies against IL-6 receptors (IL-6R, green staining) but weak expression of leptin receptors (ObR, red staining) and little co-localization (yellow staining, n=3 colons) in the merged image. Scale bar: 20µm. **B:** The dot plot and trace demonstrate that the IL-6- evoked calcium response in myenteric neurons is not significantly changed by leptin. **C:** The histogram of the area under the curve (AUC) in arbitrary units (au) and traces illustrate how leptin (1nM,

20min) inhibits IL-6 (1nM, 20min)-evoked circular muscle contractile activity in circular and **D:** longitudinal muscle (n=5 colons). **E:** The histogram and trace illustrates that TTX (100nM, 20min) does not change the stimulatory effect of leptin on circular muscle or **F:** longitudinal contractile activity (n=5). *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Figure 5: Leptin levels are elevated in IBS plasma

A: The representative immunofluorescence images and histogram illustrates expression of the early transgene, cFos in the leptin-expressing submucosal neurons exposed to plasma from HCs (n=6 pooled samples, 15 minutes exposure) and IBS patients (n=6 pooled samples from all IBS subsets, 15 minutes exposure). **B:** The dot plot and representative trace illustrates suppression of IBS plasma-evoked calcium responses in submucosal neurons by co-application of leptin (n=26). **C:** The dot plot and representative trace illustrates the robust calcium response evoked by IBS plasma (1:250 dilution) in myenteric neurons is reduced by co-application of leptin (1nM, 3min, n=45). **D:** The dot plots illustrate the response of submucosal (n=26) and myenteric (n=46) neurons to IBS plasma in the presence of either recombinant leptin or anti-leptin antibody (xleptin). *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively.