

Modulation of enteric neurons by interleukin-6 and corticotropin-releasing factor contributes to visceral hypersensitivity and altered colonic motility in a rat model of irritable bowel syndrome

Maria M. Buckley^{1,2}, Ken D. O'Halloran², Mark G. Rae², Timothy G. Dinan^{1,3} and Dervla O'Malley^{1,2}

¹Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland

²Department of Physiology, University College Cork, Cork, Ireland

³Department of Psychiatry, University College Cork, Cork, Ireland

Key points

- Hyperactivity of the stress system and low-grade immune activation characterize the functional bowel disorder irritable bowel syndrome (IBS).
- These studies show that interleukin (IL)-6 and IL-8 and the stress hormone corticotropin-releasing factor (CRF), present in IBS plasma, have functional effects on gastrointestinal activity by stimulating myenteric neurons and colonic contractions.
- Moreover, in the Wistar Kyoto rat model of IBS, which exhibits altered gastrointestinal motility and visceral pain sensitivity, blocking IL-6 and/or CRF1 receptors alleviates these IBS-like symptoms.
- Underlying these effects are altered colonic protein expression of tight junction proteins which regulate gut barrier function and the T-type calcium channel $Ca_v3.2$, which has been linked to visceral pain.
- These findings demonstrate the importance of the enteric nervous system and intestinal physiology in bowel dysfunction.

Abstract The search for effective therapeutic strategies for irritable bowel syndrome (IBS) is hampered by an incomplete understanding of its underlying pathophysiology. Stress and altered plasma cytokine profiles indicative of immune activation are characteristic of the disorder. The neuromodulatory effects of interleukin-6 (IL-6) and corticotropin-releasing factor receptor (CRFR) 1 in visceral pain and stress-induced defecation in the Wistar Kyoto (WKY) rat model of IBS were investigated. Sprague Dawley and WKY rats were administered anti-IL-6 receptor antibodies (α IL-6R, 0.5 mg kg⁻¹ i.p.) with or without the CRFR1 antagonist antalarmin (10 mg kg⁻¹ i.p.). Post-intervention, the pain threshold to colorectal distension and stress-induced faecal output were compared and changes in colonic mucosal protein expression were investigated. The neuro-stimulatory effects of IBS plasma on the myenteric plexus is mediated by IL-6, IL-8 and CRF. The stimulatory effects of these soluble factors on myenteric neuron excitability and colonic contractility were additive. Moreover, inhibition of IL-6 and CRF1 receptors *in vivo* in the WKY IBS rat model normalized stress-induced defecation ($P < 0.01$) and visceral pain sensitivity ($P < 0.001$) with associated changes in protein expression of the tight junction proteins occludin and claudin 2, the visceral pain-associated T-type calcium channel $Ca_v3.2$ and intracellular signalling molecules STAT3, SOCS3 and ERK1/2. These studies demonstrate the additive effects of immune and stress factors on myenteric neuronal excitability. Moreover, combined targeting of peripheral IL-6 and CRF1 receptors is effective in alleviating IBS-like symptoms in the WKY

rat. Thus, crosstalk between stress and immune factors during IBS flares may underlie symptom exacerbation.

(Received 23 June 2014; accepted after revision 19 September 2014; first published online 25 September 2014)

Corresponding author D. O'Malley: Department of Physiology, 4.23 Western Gateway Building, University College Cork, Cork, Ireland. Email: d.omalley@ucc.ie

Abbreviations CRD, colorectal distension; CRF, corticotropin-releasing factor; CRFR, CRF receptor; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ERK-MAPK, extracellular signal-regulated kinase-mitogen activated protein kinase; GI, gastrointestinal; HPA, hypothalamic-pituitary-adrenal; IBS, irritable bowel syndrome; IKK₂, IκappaB kinase₂; IL, interleukin; IL-6R, interleukin-6 receptor; JAK-STAT, janus tyrosine kinase and signal transducers and activators of transcription; LMMP, longitudinal muscle myenteric plexus; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; OF, open field; SD, Sprague Dawley; SOCS, suppressor of cytokine signalling; TER, trans-epithelial resistance; WKY, Wistar Kyoto; xIL-6R, anti-IL-6R.

Introduction

Irritable bowel syndrome (IBS) is a highly prevalent functional gastrointestinal (GI) disorder affecting up to 15–20% of the population (Lovell & Ford, 2012). Characterized by chronic episodic bouts of abdominal pain, bloating and altered bowel habit (Longstreth *et al.* 2006), IBS substantially impairs sufferers' quality of life and confers a heavy economic and social burden on society. The underlying pathophysiology of this functional bowel disorder is poorly understood and a lack of reproducible biomarkers hampers diagnosis and therapeutic target identification. Nonetheless it is generally accepted that dysfunction of the bidirectional communication system between the brain and the gut contributes to symptom manifestation (Shanahan & Anton, 1988; Cryan & O'Mahony, 2011). Integral to the initiation, prolongation and persistence of IBS symptom flares is activation of the hypothalamic–pituitary–adrenal (HPA) stress axis (Bohmelt *et al.* 2005; Dinan *et al.* 2006; Chang *et al.* 2009). Indeed, mimicking the effects of stress, the hypothalamic hormone corticotropin-releasing factor (CRF) induces pathophysiological changes in GI function (Mawdsley & Rampton, 2005). CRF exerts its biological effects through activation of the CRF1 and CRF2 receptors (CRFR1 and CRFR2), which are expressed in both the CNS and the enteric nervous system of the GI tract (Tache & Bonaz, 2007; O'Malley *et al.* 2010b, 2011b).

The importance of immune system activation in the development of IBS is well recognized with a bout of inflammatory gastroenteritis conferring increased susceptibility to develop IBS (Spiller & Garsed, 2009). IBS mucosal biopsies display more immune infiltrates such as T-cells, intra-epithelial lymphocytes and mast cells (Chadwick *et al.* 2002) and soluble mediators secreted from IBS biopsies have excitatory actions on human enteric neurons (Buhner *et al.* 2009). Moreover, we previously demonstrated that colonic secretions from the Wistar Kyoto (WKY) rat model of IBS stimulated naïve submucosal neurons to a greater extent than secretions from low anxiety Sprague Dawley (SD) controls, an

effect mediated in part by the pro-inflammatory cytokine interleukin-6 (IL-6) (O'Malley *et al.* 2011a). IL-6, along with other cytokines such as IL-1β and tumour necrosis factor α, are elevated in IBS peripheral mononuclear cells (Liebregts *et al.* 2007) and circulating levels of IL-6 and IL-8 have reproducibly been found to be raised in IBS patients (Dinan *et al.* 2006; Liebregts *et al.* 2007; Dinan *et al.* 2008). However, more than simply being a biomarker for IBS (Clarke *et al.* 2009), IL-6 activates submucosal secretomotor neurons (Xia *et al.* 1999; O'Malley *et al.* 2011c) and modulates mucosal ion transport and epithelial permeability (Natale *et al.* 2003; Kindt *et al.* 2010) suggesting a causative role for IL-6 in this disorder.

Both psychological stress and immune activation have been proposed as contributory factors in symptom flares, and therefore crosstalk between these systems may exacerbate symptoms (O'Malley *et al.* 2011d, 2013). In support of this, low-grade inflammatory changes correlate with alterations in the stress axis in IBS (Heitkemper *et al.* 1996; Bohmelt *et al.* 2005; Dinan *et al.* 2006) and IBS patients often exhibit concurrent increases in markers of a hyperactive stress response and immune upregulation (Dinan *et al.* 2006). To test the hypothesis that crosstalk between stress and immune factors results in exacerbation of IBS-like symptoms, such as visceral pain and altered bowel habit, the WKY rat model of IBS was treated with monoclonal anti-IL-6 receptor (xIL-6R) antibodies with and without the CRFR1 antagonist antalarmin.

Methods

Ethical approval

All experiments involving animals were conducted in full accordance with the European Community Council Directive (86/609/EEC) following University College Cork animal ethics committee approval.

The study protocol (APC020, 2009) for collecting blood samples from IBS patients and healthy volunteers was approved by the University College Cork Clinical Research

Ethics committee of the Cork University Hospital. Informed consent was obtained from all participants.

Animals and chronic treatments

SD and WKY rats (200–250 g), purchased from Harlan, Derbyshire, UK, were group-housed six per cage and maintained on a 12/12 h light–dark cycle (08:00–20:00 h) with a room temperature of $22 \pm 1^\circ\text{C}$. Rats were handled for at least 5 days by the same researcher prior to behavioural assessment. Food and water were available *ad libitum*. For the *in vivo* open field and colorectal distension (CRD) studies, SD and WKY rats were allocated to one of three groups: control, xIL-6R or xIL-6R plus antalarmin ($n = 12$ per group). All animal groups received an I.P. injection of anti-IgG (Jackson Immunoresearch, Westgrove, PA, USA, 1 mg kg^{-1}) on day 0 to induce antibody tolerance. Group 1 were administered saline and acted as the control group. Group 2 received an I.P. injection of monoclonal xIL-6R antibodies (1 mg kg^{-1} on day 0 and 0.5 mg kg^{-1} on days 3 and 10). Group 3 also received xIL-6R on days 0, 3 and 10 but additionally received the CRFR1 antagonist antalarmin (10 mg kg^{-1}) 1 h prior to the open field trial on day 10 and CRD on day 14. Following CRD, all animals were killed by decapitation. Trunk blood samples were centrifuged at 1600 g and plasma samples were stored at -80°C for later analysis. Sections of colon (2 cm long) were excised and snap frozen in liquid nitrogen for Western blotting analysis. Tissue was stored at -80°C for later processing.

Study participants and plasma samples

IBS patients aged 18–65 years who satisfied Rome II criteria for the diagnosis of IBS (Thompson *et al.* 1999) were recruited from gastroenterology clinics at Cork University Hospital, Cork, Ireland. Healthy controls were recruited from the research institute (Alimentary Pharmabiotic Centre) or hospital staff. Bowel habit for IBS patients was defined as constipation-predominant (IBS-C), diarrhoea-predominant (IBS-D) or alternating (IBS-A). Individuals with a history of psychiatric illness, inflammatory bowel disease, celiac disease, lactose intolerance, immunodeficiency or abdominal surgery were excluded. No patient was categorized as having post-infectious IBS. 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. Plasma samples were selected from a larger study previously published (McKernan *et al.* 2011b).

Twenty millilitres 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

400 g (30 min, room temperature). Consistent with previous studies in the laboratory (Dinan *et al.* 2006, 2008), plasma was collected and stored at -80°C . To maintain a consistency of response, pooled plasma samples ($n = 6$ samples in each group) from both healthy volunteers and IBS patients (IBS-D, IBS-C and IBS-A) were applied to the neurons and the responses collated.

Behavioural measurements

Open field trial. As previously described (O'Mahony *et al.* 2009, 2010a), animals were exposed to the psychological stress of being placed in the centre of a brightly illuminated (~ 800 lux), white open field (OF) arena (0.9 m in diameter, 38 cm in height) for a 10 min trial. Trials were conducted daily between 09:00 and 13:00 h and the arena was cleaned with 70% ethanol between trials. The number of faecal pellets excreted during the 10 min exposure was recorded as an indication of stress-induced defecation. Trials were recorded and the distance and velocity of movement was analysed offline.

Colorectal distension. Rats were fasted for 24 h before CRD and acclimatized to the testing room for 30 min prior to being lightly anaesthetized (Isoflurane, Abbott Animal Research, Ireland). As previously described (O'Mahony *et al.* 2009), a latex balloon (6 cm in length, Durex, Union, NJ, USA) was inserted into the colon, 1 cm from the anus. The animals were permitted a recovery time of 10 min before the CRD procedure was initiated. A ramp distension protocol was used where the balloon was distended from 0 to 80 mmHg over an 8 min period (increasing by 10 mmHg each minute). During this period two parameters were measured. The first was the threshold pressure that evoked visibly identifiable pain behaviours. Behaviours included were stretching, abdominal retractions and/or abdominal withdrawal reflex as previously described (O'Mahony *et al.* 2010). Secondly, the cumulative number of visceral pain behaviours over the course of the trial was counted. The animals were tested in a random fashion by a single blinded investigator.

Immunoassays

Western blotting. As previously described (O'Malley *et al.* 2011b), the supernatants from homogenized colonic mucosal samples taken from rats 1 h post-CRD were separated on 12% SDS polyacrylamide gels. Proteins were electro-transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked and incubated with primary antibodies against phosphoERK1/2, total ERK1/2, phosphoSTAT3, total STAT3 (all Cell Signaling Technology, Danvers, MA, USA), occludin (Abcam, Cambridge, UK), claudin 2 (Santa Cruz Biotechnology, Dallas, TX, USA) (all at 1:1000), suppressor of cytokine

signalling 3 (SOCS3; Everest Biotech, Upper Heyford, UK) (1:500) and Cav3.2 (Merck Millipore, Temecula, CA, USA) (1:200, overnight at 4 °C). Complimentary horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) (1:5000) and an ECL detection system (Pierce ECL Western Blotting Substrate, Rockford, IL, USA) were used to visualize the protein bands. β -Actin (Cell Signaling Technology) (1:1000) was used as a reference protein for occludin, claudin 2, SOCS3 and Cav3.2. Images were captured using a GelDoc Image Reader (Las3000; Fujifilm, Tokyo, Japan) and analysed using Multigauge v2.2 (Fujifilm) software. Protein densitometry was used to measure expression levels in arbitrary units as a ratio of the loading controls. Protein bands are comparable only between each sub-group which was analysed at the same time, under identical conditions. Protein samples for each experimental group (SD saline, WKY saline, WKY xIL-6R, WKY xIL-6R & antalarmin) were run in a single tank and probed under identical conditions.

ELISA. A sandwich ELISA (Legend MaxTM Rat IL-6 ELISA kit; BioLegend, San Diego, CA, USA) was carried out to determine IL-6 levels in plasma samples from control WKY ($n = 7$) and SD ($n = 7$) animals, and from WKY rats treated with either anti-IL-6R ($n = 8$) or anti-IL-6R and antalarmin ($n = 12$), according to the manufacturer's guidelines. The test was carried out in duplicate and the plate read on a fluorescent plate reader (Biotek Synergy HT plate reader, Gen5 data analysis software). A standard curve was generated from known IL-6 concentrations and sample IL-6 concentrations were extrapolated from the curve.

Calcium imaging in myenteric neurons. During an action potential, Ca^{2+} enters the neuron via voltage-gated channels and at the post-synaptic membrane neurotransmitters induce an influx of calcium via receptor-mediated receptors such as *N*-methyl-D-aspartate (NMDA) receptors, thereby contributing to dendritic action potentials. Thus, neuronal changes in calcium levels as imaged using a calcium-sensitive dye (Fura-2AM) are indicative of changes in neuronal excitability. Changes in intracellular calcium were recorded from myenteric neuronal cell bodies as reported (O'Malley *et al.* 2011c), SD rats were killed via an overdose of CO_2 and the distal colon (<4 cm from the anus) was excised and placed in ice-cold, 95% O_2 /5% CO_2 bubbled Krebs saline solution consisting of (mmol l^{-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 (11 with 1 μM nifedipine to inhibit smooth muscle contractions). Whole mount preparations of longitudinal muscle myenteric plexus (LMMP) were prepared by removing the mucosa and circular muscle, pinning the tissue out in Sylgard-lined petri dishes and

loading with Fura-2AM (7 μM , 1 h). Images were acquired at 3 Hz using a xenon/mercury arc burner (Olympus, Melville, NY, USA), a charge-coupled device digital camera (F-view II; Olympus Soft Imaging Solutions, Munster, Germany) and a 40 \times water-immersion objective on a fixed stage upright microscope (Olympus BX51WI). Ganglionic neurons were identified based on morphology and responsiveness to 75 mM KCl. Cell R software (Olympus Soft Imaging Solutions, 1986–2009) was used to record excitation and emission wavelengths of 340/380 and 510 nm, respectively, permitting ratiometric imaging. Responses are reported as a change in ratio. Responding neurons were defined as those with increases in intracellular calcium $[\text{Ca}^{2+}]_i$ greater than two standard deviations from baseline (calculated as the average ratio during the 150 s preceding stimulus application). A perfusion system continuously superfused the colonic tissue with carbogen-bubbled Krebs-buffered saline.

Antibody neutralization of IBS plasma (pooled from all IBS subtypes) with human neutralizing anti-IL-6 (1:100), anti-IL-8 (1:250), anti-C-reactive protein (anti-CRP, 1:200) and anti-IgG (1:200, all from R&D systems, Minneapolis, MN, USA) antibodies (1 h at 37 °C) was carried out prior to neuronal application.

Colonic contractile function. To measure circular muscle contractile activity from SD distal colons, mucosa was removed and, with the circular muscle orientated lengthwise, the tissue was suspended from a tension transducer under 1 g of tension in a bath of carbogen-bubbled Krebs saline at 37°C and allowed to equilibrate (20–30 min). Changes in tension were amplified, recorded and analysed using Powerlab and LabChart7 (both AD Instruments, Colorado Springs, CO, USA). Responses are reported as a percentage of the maximal response evoked by the muscarinic agonist carbachol (100 μM) in each experiment.

Statistics

Data are represented as mean \pm SEM. Student's *t*-tests and repeated measures one-way or two-way ANOVAs with Neumann Keuls *post-hoc* tests and Chi squared tests were used where appropriate. $P \leq 0.05$ was considered significant.

Results

IL-6 and CRF activate myenteric neurons and stimulate colonic contractility

We have previously demonstrated that the pro-inflammatory cytokine IL-6 stimulates increases in $[\text{Ca}^{2+}]_i$ in the submucosal plexus (O'Malley *et al.* 2011c) and that this is further modulated by the presence of CRF (O'Malley *et al.* 2013). We have interpreted this

rise in Ca^{2+} , which regulates the release of synaptic neurotransmitters and post-synaptic excitability, as an indicator of neuronal excitability. In total, 46% of LMMP neurons ($n = 35$ neurons from three rats) prepared from naïve SD rats were activated by IL-6 (1 nM, 3 min). Moreover, CRF (100 nM, 3 min), which has a higher affinity for CRFR1, activated 34% of the same neurons and co-application of IL-6 plus CRF activated 54% of these neurons and evoked a larger calcium response on average than either reagent alone (Fig. 1A).

The functional consequences of activating myenteric neurons on colonic contractility were investigated. Control experiments using the cholinergic agonist carbachol (100 μM) at the beginning and the end of the experiment demonstrated that the evoked contractions were not diminished following multiple stimulations (0.074 ± 0.004 vs. 0.076 ± 0.008 , $P > 0.05$). IL-6- and CRF-evoked contractions were expressed as a percentage of this maximal carbachol response. IL-6 (10 nM, 20 min)

induced a small but robust increase in circular muscle contractile activity ($n = 5$), whereas CRF evoked a larger contraction ($n = 4$). However, unlike the results in the LMMP, addition of both agonists did not have an additive effect on muscle contractility ($n = 4$, $P > 0.05$, Fig. 1B). Consistent with a neuronal contribution to contractile activity, the Na^+ channel blocker TTX (100 nM, 20 min) diminished the IL-6 ($n = 5$, $P < 0.01$, Fig. 1C) and CRF ($n = 4$, $P < 0.01$, Fig. 1D) effects, although incomplete abolition of the responses suggests additional direct actions of IL-6 and CRF on the musculature.

IL-8 activates myenteric neurons and stimulates colonic contractility

The chemokine IL-8 is also elevated in IBS plasma (Dinan *et al.* 2006; McKernan *et al.* 2011b) and like IL-6 it also has functional effects on colonic neurons. IL-8 (1 nM, 3 min) increased $[\text{Ca}^{2+}]_i$ in 58% of myenteric neurons

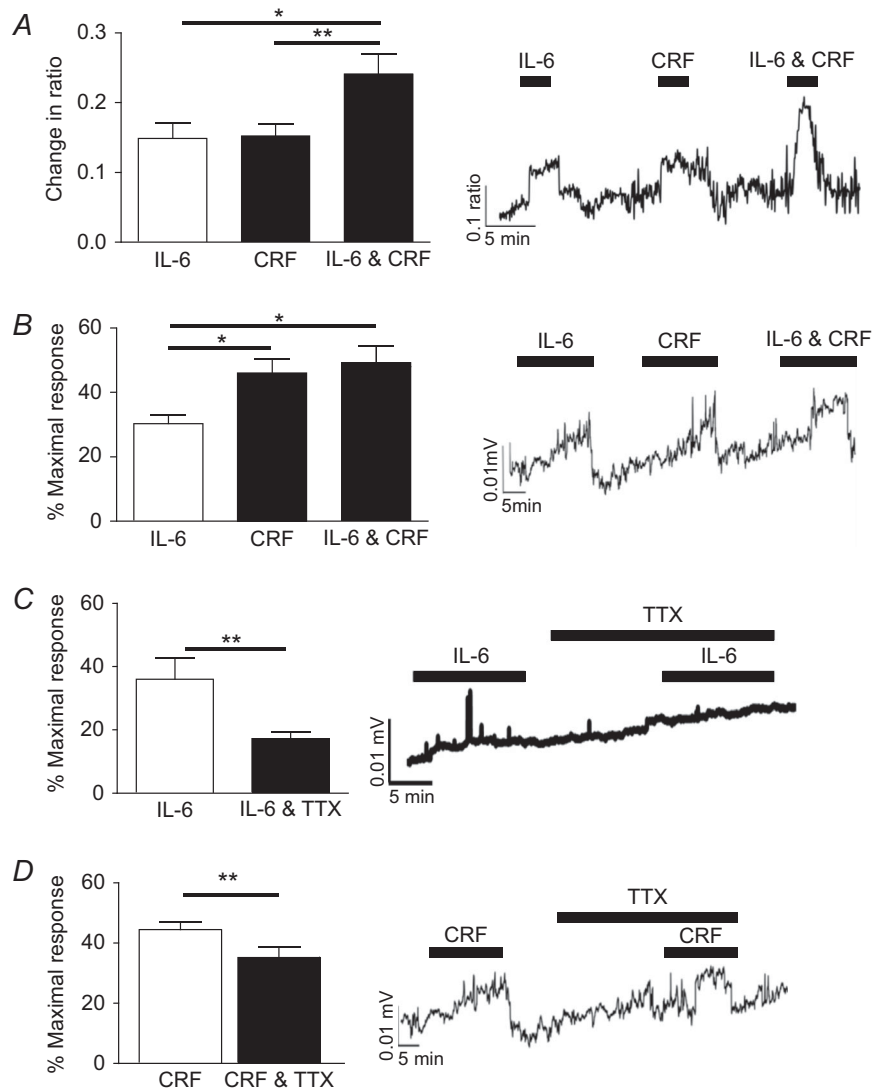


Figure 1. IL-6 and CRF activate myenteric neurons and stimulate colonic contractility

A, bar chart and representative trace illustrating calcium responses in myenteric neurons from SD distal colon in response to IL-6, CRF and IL-6 & CRF ($n = 35$). B–D, bar charts and representative traces illustrating changes in circular muscle contractile activity evoked by IL-6, CRF and IL-6 & CRF (B, $n = 4$), IL-6 (C, $n = 4$) CRF (D, $n = 4$) in the absence or presence of TTX, as a percentage of a maximal contraction evoked by carbachol (100 μM). Asterisks indicate * $P < 0.05$ and ** $P < 0.01$.

($n = 33$) tested. The amplitude of the response was similar to CRF-mediated effects ($P > 0.05$). In total, 34% of neurons responded to CRF alone, whereas 71% of neurons were activated by the co-application of IL-8 and CRF, resulting in a significantly enhanced response ($P < 0.01$, Fig. 2A). When compared with recombinant IL-6, IL-8 evoked a calcium response of similar amplitude but the number of neurons sensitive to IL-8 (58%, $n = 33$) was greater than the percentage sensitive to IL-6 (46%; $\chi^2 = 4.8$, d.f. = 2, $P < 0.05$). However, the combination of IL-8 and IL-6 activated an even greater percentage of neurons (65%; $\chi^2 = 6.2$, d.f. = 2, $P < 0.05$) and increased the amplitude of the calcium response compared with either IL-6 ($P < 0.001$) or IL-8 ($P < 0.01$, Fig. 2B) alone. Application of IL-6, IL-8 and CRF stimulated 55% of the

neurons ($n = 33$) and, although the amplitude of the response was greater than IL-6 plus IL-8 ($P < 0.05$), it was similar to the response evoked by IL-8 plus CRF ($P > 0.05$).

IL-8 evoked large, repetitive colonic contractions ($n = 5$, Fig. 2C). While the amplitude of contractions was attenuated in the presence of TTX, the frequency of contractions increased from 0.48 ± 0.1 to 0.84 ± 0.1 Hz ($n = 5$, $P < 0.01$). The amplitude of IL-8-evoked contractions ($n = 5$) was greater than IL-6-evoked contractions ($n = 5$, $P < 0.001$) but no further change was noted when IL-8 and IL-6 were co-applied ($n = 5$, $P > 0.05$, Fig. 2D). However, the amplitude of contractions evoked by co-application of IL-6, IL-8 and CRF together ($n = 4$) was larger than IL-6 ($P < 0.001$), IL-8 ($P < 0.001$) or CRF ($P < 0.001$) responses alone (Fig. 2E).

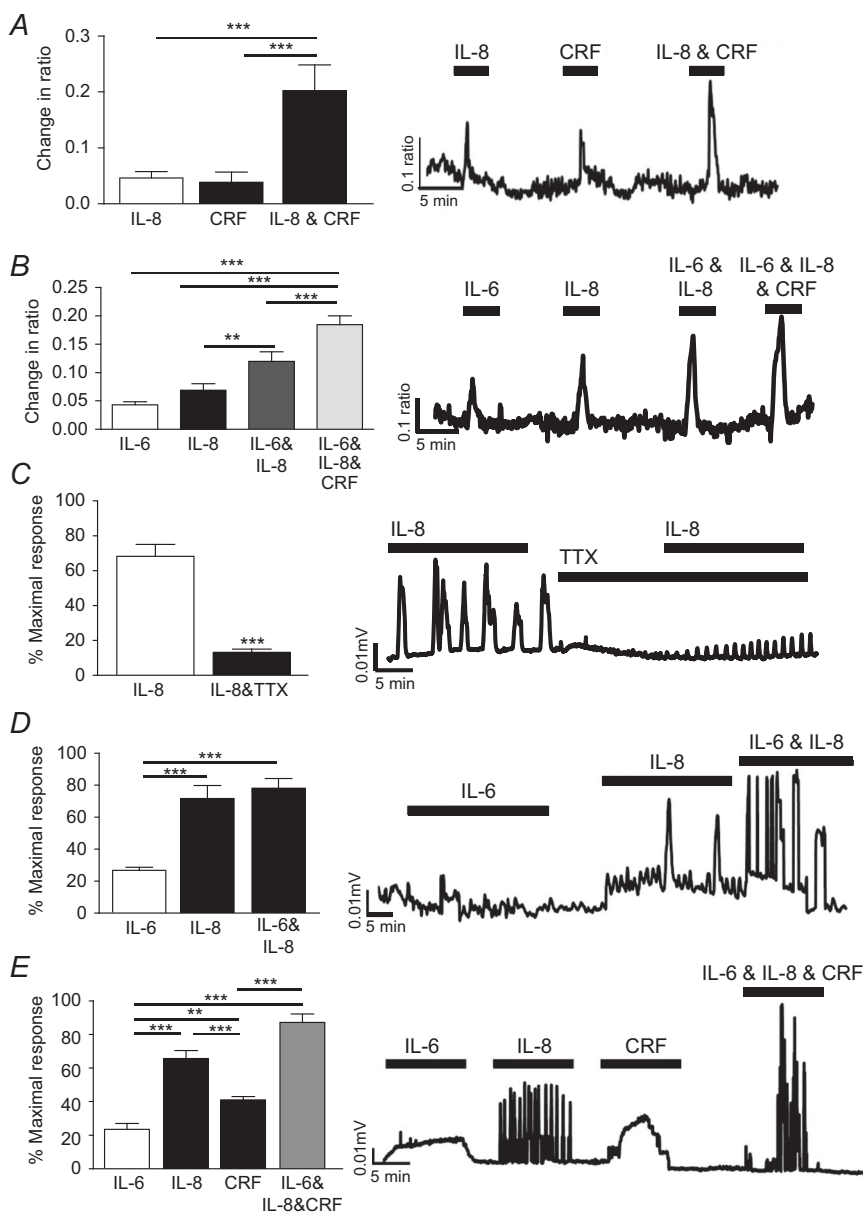


Figure 2. IL-8 activates myenteric neurons and stimulates colonic contractility

A and B, bar charts and traces illustrating calcium responses in SD myenteric neurons following exposure to IL-8 and/or CRF (A, $n = 24$) and IL-6 and/or IL-8 and/or CRF (B, $n = 33$). C and D, bar charts and original traces illustrating circular muscle contractile responses to IL-8 in the absence or presence of TTX (C, $n = 5$) and IL-6 \pm IL-8 (D, $n = 5$) as a percentage of a maximal contraction evoked by carbachol ($100 \mu\text{M}$). E, bar chart and original trace illustrating circular muscle contractile responses to IL-6, IL-8, CRF or all three ($n = 4$). Asterisks indicate $**P < 0.01$ and $***P < 0.001$.

Soluble factors in IBS plasma activate myenteric neurons

Thus far, we have demonstrated neuro-immune and neuro-endocrine interactions between IL-6, IL-8 and CRF in myenteric neurons and on colonic contractile activity. To relate these findings to human IBS, SD rat myenteric neurons were exposed to patient and healthy control plasma samples. Soluble factors in plasma samples from

healthy controls ($n = 6$ pooled samples) induced a small increase in $[Ca^{2+}]_i$ in naïve rat myenteric neurons ($n = 43$, Fig. 3A). However, IBS plasma ($n = 6$ pooled samples from all subtypes) evoked a larger response ($n = 43$, $P < 0.001$, Fig. 3A).

As cytokine profiles in plasma from IBS patients are altered (McKernan *et al.* 2011b), we investigated whether the aforementioned immune molecules contributed to the neuroexcitatory effect of IBS plasma. Control experiments,

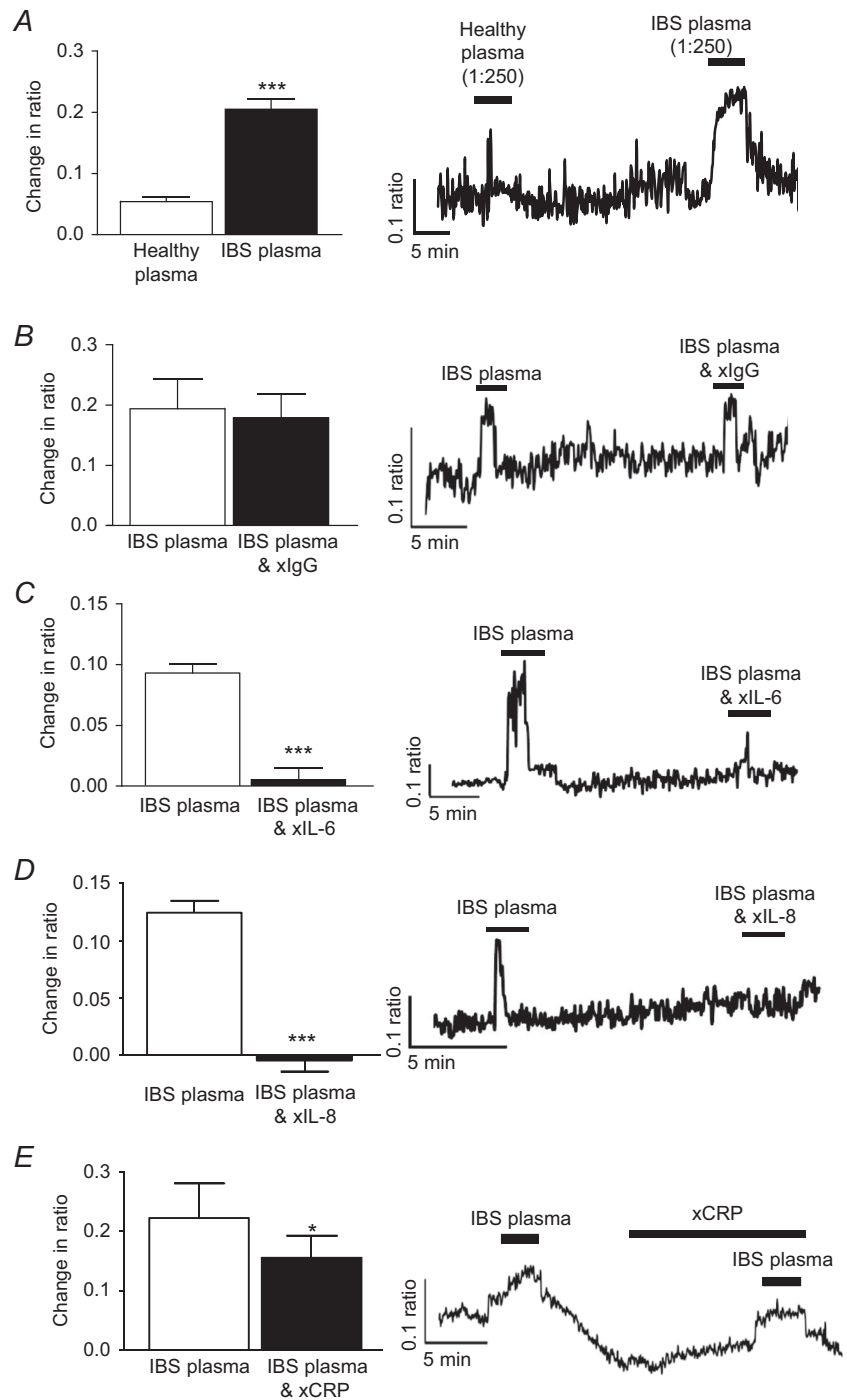


Figure 3. Immune factors in human IBS plasma activates myenteric neuron
 A, bar chart and original trace showing calcium responses in SD myenteric neurons to pooled plasma samples from healthy controls and IBS patients ($n = 43$). B–E, bar charts and original traces showing the effects of antibody neutralization of IgG (B, $n = 45$), IL-6 (C, $n = 71$), IL-8 (D, $n = 70$) and CRP (E, $n = 38$) on IBS plasma-evoked calcium responses in SD myenteric neurons. Asterisks indicate * $P < 0.05$ and *** $P < 0.001$.

where IgG was neutralized, had no effect on the calcium responses evoked by IBS plasma ($n = 45$, $P > 0.05$, Fig. 3B). However, neutralization of IL-6 ($n = 71$, $P < 0.001$, Fig. 3C) and IL-8 ($n = 70$, $P < 0.001$, Fig. 3D) attenuated the initial IBS plasma-evoked response. The inflammatory marker CRP is also elevated in these plasma samples (McKernan *et al.* 2011a) and neutralizing antibodies directed against this protein also reduced the IBS-evoked response ($n = 38$, $P < 0.05$, Fig. 3E) although to a lesser extent than the cytokines. Antalarmin ($1 \mu\text{M}$, 10 min), the CRFR1 antagonist, blocked IBS plasma-evoked neuronal activation ($n = 44$, $P < 0.001$, Fig. 4A), whereas astressin 2B, a CRFR2 inhibitor, had no effect ($n = 53$, $P > 0.05$, Fig. 4B).

Incubation of myenteric neurons with the specific STAT3 inhibitor WP1006 ($10 \mu\text{M}$, 10 min) attenuated the IBS plasma-evoked response ($n = 48$, $P < 0.01$, Fig. 5A) as did the extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) inhibitor PD98059 ($10 \mu\text{M}$, 10 min, $n = 40$, $P < 0.001$, Fig. 5B). In contrast, the phosphatidyl 3-kinase inhibitor wortmannin ($10 \mu\text{M}$, 10 min) had no effect on the IBS plasma responses ($n = 48$, $P > 0.05$, Fig. 5C). Finally the role of nuclear factor κ -light-chain-enhancer of activated B cells (NF κ B) was tested using the NF κ B antagonist I κ B Kinase₂ (IKK₂) ($5 \mu\text{M}$, 10 min). Interestingly, IKK₂ stimulated an increase in the amplitude of the IBS plasma-evoked calcium response ($n = 37$, $P < 0.05$, Fig. 5D).

Monoclonal xIL-6R antibodies and antalarmin inhibit defecation in WKY rats

The above studies demonstrate a functional role for IL-6 and CRF at a cellular level, but *in vivo* studies were necessary to determine if these observations had translational potential. Thus, stress-sensitive WKY rats, which

defecate more in the anxiogenic OF arena compared with SD controls (O'Malley *et al.* 2010a) and display visceral hypersensitivity to CRD (Gunter *et al.* 2000; O'Mahony *et al.* 2010) were treated with neutralizing xIL-6R antibodies and antalarmin. Control IgG- and saline-treated WKY rats excreted more boli in the OF than SD rats ($n = 12$, $P < 0.001$, Fig. 6A). The previously described anxious phenotype of WKY rats (O'Malley *et al.* 2010a) was further evidenced by decreased exploration of the exposed inner zone of the OF arena compared with SD ($P < 0.001$). Targeting IL-6 signalling with xIL-6R monoclonal antibodies did not affect stress-induced defecation in SD rats in the OF, but the number of faecal pellets excreted by WKY rats was reduced ($n = 12$, $P < 0.05$). However, this remained higher than the low-anxiety SD comparator ($n = 12$, $P < 0.01$, Fig. 6A). Co-application of xIL-6R with the CRFR1 antagonist antalarmin, which can modulate GI function (O'Malley *et al.* 2013), decreased faecal output further ($n = 12$, $P < 0.001$) to levels equivalent to SD controls ($P > 0.05$, Fig. 6A).

xIL-6R and antalarmin improve visceral pain sensitivity

CRD (0–80 mmHg over 8 min) evoked pain behaviours at lower pressures in control WKY as compared with SD rats ($n = 10$, $P < 0.001$, Fig. 6B). However, neutralizing IL-6Rs increased the pain threshold to CRD in WKY rats ($n = 9$, $P < 0.05$, Fig. 6B) and this was further improved by co-application of antalarmin with xIL-6R ($n = 12$, $P < 0.01$), such that visceral sensitivity in WKY rats was equivalent to SD controls ($n = 10$, $P > 0.05$). Interestingly, pain thresholds in SD rats following treatment with xIL-6R ($n = 8$, $P < 0.01$) and xIL-6R plus antalarmin ($n = 8$, $P < 0.01$) were also increased (Fig. 6B).

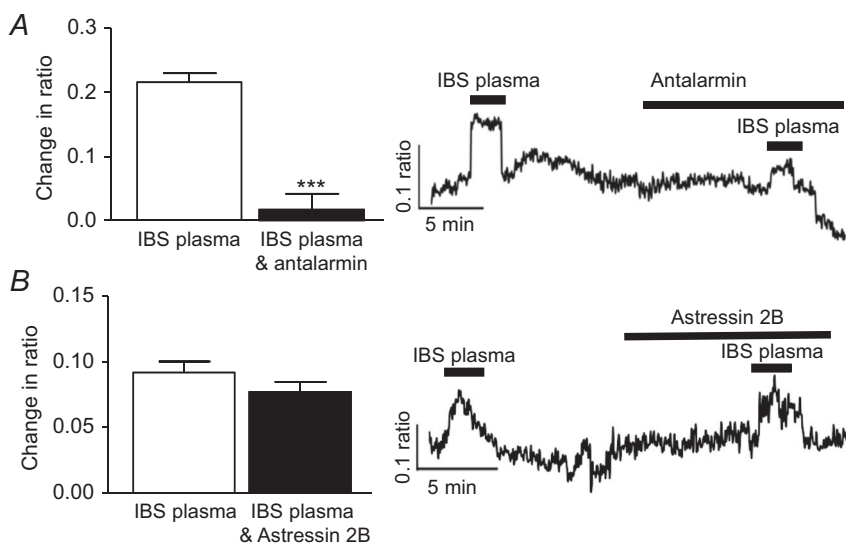


Figure 4. Stress factors in human IBS plasma activate myenteric neurons Bar charts and original traces illustrating the effects of the CRFR1 antagonist antalarmin (A, $n = 44$) and the CRFR2 antagonist astressin 2B (B, $n = 53$) on IBS plasma-evoked calcium responses in SD myenteric neurons. *** $P < 0.001$.

ELISA analysis of plasma IL-6 concentrations revealed no differences between saline-treated control WKY and SD rats ($P > 0.05$) and no changes following treatment with xIL-6R ($P > 0.05$) or xIL-6R plus antalarmin ($P > 0.05$, data not shown).

Colonic mucosal protein expression is altered by xIL-6R and antalarmin treatment

Underlying the beneficial effects of treatment of GI function were changes in colonic protein expression. Depletion of the tight junction protein occludin is linked with increased intestinal barrier permeability (Al-Sadi *et al.* 2011), and thus the observed increase in occludin expression in WKY control tissues ($P < 0.05$) suggests tighter barrier control. This was unchanged by combined treatment with xIL-6R plus antalarmin, although xIL-6R

treatment alone reduced occludin expression levels ($P < 0.05$, $n = 5$ rats, Fig. 7A). In contrast, increased expression of another tight junction protein, claudin 2, is thought to reduce barrier tightness (Amasheh *et al.* 2002) and expression of this protein was also elevated in WKY colons ($P < 0.05$) but reduced following treatment with xIL-6R alone or xIL-6R and antalarmin ($P < 0.05$, $n = 5$ rats, Fig. 7B). Given that we have previously found that transepithelial resistance, as an indicator of colonic permeability, is comparable between SD and WKY rats (O'Malley *et al.* 2012) it may be that the sum effect of increased expression of claudin 2 and occludin is no net change in permeability. Consistent with the visceraally sensitive phenotype of the WKY rat, $Ca_v3.2$, T-type calcium channels linked to visceral pain sensitivity (Marger *et al.* 2011), were also elevated in WKY tissue ($P < 0.01$). Moreover, consistent with amelioration of the visceral sensitivity following treatment with xIL-6R alone

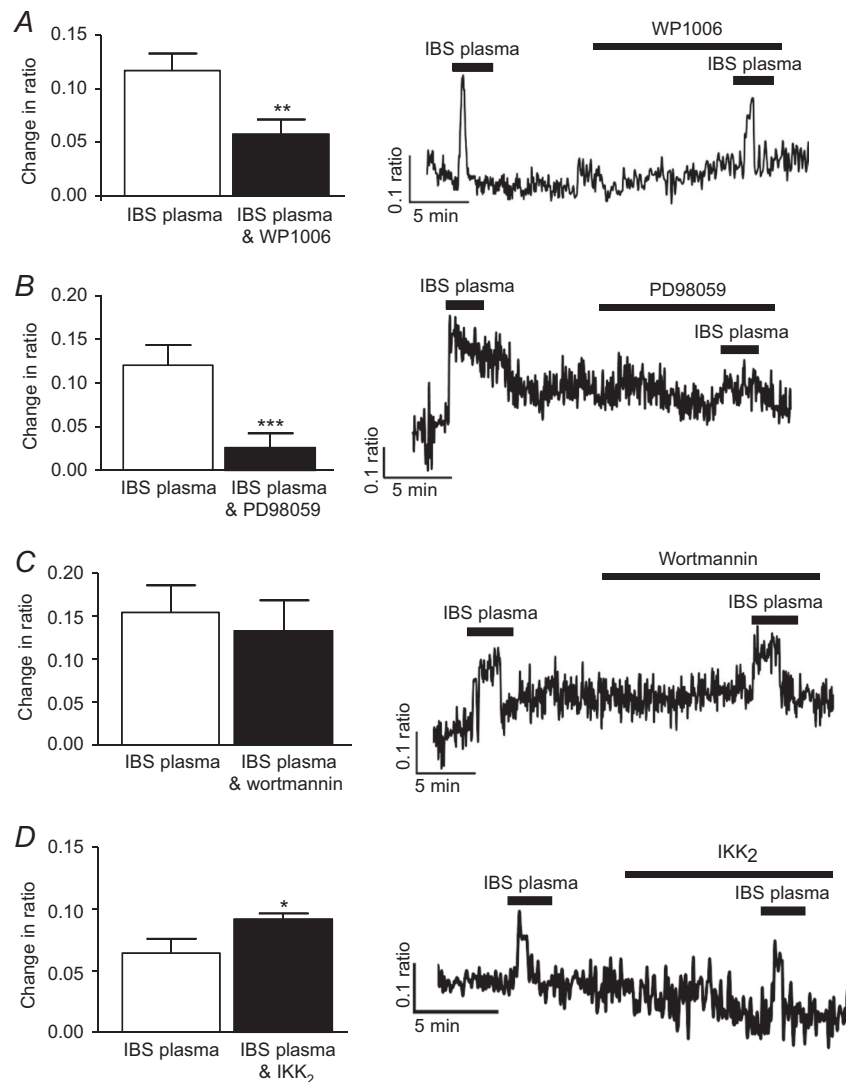


Figure 5. Intracellular signalling molecules activated by IBS plasma

Bar charts and original traces illustrating the effects of the STAT3 inhibitor WP1006 (A, $10 \mu\text{M}$, $n = 48$), the ERK-MAPK inhibitor PD98059 (B, $10 \mu\text{M}$, $n = 40$), the PI 3-kinase inhibitor wortmannin (C, $10 \mu\text{M}$, $n = 48$) and the $\text{NF}\kappa\text{B}$ antagonist IKK_2 (D, $5 \mu\text{M}$, $n = 37$) on calcium signals evoked by IBS plasma in SD myenteric neurons. Asterisks indicate $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

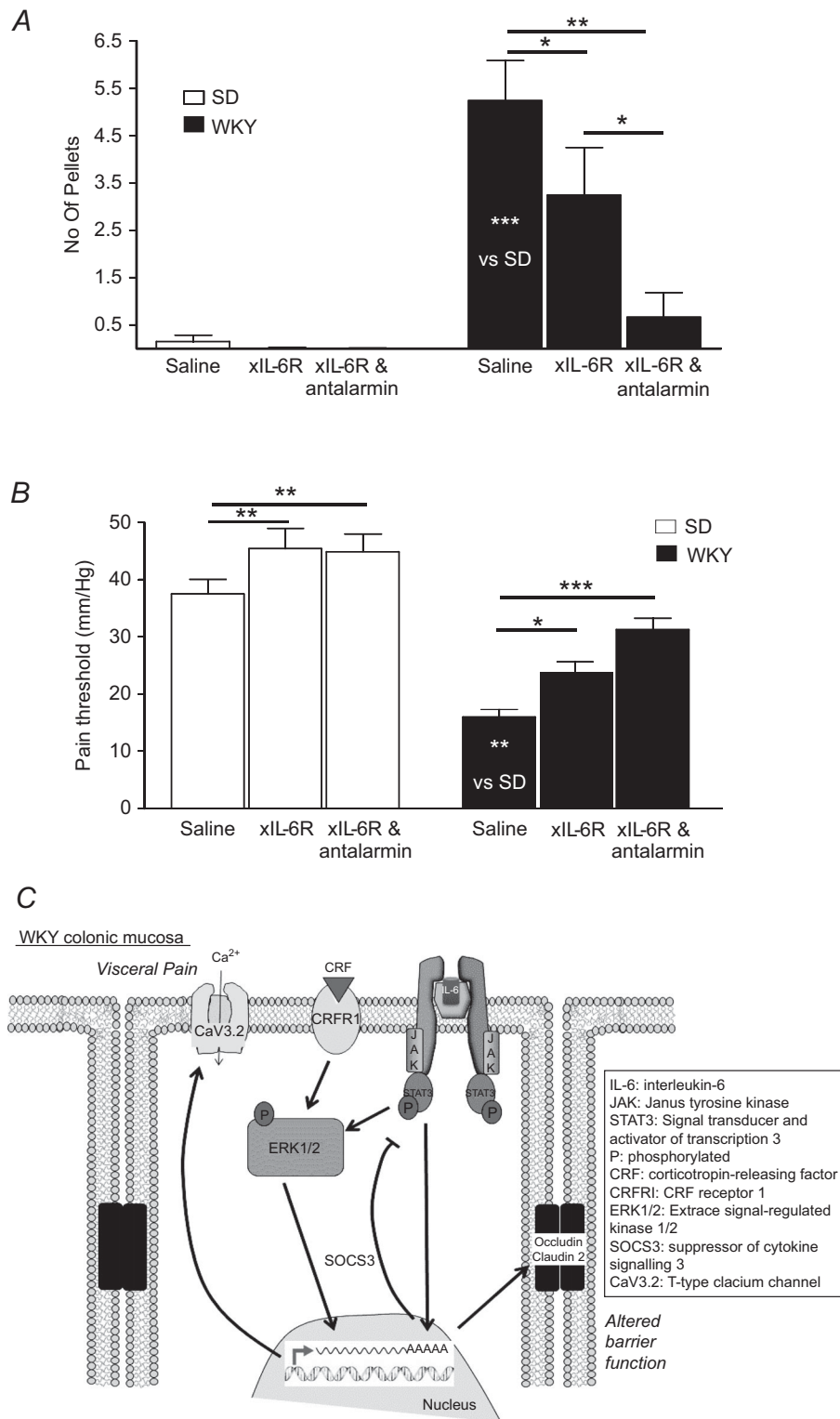


Figure 6. IL-6R neutralization and antalarmin inhibit defecation in WKY rats

A, bar chart illustrating the number of faecal pellets excreted by SD ($n = 7, 7$ and 9) and WKY ($n = 10, 9$ and 11) rats in the OF when administered saline, anti-IL-6 receptor antibodies (xIL-6R) or xIL-6R and antalarmin. *B*, bar chart showing the pressure (mmHg) at which SD ($n = 7, 7$ and 9) and WKY ($n = 10, 9$ and 11) rats display pain behaviours in response to colorectal distension when treated with saline, xIL-6R or xIL-6R and antalarmin. *C*, schematic illustration of potential intracellular signalling mechanisms activated by CRF and IL-6 binding to their receptors. Asterisks indicate $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

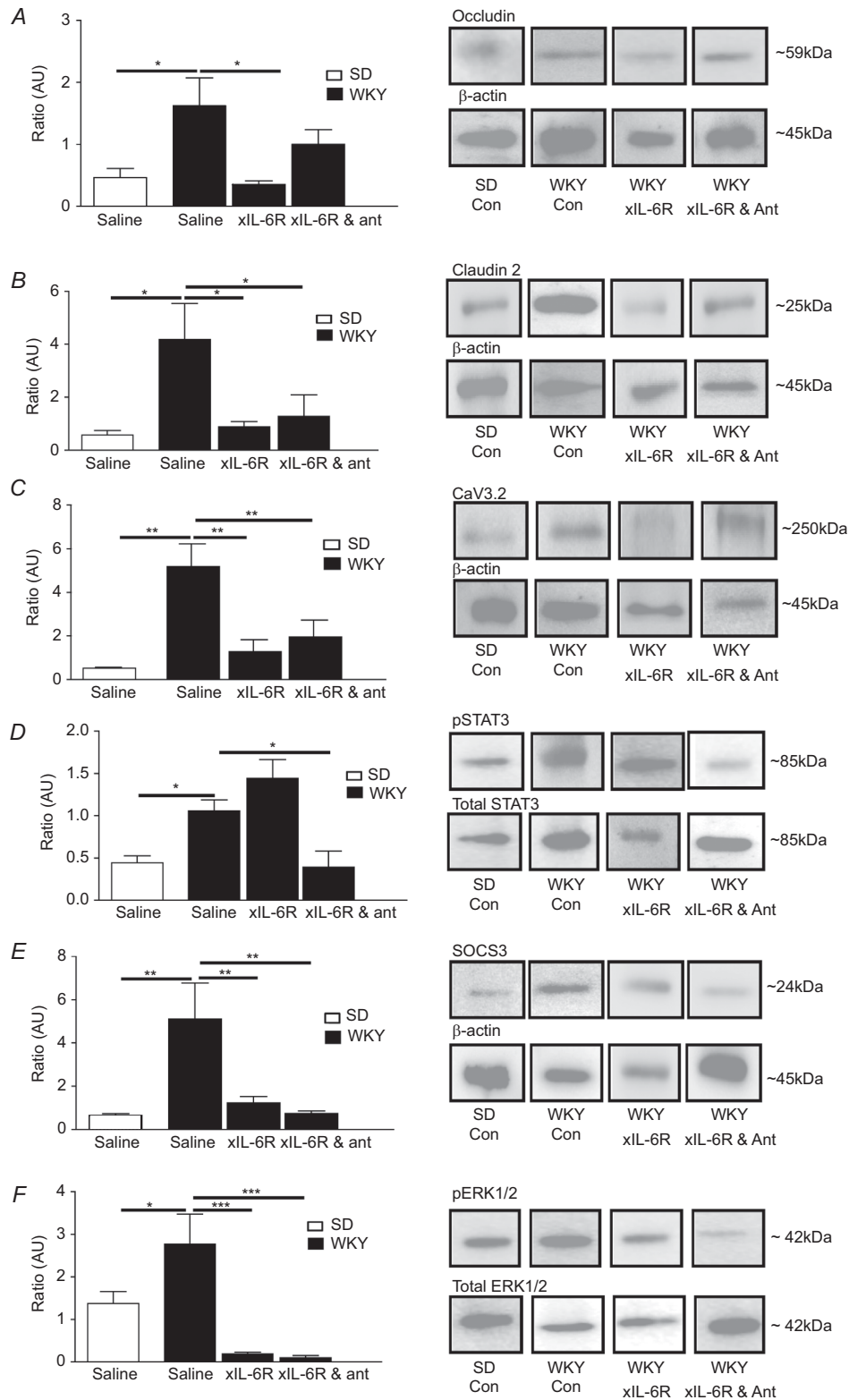


Figure 7. IL-6R neutralization and antalarmin alter colonic mucosal protein expression
 Bar charts illustrating the ratios of expression of occludin (A, $n = 5$), claudin 2 (B, $n = 5$) and $Ca_v3.2$ (C, $n = 5$) over the β actin loading control, phosphoSTAT3 as a fraction of total STAT3 (D, $n = 5$), SOCS3 as a fraction of the β actin loading control (E, $n = 5$) and phosphoERK1/2 over total ERK1/2 (F, $n = 5$) in mucosal samples from the distal colon of SD and WKY rats treated with saline, anti-IL-6 receptor antibody (xIL-6R) and xIL-6R with antalarmin (ant). Asterisks indicate * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

and xIL-6R plus antalarmin, expression of $Ca_v3.2$ was also reduced ($P < 0.01$, $n = 5$ rats, Fig. 7C).

IL-6 activates the intracellular janus tyrosine kinase and signal transducers and activators of transcription (JAK-STAT) signalling cascade and indeed phosphorylation of STAT3 was elevated in WKY rats which have elevated levels of IL-6 as compared with SD colons ($P < 0.05$). Treatment with xIL-6R had no effect on pSTAT3 expression ($P > 0.05$), whereas co-administration of xIL-6R and antalarmin suppressed pSTAT3 expression ($P < 0.05$, $n = 5$ rats, Fig. 7D). SOCS3, a suppressor of cytokine signalling, was also elevated in WKY controls ($P < 0.01$), and was reduced to levels equivalent to SD controls following xIL-6R ($P > 0.05$) and xIL-6R plus antalarmin ($P > 0.05$, $n = 5$ rats, Fig. 7E) treatment. IL-6 binding also activates the intracellular signalling molecule pERK1/2 and this was greater in control WKY compared with SD colons ($P < 0.05$), but was markedly reduced by xIL-6R ($P < 0.001$) and xIL-6R plus antalarmin ($P < 0.001$, $n = 5$ rats, Fig. 7F) treatment.

Discussion

Neuroimmune and neuroendocrine interactions contribute to IBS pathophysiology

The importance of immune factors such as IL-6 (Liebregts *et al.* 2007), IL-8 (Dinan *et al.* 2006, 2008; McKernan *et al.* 2011b) and the stress hormone CRF (Mayer, 2000; Bohmelt *et al.* 2005; Dinan *et al.* 2006; Chang *et al.* 2009) in the aetiology of human IBS has been reported. Moreover, we have previously reported the effects of these factors on the excitability of submucosal neurons leading to functional changes in secretory activity (O'Malley *et al.* 2011c, 2013). The studies reported here have identified that the pro-inflammatory cytokines IL-6 and IL-8 are acting as neuromodulators, which is consistent with the work of Kelles *et al.* (2000), who described the stimulation of guinea-pig ileum myenteric neurons by IL-1 β and IL-6. However, we have also determined that the stress factor CRF activates myenteric neurons and that interaction between the immune and stress factors may occur, thereby contributing to bowel symptom flares. Myenteric neurons are associated with GI motility and in terms of pathophysiology, GI spasm and abdominal pain (Sarna, 2007). Indeed, consistent with recent reports describing how recombinant IL-6 (Zhang *et al.* 2013) and an acute stressor (Liang *et al.* 2012) increased contractile activity, we found that IL-6, IL-8 and CRF induced colonic contractions. IL-8, which had minimal effects on submucosal excitability (O'Malley *et al.* 2011c), evoked robust responses in myenteric neurons and stimulated large colonic contractions. The amplitude of contractions evoked by IL-6 and IL-8 was greatly reduced by TTX, whereas the inhibitory effect was less for CRF-evoked

contractions, which may be acting directly on smooth muscle cells or interstitial cells of Cajal. The observed increase in the frequency of GI smooth muscle basal contractility in the presence of TTX may due to endogenous inhibitory neural tone (Huizinga *et al.* 1990). Furthermore, the stimulatory effects of the immune and stress factors were additive, with the largest neuronal and contractile response being evoked by the combined presence of all three factors. These findings are consistent with our proposal that hyperactivity of the stress response, in conjunction with low-grade immune activation, may underlie symptom flares in functional bowel disorders (O'Malley *et al.* 2011d).

To test this hypothesis further, an LMMP preparation was exposed to clinical plasma samples from IBS patients and healthy controls. The much larger neuronal responses to IBS plasma indicates that certain soluble mediators present in this plasma have neuroexcitatory effects. Neutralizing IL-6 and IL-8 and the CRFR1 antagonist antalarmin attenuated the IBS plasma-evoked neuronal responses, thereby demonstrating the importance of these factors to the neuroexcitatory actions of IBS plasma. Indeed, IL-6 has previously been shown to act as a neuromodulator in myenteric S and AH neurons (Kelles *et al.* 2000). Although others have described the neuroexcitatory effects of other mucosally secreted soluble factors, such as serotonin, histamine and proteases in submucosal neurons (Buhner *et al.* 2009, 2012), our findings demonstrate that blood-borne IL-6, CRF, IL-8 and, to a lesser extent, CRP activate the myenteric neurons.

IBS plasma also activated JAK-STAT and ERK-MAPK intracellular signalling cascades. Interestingly, inhibiting the NF κ B signalling pathway, which normally induces transcription of immune and inflammatory factors, potentiated the neuroexcitatory actions of IBS plasma, contrasting with its inhibitory effects on recombinant IL-6 in submucosal neurons (O'Malley *et al.* 2011c). This may be due to an interaction with one of the other neuroexcitatory mediators present in the plasma.

Inhibiting both IL-6 and CRF1 receptors improves IBS-like symptoms in WKY rats

Consistent with the variable phenotype and multi-factorial nature of IBS, our studies have identified several elements which contribute to altered bowel motility and visceral pain sensitivity in the WKY rats. As previously reported (Martinez *et al.* 2007; O'Mahony *et al.* 2010; O'Malley *et al.* 2010b), WKY rats defecated more in the anxiogenic OF arena and have lower pain thresholds to CRD, thereby mimicking symptoms of altered bowel habit and visceral pain in humans. However, these IBS-like symptoms were ameliorated by blocking IL-6R signalling and normalized to control levels when

both α IL-6R and antalarmin were co-administered. Moreover, consistent with evidence that psychological stress elevates IL-6 in humans, our findings illustrate the intricate relationship between immune and stress factors (O'Malley *et al.* 2011d, 2013). Perhaps overlooking the importance of pro-inflammatory cytokines in symptom flares may account for the disappointing outcomes of clinical trials of CRFR1 antagonists in IBS patients (Tache *et al.* 2009).

Underlying the improvements in GI function caused by α IL-6R and antalarmin co-treatment, were dramatic changes in mucosal protein expression. Figure 6C summarizes a potential signalling cascade underlying the IBS-like symptoms exhibited by WKY rats. Downstream of IL-6Rs, which comprise α -chains and the signal transduction gp130 subunit, is the JAK-STAT signalling cascade (Hemmann *et al.* 1996). STAT3 phosphorylation was elevated in WKY colonic tissue, perhaps due to raised mucosal levels of IL-6 (O'Malley *et al.* 2011a). However, other cytokines or growth factors may also activate this transcription molecule as STAT3 phosphorylation was not altered following blockade of IL-6 signalling. Interestingly, given that CRFR1 activation is not associated with JAK-STAT signalling, co-treatment with antalarmin normalized pSTAT3 levels, demonstrating a complex interaction between the immune and stress factor. SOCS3, which terminates JAK/STAT signalling via negative feedback following STAT3 activation (Crocker *et al.* 2003), was elevated in control WKY colons, and expression of this downstream regulatory molecule was inhibited by treatment with α IL-6R or α IL-6R plus antalarmin. Activation of the ERK 1/2 signalling cascade by either CRF (Stengel & Tache, 2009) or IL-6 (Hoffmann *et al.* 2002) was evident in the WKY colonic mucosa and this was reduced following treatment with α IL-6R or α IL-6R plus antalarmin. This is consistent with previous studies in submucosal neurons which demonstrated that CRF tends to drive IL-6-mediated signalling via the ERK/MAPK cascade (O'Malley *et al.* 2013), possibly due to ERK activation antagonizing JAK-STAT signalling (Bonni *et al.* 1997).

Both JAK-STAT and ERK/MAPK signalling evoke transcription and *de novo* protein synthesis. In WKY colonic mucosa, expression of two tight junction proteins, occludin and claudin 2, which are crucial to GI barrier function and permeability, were both increased. Functionally, transepithelial resistance (TER) is equivalent between WKY and SD rats (O'Malley *et al.* 2012) but given that increased expression of occludin is thought to induce tighter barrier control and claudin 2 is thought to reduce barrier tightness (Amasheh *et al.* 2002) the sum effect of elevated mucosal levels of both proteins may be no net change in permeability, although with the presence of many additional tight junction proteins, this requires further research. Expression of claudin 1 and 2 is also

increased in IBS (Martinez *et al.* 2013). Conversely, others have demonstrated decreased expression of occludin in bowel inflammation (Poritz *et al.* 2011) and, in caco cells, exposure to IL-6 decreased claudin 2 expression resulting in decreased TER (Suzuki *et al.* 2011). We have previously demonstrated that acute administration of IL-6 increases TER in WKY colons (O'Malley *et al.* 2012) and others have shown that chronic exposure to IL-6 increases gut permeability (Natale *et al.* 2003). Our study has provided evidence that blocking IL-6Rs decreased expression of both occludin and claudin 2, and the addition of antalarmin reduced expression of claudin 2. The decreases in tight junction protein expression indicate a possible change in TER, although functional studies will be required to certify this.

Finally, expression of the T-type calcium channel $Ca_v3.2$, which is linked to visceral pain in a rodent model of IBS (Marger *et al.* 2011), was increased in WKY colons. Consistent with improvements in pain threshold to CRD, treatment with α IL-6R and antalarmin resulted in reduced expression of $Ca_v3.2$. CRFR1 antagonists alleviate visceral sensitivity in the WKY rat and CRFR1 has been shown to functionally couple to $Ca_v3.2$ in a cell line, inhibiting the calcium current (Tao *et al.* 2008). Thus, as has recently been proposed (Beyder *et al.* 2014), specific ion channelopathies may contribute to visceral pain in some IBS patients. However, to our knowledge, this is the first study to link IL-6 signalling with $Ca_v3.2$ expression and visceral pain sensitivity.

Thus, in a multifactorial disorder such as IBS, where the stress system is chronically activated and cytokine levels of IL-6 and IL-8 are elevated, interaction and crosstalk between these biologically active factors results in increased stimulation of myenteric neurons, which subsequently affects contractile activity. Indeed, the demonstrated effectiveness in ameliorating IBS-like pathophysiology, such as defecation patterns and visceral pain sensitivity, in the WKY rat by targeting IL-6 and CRF1 and possibly also IL-8 receptors establishes that these immune and stress molecules do indeed contribute to these symptoms. Moreover, we have determined that the neuroexcitatory effects of human IBS plasma are evoked primarily by the immune mediators IL-6 and IL-8 and the stress hormone CRF. These studies in the WKY rat, which mimics symptoms of IBS, offers proof of principle that combined targeting of both immune and stress factors may be a viable therapeutic strategy for the treatment of IBS.

References

- Al-Sadi R, Khatib K, Guo S, Ye D, Youssef M & Ma T (2011). Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. *Am J Physiol Gastrointest Liver Physiol* **300**, G1054–1064.

- Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD & Fromm M (2002). Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci* **115**, 4969–4976.
- Beyder A, Mazzone A, Strege PR, Tester DJ, Saito YA, Bernard CE, Enders FT, Ek WE, Schmidt PT, Dlugosz A, Lindberg G, Karling P, Ohlsson B, Gazouli M, Nardone G, Cuomo R, Usai-Satta P, Galeazzi F, Neri M, Portincasa P, Bellini M, Barbara G, Camilleri M, Locke GR 3rd, Talley NJ, D'Amato M, Ackerman MJ & Farrugia G (2014). Loss-of-function of the voltage-gated sodium channel Nav1.5 (Channelopathies) in patients with irritable bowel syndrome. *Gastroenterology* **146**, 1659–1668.
- Bohmelt AH, Nater UM, Franke S, Hellhammer DH & Ehlert U (2005). Basal and stimulated hypothalamic–pituitary–adrenal axis activity in patients with functional gastrointestinal disorders and healthy controls. *Psychosom Med* **67**, 288–294.
- Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD & Greenberg ME (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science (New York, NY)* **278**, 477–483.
- Buhner S, Li Q, Berger T, Vignali S, Barbara G, De Giorgio R, Stanghellini V & Schemann M (2012). Submucosal rather than myenteric neurons are activated by mucosal biopsy supernatants from irritable bowel syndrome patients. *Neurogastroenterol Motil* **24**, 1134–e572.
- Buhner S, Li Q, Vignali S, Barbara G, De Giorgio R, Stanghellini V, Cremon C, Zeller F, Langer R, Daniel H, Michel K & Schemann M (2009). Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. *Gastroenterology* **137**, 1425–1434.
- Chadwick VS, Chen W, Shu D, Paulus B, Bethwaite P, Tie A & Wilson I (2002). Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* **122**, 1778–1783.
- Chang L, Sundaresh S, Elliott J, Anton PA, Baldi P, Licudine A, Mayer M, Vuong T, Hirano M, Naliboff BD, Ameen VZ & Mayer EA (2009). Dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis in irritable bowel syndrome. *Neurogastroenterol Motil* **21**, 149–159.
- Clarke G, Quigley EM, Cryan JF & Dinan TG (2009). Irritable bowel syndrome: towards biomarker identification. *Trends Mol Med* **15**, 478–489.
- Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, Robb L, Greenhalgh CJ, Forster I, Clausen BE, Nicola NA, Metcalf D, Hilton DJ, Roberts AW & Alexander WS (2003). SOCS3 negatively regulates IL-6 signaling in vivo. *Nature Immunol* **4**, 540–545.
- Cryan JF & O'Mahony SM (2011). The microbiome–gut–brain axis: from bowel to behavior. *Neurogastroenterol Motil* **23**, 187–192.
- Dinan TG, Clarke G, Quigley EM, Scott LV, Shanahan F, Cryan J, Cooney J & Keeling PW (2008). Enhanced cholinergic-mediated increase in the pro-inflammatory cytokine IL-6 in irritable bowel syndrome: role of muscarinic receptors. *Am J Gastroenterol* **103**, 2570–2576.
- Dinan TG, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F & Keeling PW (2006). Hypothalamic–pituitary–gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* **130**, 304–311.
- Gunter WD, Shepard JD, Foreman RD, Myers DA & Greenwood-Van Meerveld B (2000). Evidence for visceral hypersensitivity in high-anxiety rats. *Physiol Behav* **69**, 379–382.
- Heitkemper M, Jarrett M, Cain K, Shaver J, Bond E, Woods NF & Walker E (1996). Increased urine catecholamines and cortisol in women with irritable bowel syndrome. *Am J Gastroenterol* **91**, 906–913.
- Hemmman U, Gerhartz C, Heesel B, Sasse J, Kurapatk G, Grotzinger J, Wollmer A, Zhong Z, Darnell JE Jr, Graeve L, Heinrich PC & Horn F (1996). Differential activation of acute phase response factor/Stat3 and Stat1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. II. Src homology SH2 domains define the specificity of stat factor activation. *J Biol Chem* **271**, 12999–13007.
- Hoffmann E, Dittrich-Breiholz O, Holtmann H & Kracht M (2002). Multiple control of interleukin-8 gene expression. *J Leukoc Biol* **72**, 847–855.
- Huizinga JD, Berezin I, Daniel EE & Chow E (1990). Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can J Physiol Pharmacol* **68**, 447–454.
- Kelles A, Janssens J & Tack J (2000). IL-1 β and IL-6 excite neurones and suppress cholinergic neurotransmission in the myenteric plexus of the guinea pig. *Neurogastroenterol Motil* **12**, 531–538.
- Kindt S, Vanden Berghe P, Boesmans W, Roosen L & Tack J (2010). Prolonged IL-1 β exposure alters neurotransmitter and electrically induced Ca²⁺ responses in the myenteric plexus. *Neurogastroenterol Motil* **22**, 321–e85.
- Liang C, Luo H, Liu Y, Cao J & Xia H (2012). Plasma hormones facilitated the hypermotility of the colon in a chronic stress rat model. *PLoS One* **7**, e31774.
- Liebrechts T, Adam B, Bredack C, Roth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ & Holtmann G (2007). Immune activation in patients with irritable bowel syndrome. *Gastroenterology* **132**, 913–920.
- Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F & Spiller RC (2006). Functional bowel disorders. *Gastroenterology* **130**, 1480–1491.
- Lovell RM & Ford AC (2012). Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. *Clin Gastroenterol Hepatol* **10**, 712–721.
- Marger F, Gelot A, Alloui A, Matricon J, Ferrer JF, Barrere C, Pizzoccaro A, Muller E, Nargeot J, Snutch TP, Eschalier A, Bourinet E & Ardid D (2011). T-type calcium channels contribute to colonic hypersensitivity in a rat model of irritable bowel syndrome. *Proc Natl Acad Sci U S A* **108**, 11268–11273.
- Martinez C, Lobo B, Pigrau M, Ramos L, Gonzalez-Castro AM, Alonso C, Guilarte M, Guila M, de Torres I, Azpiroz F, Santos J & Vicario M (2013). Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier. *Gut* **62**, 1160–1168.

- Martinez V, Ryttinger M, Kjerling M & Astin-Nielsen M (2007). Characterisation of colonic accommodation in Wistar Kyoto rats with impaired gastric accommodation. *Naunyn Schmiedebergs Arch Pharmacol* **376**, 205–216.
- Mawdsley JE & Rampton DS (2005). Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut* **54**, 1481–1491.
- Mayer EA (2000). The neurobiology of stress and gastrointestinal disease. *Gut* **47**, 861–869.
- McKernan DP, Dennison U, Gaszner G, Cryan JF & Dinan TG (2011a). Enhanced peripheral toll-like receptor responses in psychosis: further evidence of a pro-inflammatory phenotype. *Transl Psychiatry* **1**, e36.
- McKernan DP, Gaszner G, Quigley EM, Cryan JF & Dinan TG (2011b). Altered peripheral toll-like receptor responses in the irritable bowel syndrome. *Aliment Pharmacol Ther* **33**, 1045–1052.
- Natale L, Piepoli AL, De Salvia MA, De Salvatore G, Mitolo CI, Marzullo A, Portincasa P, Moschetta A, Palasciano G & Mitolo-Chieppa D (2003). Interleukins 1 beta and 6 induce functional alteration of rat colonic motility: an in vitro study. *Eur J Clin Invest* **33**, 704–712.
- O'Mahony SM, Bulmer DC, Coelho AM, Fitzgerald P, Bongiovanni C, Lee K, Winchester W, Dinan TG & Cryan JF (2010). 5-HT_{2B} receptors modulate visceral hypersensitivity in a stress-sensitive animal model of brain-gut axis dysfunction. *Neurogastroenterol Motil* **22**, 573–578.
- O'Mahony SM, Marchesi JR, Scully P, Codling C, Coelho AM, Quigley EM, Cryan JF & Dinan TG (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* **65**, 263–267.
- O'Malley D, Cryan JF & Dinan TG (2013). Crosstalk between interleukin-6 and corticotropin-releasing factor modulate submucosal plexus activity and colonic secretion. *Brain Behav Immun* **30**, 115–124.
- O'Malley D, Dinan TG & Cryan JF (2011a). Altered expression and secretion of colonic interleukin-6 in a stress-sensitive animal model of brain-gut axis dysfunction. *J Neuroimmunol* **235**, 48–55.
- O'Malley D, Dinan TG & Cryan JF (2011b). Neonatal maternal separation in the rat impacts on the stress responsivity of central corticotropin-releasing factor receptors in adulthood. *Psychopharmacology* **214**, 221–229.
- O'Malley D, Dinan TG & Cryan JF (2012). Interleukin-6 modulates colonic transepithelial ion transport in the stress-sensitive Wistar Kyoto rat. *Front Pharmacol* **3**, 190.
- O'Malley D, Julio-Pieper M, Gibney SM, Dinan TG & Cryan JF (2010a). Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. *Stress* **13**, 114–122.
- O'Malley D, Julio-Pieper M, Gibney SM, Gosselin RD, Dinan TG & Cryan JF (2010b). Differential stress-induced alterations of colonic corticotropin-releasing factor receptors in the Wistar Kyoto rat. *Neurogastroenterol Motil* **22**, 301–311.
- O'Malley D, Liston M, Hyland NP, Dinan TG & Cryan JF (2011c). Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism. *Am J Physiol* **300**, G241–252.
- O'Malley D, Quigley EM, Dinan TG & Cryan JF (2011d). Do interactions between stress and immune responses lead to symptom exacerbations in irritable bowel syndrome? *Brain Behav Immun* **25**, 1333–1341.
- Poritz LS, Harris LR 3rd, Kelly AA & Koltun WA (2011). Increase in the tight junction protein claudin-1 in intestinal inflammation. *Digest Dis Sci* **56**, 2802–2809.
- Sarna SK (2007). Enteric descending and afferent neural signaling stimulated by giant migrating contractions: essential contributing factors to visceral pain. *Am J Physiol* **292**, G572–581.
- Shanahan F & Anton P (1988). Neuroendocrine modulation of the immune system. Possible implications for inflammatory bowel disease. *Digest Dis Sci* **33**, 41S–49S.
- Spiller R & Garsed K (2009). Postinfectious irritable bowel syndrome. *Gastroenterology* **136**, 1979–1988.
- Stengel A & Tache Y (2009). Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. *Annu Rev Physiol* **71**, 219–239.
- Suzuki T, Yoshinaga N & Tanabe S (2011). Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J Biol Chem* **286**, 31263–31271.
- Tache Y & Bonaz B (2007). Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. *J Clin Invest* **117**, 33–40.
- Tache Y, Kiank C & Stengel A (2009). A role for corticotropin-releasing factor in functional gastrointestinal disorders. *Curr Gastroenterol Rep* **11**, 270–277.
- Tao J, Hildebrand ME, Liao P, Liang MC, Tan G, Li S, Snutch TP & Soong TW (2008). Activation of corticotropin-releasing factor receptor 1 selectively inhibits CaV3.2 T-type calcium channels. *Mol Pharmacol* **73**, 1596–1609.
- Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ & Muller-Lissner SA (1999). Functional bowel disorders and functional abdominal pain. *Gut* **45**(Suppl 2), II43–47.
- Xia Y, Hu HZ, Liu S, Ren J, Zafirov DH & Wood JD (1999). IL-1 β and IL-6 excite neurons and suppress nicotinic and noradrenergic neurotransmission in guinea pig enteric nervous system. *J Clin Invest* **103**, 1309–1316.
- Zhang L, Hu L, Chen M & Yu B (2013). Exogenous interleukin-6 facilitated the contraction of the colon in a depression rat model. *Dig Dis Sci* **58**, 2187–2196.

Additional information

Competing interests

The authors declare that there are no competing interests regarding this publication.

Author contributions

M.M.B.: acquisition of data; analysis and interpretation of data; drafting of the manuscript; K.D.O'H.: critical revision of the manuscript for important intellectual content; M.G.R.: critical revision of the manuscript for important intellectual content; T.G.D.: critical revision of the manuscript for important intellectual content; D.O'M.: study concept and design, critical revision of the manuscript for important intellectual content; obtained funding, study supervision.

Funding

This publication emanated from research supported by the Health Research Board (HRA.POR/2010/52) and by a research grant from the Science Foundation Ireland (SFI) under Grant Number 07/CE/B1368 in the Alimentary Pharmabiotic Centre. M.M.B. was part funded by the Department of Physiology, UCC.

Acknowledgements

We thank Drs Declan McKernan, Gabor Gaszner, Sinead Heuston and Rachel Moloney, Colette Manley and Patrick Fitzgerald for assistance with various parts of this study.