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Immune derived opioidergic inhibition of viscerosensory afferents is decreased in Irritable Bowel Syndrome patients

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Abbreviations:

IBS: Irritable Bowel Syndrome

IBS-D: IBS diarrhoea predominant

IBS-C: IBS constipation predominant

IBS-A: IBS alternating

PBMC: peripheral blood mononuclear cell

MALDI-TOF: Matrix-Assisted Laser Desorption / Ionization-Time of Flight

MOR: μ-opioid receptor

TNBS: trinitrobenzene sulphonic acid

TRP: Transient Receptor Potential

CVH: chronic visceral hypersensitivity

HS: healthy subjects

LPS: Lipopolysaccharide

PMA: phorbol 12-myristate 13- acetate

DAMGO: [D-Ala2, N-MePhe4, Gly-ol]-enkephalin

qRT-PCR: quantitative RT-PCR

DRG: dorsal root ganglia.

Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

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Abstract

Alterations in the neuro-immune axis contribute towards viscerosensory nerve sensitivity and symptoms in Irritable Bowel Syndrome (IBS). Inhibitory factors secreted from immune cells inhibit colo-rectal afferents in health, and loss of this inhibition may lead to hypersensitivity and symptoms. We aimed to determine the immune cell type(s) responsible for opioid secretion in humans and whether this is altered in patients with IBS. The β-endorphin content of specific immune cell lineages in peripheral blood and colonic mucosal biopsies were compared between healthy subjects (HS) and IBS patients. Peripheral blood mononuclear cell (PBMC) supernatants from HS and IBS patients were applied to colo-rectal sensory afferent endings in mice with post-inflammatory chronic visceral hypersensitivity (CVH). β-endorphin was identified predominantly in monocyte / macrophages relative to T or B cells in human PBMC and colonic lamina propria. Monocyte derived β-endorphin levels and colonic macrophage numbers were lower in IBS patients than healthy subjects. PBMC supernatants from healthy subjects had greater inhibitory effects on colo-rectal afferent mechanosensitivity than those from IBS patients. The inhibitory effects of PBMC supernatants were more prominent in CVH mice compared to healthy mice due to an increase in μ-opioid receptor expression in dorsal root ganglia neurons in CVH mice. Monocyte / macrophages are the predominant immune cell type responsible for β-endorphin secretion in humans. IBS patients have lower monocyte derived β-endorphin levels than healthy subjects, causing less inhibition of colonic afferent endings. Consequently, altered immune function contributes toward visceral hypersensitivity in IBS.

Introduction

Irritable Bowel Syndrome (IBS) is a prevalent functional disorder of the gastrointestinal tract estimated to affect more than 10% of the population (Longstreth et al., 2006). IBS patients are defined by symptoms of pain from the lower abdominal region that is associated with altered bowel habit and which occurs in the absence of readily identifiable pathophysiology, a clear differential diagnosis from Inflammatory Bowel Disease (Longstreth et al., 2006). These patients may be further characterized according to bowel habit as diarrhoea predominant (IBS-D), constipation predominant (IBS-C), or alternating between these states (IBS-A). The symptom of pain crosses all these subtypes and has the greatest impact on quality of life, but remains the most difficult to treat (Longstreth et al., 2006). Little is known of the mechanisms underlying IBS and it has historically been viewed as a neurological motility disorder involving alterations in the brain-gut axis. However there is increasing evidence that the immune system is altered in these patients, and that these alterations contribute toward symptoms (Hughes et al., 2013b).

Distension of the colo-rectum is sensed by mechanosensitive extrinsic primary sensory afferent nerves, which are best characterized in the mouse (Brierley et al., 2004; Hughes et al., 2009b). Muscular / mucosal afferents respond to fine tactile stimuli and low intensity circular stretch, and in the colo-rectum are unique to the pelvic afferent pathway where they comprise approximately 25% of the total afferent population (Brierley et al., 2004; Feng et al., 2010; Hughes et al., 2009b). These afferent nerves respond linearly to increasing levels of distension and signal into the noxious range. They also express putative nociceptive channels, including members of the Transient Receptor Potential (TRPV1, TRPA1) and Acid Sensing Ion Channel

(ASIC3) families, implying they act as intensity encoders and modulate the sensory processing of pain (Brierley et al., 2009; Brookes et al., 2013; Gebhart, 2000; Jones et al., 2005). Immune derived mediators are known to excite viscerosensory nerves and have previously been implicated in the heightened sensitivity to distension of the colo-rectum experienced by IBS patients (Hughes et al., 2009a; Hughes et al., 2013b). However, the generation and propagation of action potentials is a dynamic process that not only results from increased excitation, but also loss of inhibition. We recently showed that muscular / mucosal afferent electrophysiological responses to distension were inhibited following incubation with unstimulated peripheral blood mononuclear cell (PBMC) supernatants from healthy subjects in a manner consistent with activation of the μ-opioid receptor (MOR) (Hughes et al., 2009c; Hughes et al., 2013a). This inhibition was lost following incubation with supernatants from IBS-C patients and switched to sensitization after incubation with supernatants from IBS-D, an effect we characterized as cytokine driven (Hughes et al., 2009c; Hughes et al., 2013a).

Opioids are well known for their analgesic properties, and exert their inhibitory effects by binding to three major receptors; μ, δ and κ. These receptors are $G_{i/o}$ members of the G-protein coupled receptor family, with binding typically decreasing neuronal excitability via inhibition of adenylyl cyclase, activation of potassium channels and inhibition of calcium channels. Immune cells are known to secrete opioids, and T cell derived β-endorphin, a MOR preferring agonist, has previously been shown to be essential for setting the colo-rectal afferent activation threshold to distension in healthy mice (Hughes et al., 2013b; Stein et al., 2003; Verma-Gandhu

et al., 2006). However, little is known of how these studies translate to humans, and opioid secretion by immune cells is yet to be directly investigated in IBS.

In rodents rectal administration of trinitrobenzene sulphonic acid (TNBS) induces an acute colitis characterized by transmural damage to the colon wall associated with an influx in neutrophils and accompanied by increased colonic myeloperoxidase concentrations. This colitis is transient, peaking several days after administration and then spontaneously healing such that by 28 days following administration the histology of the colon and myeloperoxidase levels do not differ from untreated animals (Hughes et al., 2009a; Hughes et al., 2009b; Krauter et al., 2007; Qin et al., 2011). However, colonic afferent nerves remain sensitized to distension long after the mucosa heals, which models aspects of the chronic visceral hypersensitivity (CVH) experienced by IBS patients (Gschossmann et al., 2004; Hughes et al., 2009a; Hughes et al., 2009b). In this model muscular / mucosal afferents are sensitized to mucosal stroking but not circular stretch, and the sensitization observed is relatively modest compared to hypersensitivity displayed by high-threshold afferents (Hughes et al., 2009b). The molecular changes underlying the sensitization of colo-rectal sensory afferents following recovery from inflammation and the contribution by opioid receptors to CVH in this model remain to be determined.

In order to investigate the interaction between the immune system and pain symptoms in IBS patients we aimed to determine the immune cell type(s) responsible for β-endorphin secretion in humans, and whether this is altered in IBS. We also aimed to determine whether the effects that PBMC supernatants from IBS

patients had on colo-rectal afferent function in CVH mice differed from those caused by supernatants from healthy subjects (HS).

MATERIALS AND METHODS

All experiments were approved by the Royal Adelaide Hospital, Flinders Medical Centre and University of Adelaide Human Ethics Committees, and the Animal Ethics Committees of SA Pathology and University of Adelaide.

Human subjects.

Two cohorts of HS and ROME II categorized IBS patients were recruited from the Department of Gastroenterology and Hepatology at the Royal Adelaide Hospital and Department of Luminal Gastroenterology, Flinders Medical Centre, South Australia. Patients were categorized according to bowel habits as either diarrhoea predominant (IBS-D), constipation predominant (IBS-C) or alternating (IBS-A) . Patients with more than 3 bowel movements per day and loose stools were categorized as having diarrhoea-predominant IBS (IBS-D) and patients with fewer than 3 movements per week and hard, lumpy stools were categorized as constipation predominant IBS (IBS-C). Patients with an alternating bowel pattern or bowel frequencies that did not exceed the earlier described limits were categorized as alternators (IBS-A). HS were recruited by advertisement. Written informed consent was obtained prior to inclusion. A comprehensive diagnostic work up, including colonoscopy and repeated stool testing, did not reveal structural lesions or any evidence for acute infection as the cause of symptoms. Patients were excluded based on concomitant chronic fatigue syndrome, fibromyalgia or a history of analgesic or immunosuppressive medication (nonsteroidal anti-inflammatory drugs, steroids and so forth) within the 3 months prior to sampling, or if results of full blood count, renal and liver function, fibrinogen and C-reactive protein level indicated medical conditions likely to confound the study

aims. 5 of the IBS-D patients in cohort 1 were considered post infectious with confirmed acute gastrointestinal infection (3 *Salmonella*, 2 *Campylobacter* enteritis) that preceded the manifestation of symptoms. The time interval between acute infection and time of the study was at least 2 years (median 29.8 months). All patients in this group had at least 3 negative stool cultures within the 6 months before inclusion in our study. There was no evidence of a preceding gastrointestinal infection in IBS-C, IBS-A or healthy subjects (HS) in cohort 1 or in any subjects in cohort 2. Abdominal symptoms were assessed utilizing a validated self-report Bowel Disease Questionnaire before study participation (Liebregts et al., 2007). The ranked symptoms of pain intensity and pain frequency were pre-selected before data analysis.

Cohort 1 Blood was taken from 35 IBS patients (58% female; median age 46.3 yr 15 IBS-D, 20 IBS-C) and from 36 HS (63% female; median age 38.9 yr) as previously described (Hughes et al., 2013a; Liebregts et al., 2007). PBMC supernatants (see below) from this cohort were used for electrophysiology experiments.

Cohort 2 Blood was taken from 27 IBS patients (63% female; median age 48.5 yr, 10 IBS-D, 6 IBS-A, 11 IBS-C) and from 18 HS (66% female; median age 49 yr) and used for flow cytometry experiments (see below). This study was powered to detect a difference between healthy subjects and IBS patients ($\alpha=0.05$, $\beta=0.2$).

Rectosigmoid mucosal biopsies were taken from some HS and IBS patients from this cohort.

Peripheral Blood Mononuclear Cell isolation and culture

PBMC were isolated from fresh blood by density centrifugation as previously described (Hughes et al., 2013a; Liebregts et al., 2007). Briefly, fresh blood was

diluted 1:2 in PBS, layered onto Lymphoprep solution (Lymphoprep, Axis Shield, Norway) and centrifuged at 850g for 20 min. PBMC were harvested, washed in sterile PBS, viability determined by trypan blue (Invitrogen, USA) exclusion and resuspended to $1x10^7$ cells/mL in complete medium (RPMI 1640, Gibco, Germany) supplemented fetal calf serum, glutamax and penicillin / streptomycin. Only PBMC with greater than 80% viability were included. PBMC from cohort 1 were cultured overnight in complete media and supernatants were collected, pooled according to IBS-D, IBS-C or HS and stored at -80°C. PBMC from cohort 2 were analyzed by flow cytometry either freshly thawed or after stimulation with 1 ng/ml Lipopolysaccharide (LPS) (Sigma, NSW, Australia) for 16 hours or 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) / 500 ng/ml ionomycin (Sigma) for 4 hours at 37°C in a humidified 5% CO_2 atmosphere. For intracellular flow cytometry experiments cells were also cultured in the presence of the protein transport inhibitor Brefeldin-A, (GolgiPlug, BD Bioscience, CA, USA).

Chronic visceral hypersensitivity model (CVH mice)

Thirteen week old male c57/Bl6 mice were fasted overnight prior to treatment with free access to 5% glucose solution for nutrition. Experiments were restricted to male mice to control for the potential effects of sex steroids on pain responses (Miranda et al., 2011; Myers et al., 2011). Colitis was induced the next morning by a single intrarectal administration of 0.1 ml trinitrobenzene sulfonic acid (TNBS) (Sigma) (130 μg/ml / 30% ethanol) via a polyethylene catheter inserted 3 cm from the anus of isoflurane-anaesthetised mice as previously described (Brierley et al., 2009; Harrington et al., 2012; Hughes et al., 2009b). After 28 days mice were used for experiments.

Electrophysiology

Colo-rectal single unit extracellular electrophysiological recordings from muscular / mucosal afferents from the pelvic nerve were performed as previously described (Brierley et al., 2009; Brierley et al., 2004; Brierley et al., 2008; Hughes et al., 2009b; Hughes et al., 2013a). Briefly the distal colo-rectum plus attached pelvic nerves were removed from the mice, the colo-rectum opened up longitudinally and pinned mucosal side up in a specialized organ bath. The colonic compartment was superfused with modified Krebs solution composed of 117.9mmol/L NaCl, 4.7mmol/L KCl, 25mmol/L $NaHCO_3$, 1.3mmol/L NaH_2PO_4, 1.2mmol/L $MgSO_4(H2O)_7$, 2.5mmol/L $CaCl_2$, 11.1mmol/L D-Glucose and bubbled with 95% O_2/5% CO_2 at 34°C, supplemented with the L-type calcium channel antagonist nifedipine (1 μmol/L) to block smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 μmol/L) to block endogenous prostaglandin activity. The nerve bundle was extended into the paraffin-filled recording compartment in which finely dissected strands were laid onto a mirror, and single fibers were recorded. Receptive fields were identified and then tested with 3 distinct mechanical stimuli to enable classification: focal compression with calibrated von Frey hairs (1000mg), mucosal stroking with calibrated von Frey hairs (10mg), and circular stretch (3g). Stretch was applied by a claw attached to the tissue near the receptive field connected to a cantilever system. Muscular / mucosal afferents were identified by their response to both low intensity mucosal stroking (10mg Von Frey Hair (VFH)) and circular stretch (3g). Following classification baseline mechanosensitivity was determined by mucosal stroking with calibrated VFH (10, 200, 500, 1000mg) and circular stretch (5gm). Afferents were then incubated in 100 μL of PBMC supernatant or 1 μM [D-

Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) (MOR selective agonist) (Sigma) for 5 minutes and mechanosensitivity re-tested. Some supernatants were added in the presence of 10 μM CTOP (MOR selective antagonist) (Sigma) that was firstly pre-incubated for 10 minutes alone and mechanosensitivity re-assessed. In all cases n=number of observations, N=number of mice.

Quantitative RT-PCR

Messenger RNA (mRNA) was extracted from L6-S1 DRG from three healthy (17 week old C57/Bl6 male) and three CVH mice using RNeasy kits (Qiagen, CA, USA). Equal mRNA amounts from each mouse were pooled and quantitative real time PCR (qRT-PCR) analysis performed using the following conditions: Reverse Transcription: 50°C (30 min); Initial PCR activation: 95°C (15 min); Annealing, denaturing and extension cycles: 94°C (15 sec), 55°C (30 sec) and 72°C (30 sec) repeated for 50 cycles. Experiments were run on a Chromo-4 real time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analyzed using Opticon Monitor software (MJ research). The abundance of *oprm-1* (primers (Geneworks, Australia): forward CTCCACAATCGAACAGCAAA, reverse TCATTCCTCTGTCCATGCAA)) was compared between healthy and CVH mice using the comparative cycle threshold method with *tubb3* (primers: forward CCAAGTTCTGGGAGGTCATC, reverse TGAGAGGAGGCCTCATTGTAG) as a housekeeper, as previously described (Brierley et al., 2009; Brierley et al., 2008; Hughes et al., 2007; Hughes et al., 2013a).

β-endorphin immunoprecipitation

Anti-human β-endorphin (ThermoScientific, MA, USA) was conjugated to magnetic beads essentially according to manufacturer's instructions. Briefly, 25μg of antibody was incubated with 5mg of M-270 Epoxy Dynabeads (Invitrogen) for 20 hours at 37°C on a roller. The antibody / bead conjugate was washed in 0.05% Tween20 and reconstituted to 10mg/mL for use in immunoprecipitation experiments. 1mg of the antibody / bead conjugate was incubated in PBS in the presence or absence of 600ng of β-endorphin peptide (Abnova, Taiwan) for 2 hours at 4°C, washed 4 x in PBS and eluted with 0.1M citric acid, pH 3.1. As a second negative control unconjugated beads were incubated with β-endorphin. 1 μL(1/10) of the eluate was measured in reflectron mode on a UltraFlex III MALDI-TOF mass spectrometer (Bruker Daltinik GmbH, Germany) as described previously (Condina et al., 2009).

Flow cytometry 2 mg/ml mouse anti-human human β-endorphin or mouse IgG_1 isotype control (X-63, in house) antibodies were directly conjugated to Alexa-fluor 647 carboxylic acid, succinimidyl ester (Invitrogen, NY, USA) according to manufacturer's instructions. Briefly antibodies were incubated with 20 fold molar excess of dimethylsulfoxide diluted dye at 4°C for 1 hour. Conjugated antibody fractions were then separated from unbound dye or antibody by low pressure chromotography and further concentrated by centrifugation (Vivaspin, GE, Australia) for use in flow cytometry experiments.

$0.5x10^6$ PBMC were stained with monoclonal antibodies against CD4, CD8, CD14 or CD19 (APC-Cy7 conjugated), or CD33 (PerCP-Cy5.5 conjugated), fixed and permeabilized (Cytofix/Cytoperm) (BD Biosciences) and then stained with monoclonal antibodies against β-endorphin (AF-647 conjugated), TNF-α (PeCy7

conjugated) or mouse α-IgG_1 (AF-647 or PeCy7 conjugated). All antibodies were purchased from BD Bioscience unless stated. Data was analyzed within 2 hours on a FACSCanto II flow cytometer (BD Bioscience) using FlowJo software (Tree Star, OR, USA). At least 20,000 events were analyzed per sample.

Immunohistochemistry 10 μm frozen sections of cryoprotected formalin fixed rectosigmoid mucosal biopsies were incubated at 4°C overnight with mouse α-β-endorphin (IgG_1) (1:200) (ThermoScientific) and rabbit α-CD3 (1:400) (Abcam, Cambridge, UK) or mouse α-CD68 (IgG_{2b}) (1:1600) (BD Bioscience) antibodies with isotype selective secondary antibodies raised in goat or chicken coupled to AF-488, 546 or 594 for visualization. Cell counts were performed independently by three blinded investigators on mucosal colonic biopsies from 9 HS (67% female, median age 43 yr.) and 14 IBS patients (75% female, median age 46.1 yr, 5 IBS-C, 5 IBS-A and 4 IBS-D). Two biopsies were counted per subject at 400X, and staining of lamina propria cells was counted from ten non-overlapping sections per biopsy. CD3 and CD68 cell counts are expressed as cell counts / mm^2 tissue. Co-localization was determined by counting the number of β-endorphin containing cells that co-expressed either CD3 or CD68 per 150 β-endorphin positive cells per subject, and the number of CD68 +ve cells that co-expressed β-endorphin per 150 CD68 +ve cells per subject.

Statistical analysis.

In all cases results are expressed as Mean±SEM, N=number of subjects and n=number of observations. Paired and unpaired *Student's* t-tests or Two-Way ANOVA determined the significance of changes in colonic afferent

mechanosensitivity. Unpaired *Student's* t-tests determined the significance of differences in immune parameters between IBS and HS, with One-Way ANOVA used to determine differences between IBS cohorts. The relationship between intracellular β-endorphin and ranked symptom scores was assessed by Spearman rank correlations. In all cases *P<0.05, **P<0.01, ***P<0001.

RESULTS

Patient characteristics

There were no differences between the self-reported symptom scores for pain intensity or pain frequency between IBS patient cohorts (pain intensity: 2.5±0.2 cohort 1 *vs* 3.0±0.3 cohort 2. pain frequency: 3.3±0.3 cohort 1 *vs* 3.8±0.3 cohort 2), or between IBS subtypes (pain intensity: IBS-D 2.6±0.2, IBS-C 2.8±0.2, IBS-A 3.0±0. pain frequency: IBS-D 3.3±0.4, IBS-C 3.8±0.3, IBS-A 3.2±0.7).

PBMC supernatants inhibit muscular / mucosal afferents in CVH mice

We assessed the effects that PBMC supernatants from HS, IBS-C and IBS-D patients had on muscular / mucosal afferent mechanosensitivity in CVH mice. Incubation with PBMC supernatants from HS (N=6, n=9) (figure 1A) and IBS-C patients (N=6, n=7) (figure 1B) caused a pronounced inhibition of responses to i) 5g circular stretch (HS p=0.036, IBS-C p=0.011) and ii) graded increases in mucosal stroking intensity (HS p=0.005, IBS-C p=0.009) in CVH mice, while PBMC supernatants from IBS-D patients (N=6, n=6) (figure 1C) had no effect on afferent responses to either i) stretch (p=0.72) or ii) stroke (p=0.16).

The effects that PBMC supernatants had on afferent mechanosensitivity in CVH mice outlined above differ considerably from those we previously characterized in healthy mice (Hughes et al., 2009c; Hughes et al., 2013a). The inhibition observed in healthy mice following incubation with PBMC supernatants from HS (N=5, n=10) was maintained in CVH mice (N=6, n=9), and while there was a trend toward a stronger inhibitory effect in response to 5g circular stretch (figure 2Ai) and 500mg vfh mucosal stroking (figure 2Aii) this did not reach statistical significance (stretch p=0.14, stroke

0.30). The inhibition of responses to circular stretch observed following incubation with PBMC supernatants from IBS-C patients in CVH mice (N=6, n=7) was substantially different (p=0.046) to responses in healthy mice (N=6, n=6), where we previously reported there was no effect (figure 2Bi). The inhibitory effect on responses to mucosal stroking in CVH mice by IBS-C supernatants did not differ significantly (p=0.43) from responses in healthy mice (figure 2Bii). The sensitizing effects PBMC supernatants from IBS-D patients had on responses to circular stretch (figure 2Ci) and mucosal stroking (figure 2Cii) observed in healthy mice (N=4, n=7) were completely lost in CVH mice (N=6, n=6) (stretch p=0.034, stroke p=0.044). Combined these results indicate PBMC supernatants have a more pronounced inhibitory effect on the mechanosensitivity of muscular / mucosal afferents in CVH mice compared to healthy mice.

Pre-incubation with the MOR selective antagonist CTOP had no effect on baseline mechanosensitivity (data not shown), but blocked the IBS-C supernatant induced inhibition of responses to 5g circular stretch (figure 3Ai) and 500mg vfh mucosal stroke (figure 3Aii) in CVH mice (N=3, n=4) (stretch p=0.60, stroke p=0.49), a similar finding to those we previously observed with HS supernatants in healthy mice (Hughes et al., 2013a). Dorsal Root Ganglia (DRG) from L6-S1 corresponds to the spinal level for the pelvic innervation of the colo-rectum (Hughes et al., 2007; Robinson et al., 2004). We found that L6-S1 DRG express MOR mRNA (Figure 3Bi), and this expression is substantially increased in L6-S1 DRG from CVH mice when compared to healthy mice (figure 3Bii). In CVH mice, incubation of afferent endings with the MOR selective agonist DAMGO (1μM) inhibited responses to circular stretch (figure 3Ci) (p=0.047) and mucosal stroking (p=0.029) (figure 3Cii) (N=5, n=6), as

previously observed in healthy mice (N=6, n=7) (figure 3Di and ii) (Hughes et al., 2013a). These results indicate the inhibitory effects we observe from IBS supernatants are again mediated by the μ-opioid receptor, which is upregulated in CVH mice.

Monocyte / macrophages are the primary immune source of β-endorphin in PBMC

Firstly we confirmed that our antibody recognizes β-endorphin using MALDI TOF mass-spectrometry analysis. The antibody against β-endorphin was covalently bound to magnetic beads and native β-endorphin protein was immunoprecipitated. The eluate was analyzed by mass-spectrometry and a single mass of 3463, the expected mass of β-endorphin, was detected (figure 4A). We confirmed the expected mass of β-endorphin by measuring β-endorphin alone (figure 4B). This mass was not present when beads conjugated with antibody were incubated without β-endorphin (figure 4C) or if the unconjugated beads were incubated with β-endorphin (figure 4D), indicating the peak is not due to non-specific binding.

Flow cytometry was used to identify the immune cell type(s) responsible for β-endorphin secretion in PBMC with a gating strategy as shown on figure 5Ai-vi. In unstimulated PBMC (figure 5B), CD14 monocytes stained for β-endorphin (figure 5Bi), but there was no observable staining above IgG control for β-endorphin in CD4, CD8 or CD19 T or B cells (figure 5Bii-iv). To investigate further we used LPS culture which is a strong stimulator of innate immune cells including monocyte / macrophages, and PMA/ionomycin which activates lymphocytes, including T cell

subsets and B cells. We observed that TNF-α production occurred in LPS stimulated CD33 monocyte / macrophages (figure 5Ci) and PMA/ionomycin stimulated CD4, CD8 and CD19 lymphocytes (figure 5Cii-iv), indicating our stimulations were successful. We also found that β-endorphin production was substantially higher than background levels in LPS stimulated CD33 monocyte / macrophages (figure 5Di), but not in PMA/ionomycin stimulated CD4, CD8 or CD19 lymphocytes (figure 5Dii-iv). These results indicate that human monocytes and LPS-stimulated CD33 monocyte / macrophages contain β-endorphin, while lymphocytes do not.

β-endorphin levels in PBMC are altered in IBS

In PBMC isolated from IBS patients (N=27) the mean fluorescence intensity (MFI) for β-endorphin in unstimulated CD14 monocytes was significantly reduced compared to HS (N=18) (24522±1493 (HS) *vs* 18111±1787 (IBS) p=0.009) (figure 6Ai). However there was no difference between the respective IBS patient cohorts based on bowel habit (data not shown). Interestingly, after LPS stimulation there was a significant increase in β-endorphin in IBS patients (N=18) compared to HS (N=12) (1783±204 (HS) *vs* 2779±337 (IBS) p=0.033) (figure 6Bi), but again no difference between IBS patient cohorts (data not shown). There were no differences in the proportions of CD14 or CD33 cells in the monocyte / macrophage gate between HS and IBS patients (CD14: 87.8%±0.5 (HS) *vs* 86.4%±0.7 (IBS) p=0.11), CD33: 38.4%±4.7 (HS) *vs* 42.6%±4.4 (IBS) p=0.37) (figure 6Aii and Bii respectively), or between IBS patient subtypes (data not shown) indicating our results are not due to differences in cell proportions. β-endorphin MFI did not correlate with symptoms of pain intensity or pain frequency reported by IBS patient cohort 2 (P>0.05 for both symptoms) in either unstimulated CD14 monocytes or LPS stimulated CD33 monocyte / macrophages

(figure 6C), indicating this assay is not likely to be useful as a biomarker or diagnostic test for IBS.

β-endorphin expression is reduced in colonic biopsies from IBS patients

We investigated β-endorphin expression in CD68 macrophages and CD3 T cells in colonic biopsies from some of the healthy and IBS blood donors (figure 7A). The majority of β-endorphin expressing cells in the colonic lamina propria were CD68 macrophages (87.8%±4.5, N=6), while only a relatively small percentage of β-endorphin expressing cells were T cells (12.1%±4.2, N=11) $p<0.001$ (figure 7B). We then asked whether the β-endorphin expressing cells are widespread throughout the CD68 lineage, or whether they represent a discrete subpopulation, and found nearly all (90.4% ± 1.5) macrophages expressed β-endorphin. When we counted the number of CD68 cells in the lamina propria we found they were decreased in IBS patients (N=14) compared to HS (N=9) (49.4±2.9 (HS) vs 40.5±1.1 (IBS), $p=0.0036$ (figure 7C). Post-hoc analysis indicated CD68 counts are unlikely to differ between IBS patient subtypes (data not shown), although it should be noted that patient numbers were low for these studies.

DISCUSSION

We show for the first time using a novel flow cytometry based intracellular staining method for β-endorphin that monocyte / macrophages are the primary immune source of β-endorphin in human PBMC and colon, and that β-endorphin production by these cells is altered in IBS patients. We also demonstrate that inhibitory signaling via MOR is up-regulated in mice that develop chronic visceral hypersensitivity following recovery from acute inflammation of the colo-rectum. These results support the theory that neuro-immune interactions are altered in IBS patients.

β-endorphin is an endogenous agonist of MOR, which we confirm is expressed and functional in colonic sensory afferents (Chatter et al., 2012; Hughes et al., 2013a; Valdez-Morales et al., 2013). The functional effects that PBMC supernatants from healthy subjects, IBS-C and IBS-D patients had in CVH mice followed a similar pattern to that we previously observed in healthy mice (Hughes et al., 2009c; Hughes et al., 2013a); whereby healthy subjects caused the most inhibition, followed by IBS-C and then IBS-D. Overall the inhibitory effects observed with all the subject cohorts were more prominent in CVH mice compared to healthy mice. MOR expression has been shown to be increased in sensory neurons in various animal models of inflammatory and neuropathic pain (Busch-Dienstfertig and Stein, 2010). Here we show acute colonic inflammation induces an up-regulation of MOR in sensory DRG neurons that is sustained long after the colon has healed. This finding provides a mechanism for the increased inhibitory effects from the human supernatants. We also found that selective MOR agonism with DAMGO mimicked the inhibitory effects of supernatants in CVH mice, which was of similar potency to

healthy mice. However the concentration of DAMGO used was high, potentially resulting in saturation of downstream second messenger pathways / effecter ion channels which may have prevented further inhibition. Despite their well-documented analgesic effects much remains to be understood regarding opioid receptor physiology in colonic extrinsic sensory afferents, with the potential for heteromeric complexes of opioid receptors to activate multiple G-protein receptor signaling pathways and potentially act on numerous effecter ion channels (Costantino et al., 2012; Wang et al., 2010). Further, mice lacking MOR have been shown to be more susceptible to TNBS induced colonic inflammation, indicating MOR activation may also have prominent effects on the immune system (Philippe et al., 2003). It is currently unclear whether these effects are due to direct MOR activation on immune cells or occur indirectly due to neuronal consequences of MOR activation.

In the current study we identified monocyte / macrophages as the predominant source of immune derived β-endorphin in human PBMC and colonic biopsies. This differs from previous mouse studies indicating T cells are the major source in the colon in health (Verma-Gandhu et al., 2006). We found monocyte derived β-endorphin is decreased in IBS patients, and this supports the loss of inhibition that PBMC supernatants from IBS patients had on mouse colo-rectal afferents. It has been suggested that endogenous opioids do not influence colo-rectal sensations in healthy humans, as infusion of 20μg/kg/hr naloxone does not alter sensitivity to rectal balloon pressure (Geeraerts et al., 2009). However, this dose is typically used to antagonize opioid induced respiratory depression, an effect predominantly mediated by the central nervous system, and may not be high enough to block the effects of endogenous opioids in opioid naive subjects. Indeed infusions of up to 4

mg/kg/hr are reportedly well tolerated in healthy humans, although sensitivity to rectal distension at high doses is yet to be investigated (Clarke et al., 2005). Interestingly, our results suggest monocyte / macrophage derived β-endorphin levels do not differ between IBS patient cohorts, indicating the differences in the functional effects that supernatants from IBS-D and IBS-C patients have on colo-rectal sensory afferents are due to excitatory actions of cytokines TNF-α, IL-1β and IL-6, which we previously showed were increased in IBS-D relative to IBS-C (Hughes et al., 2013a; Liebregts et al., 2007). The lack of difference between patients based on bowel habit is perplexing however, as monocyte / macrophages are likely to be in close contact with the enteric nervous system where MOR agonists are well known to induce constipation. Indeed the weak MOR agonist Loperamide normalizes diarrhoea in IBS-D, but only while taken (Talley, 2003). The enteric nervous system was not investigated as part of these studies, but our own anecdotal evidence does not suggest CVH mice are constipated. However, investigations during acute TNBS colitis indicate inhibitory mechanisms are upregulated, with colonic motility slowed at sites of ulceration and reductions in the mechanosensitivity of high threshold colo-rectal afferents from the pelvic pathway (Hughes et al., 2009b; Strong et al., 2010). Secretion of opioids by immune cells may contribute toward these findings but are yet to be investigated. Recent studies with new peripherally restricted therapies targeting opioidergic mechanisms are showing promise for IBS treatment, particularly IBS-D, although it is currently unknown whether alterations in enteric or extrinsic neural opioid pathways underlie symptoms in IBS patients (Dove et al., 2013; Mangel and Hicks, 2012).

Monocytes are the precursors of macrophages and dendritic cells but also have important immune properties in their own right including phagocytosis and cytokine secretion (Auffray et al., 2009). Afferent endings are exposed to monocyte derived factors as they frequently line blood vessel walls which monocytes move through as they migrate from the blood stream into the colon (Song et al., 2009). The intestinal immune system is in a constant state of activation, and monocytes frequently migrate to the intestine in health (MacDonald et al., 2011). Monocytic expression of the gram positive bacterial sensor toll like receptor (TLR)-2 is increased in IBS patients, indicating an increased activation profile (Ohman et al., 2012). However this study focused on CD11c monocytes, presumably dendritic cell precursors, which represent only a subpopulation of the monocytes investigated in our study. Once they enter the lamina propria, monocytes mature into macrophages in response to signals from the local environment. Expression of monocyte attracting chemokines CCL2 and CXCL-10 are decreased in the colon wall in IBS relative to health, and we confirm previous findings indicating colonic lamina propria macrophage numbers are also reduced in IBS patients based on CD68 staining (Braak et al., 2012; Macsharry et al., 2008; Spiller et al., 2000). Interestingly in one of these papers Spiller *et. al.* also suggested calprotectin positive macrophage numbers were increased in IBS, despite the decrease in CD68 macrophages (Spiller et al., 2000), raising the possibility that discrete subsets of macrophages may be activated in IBS.

Macrophages may be categorized according to function as belonging to M1 or M2 subsets, with M1 promoting inflammation and M2 promoting repair, although distinctly identifying these is currently challenging (Murray and Wynn, 2011). CD68 does not differentiate between subsets, and we found the majority of CD68 stained macrophages expressed β-endorphin, indicating it is unlikely to be restricted to a

particular macrophage subtype. Taken together these studies indicate that decreased numbers of β-endorphin containing cells migrate to the colon in IBS, and those that do migrate contain less β-endorphin than those in healthy subjects.

LPS is a powerful stimulator of innate cells and drives the *in vitro* maturation of monocytes into macrophages. When we stimulated PBMC with LPS we found β-endorphin levels in macrophages were increased in IBS patients compared to health. These results are particularly striking given that β-endorphin levels started from a lower baseline in IBS patients than in healthy subjects, indicating strongly stimulated macrophages are in a more responsive state in IBS. This suggestion is supported by previous reports demonstrating LPS stimulated PBMC from IBS patients secrete higher concentrations of macrophage derived cytokines including TNF-α, IL-1β and IL-6 relative to health (Hughes et al., 2013a; Liebregts et al., 2007; McKernan et al., 2011). As LPS is such a strong stimulator this type of response may be similar to that observed during overt inflammatory conditions, such as Inflammatory Bowel Disease (IBD). Indeed patients with Crohn's disease and Ulcerative Colitis experience hyposensitivity to rectal balloon pressure, which has previously been attributed to alterations in the central nervous system processing and descending inhibitory nervous pathways (Bernstein et al., 1996; Chang et al., 2000). Our studies indicate immune derived β-endorphin may also provide inhibitory influences in IBD , as has been described in mouse models of colonic inflammation, however this is yet to be investigated in humans (Valdez-Morales et al., 2013; Verma-Gandhu et al., 2007).

In conclusion we identify monocyte / macrophages as the main immune cell type responsible for β-endorphin secretion in humans. We demonstrate that immune derived β-endorphin is decreased in IBS patients, but that this is dynamic and following stimulation increases to levels greater than those in healthy subjects. We also show acute inflammatory insults can induce long lasting changes in opioid receptor expression on colonic sensory afferents. Combined these results support the concept that immune function is altered in IBS, and demonstrates that these alterations have functional consequences for viscerosensory afferent nerves.

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Figure Legends

Figure 1: Effects of Peripheral Blood Mononuclear Cell (PBMC) supernatants from humans on muscular / mucosal afferent mechanosensitivity.

PBMC supernatants from (**A**) HS (N=6, n=9) and (**B**) IBS-C (N=6, n=7) inhibited muscular / mucosal afferent mechanosensitivity to (**i**) 5g circular stretch (paired t-test) and (**ii**) mucosal stroking of graded intensity (10-1000mg vfh) (2-way ANOVA with Bonferroni post-hoc), while supernatants from (**C**) IBS-D (N=6, n=6) were without effect. Also shown are representative examples of responses to (**i**) 5g stretch and (**ii**) 500mg vfh mucosal stroking before and after incubation with supernatant for PBMC supernatants from (**A**) HS, (**B**) IBS-C and (**C**) IBS-D. N=number of animals, n=number of observations. All data expressed as Mean±SEM. *n.s.* not significant *, # $P<0.05$, ** $P<0.01$.

Figure 2: Comparison of effects of PBMC supernatants from humans on muscular / mucosal afferents.

Responses to (**i**) 5g circular stretch and (**ii**) 500mg vfh mucosal stroking in healthy and chronic visceral hypersensitivity (CVH) mice. PBMC supernatants from (**A**) HS trended toward more inhibition in CVH mice (N=6, n=9) compared to healthy mice (N=5, n=10) (unpaired t-test). PBMC supernatants from (**B**) IBS-C patients caused more inhibition in CVH mice (N=6, n=7) compared to healthy mice (N=6, n=6) (unpaired t-test). The sensitizing effect of PBMC supernatants from (**C**) IBS-D patients in healthy mice (N=4, n=7) were lost in CVH mice (N=6, n=6) (unpaired t-test). (**D**) Table summarizing the effects that PBMC supernatants from HS and IBS patients had on responses by muscular / mucosal afferents to stretch and stroking in healthy mice and CVH mice. Overall, PBMC supernatants have a more inhibitory

effect in CVH mice compared to healthy mice. Data from healthy mice previously published in a different form of analysis (Hughes et al., 2009c; Hughes et al., 2013a)). N=number of animals, n=number of observations. All data expressed as Mean±SEM. *n.s.* not significant *P<0.05.

Figure 3: Inhibition in CVH mice is mediated by μ-opioid receptor.

(**A**) The inhibitory effect that PBMC supernatants from IBS-C patients has on responses to (**i**) 5g circular stretch and (**ii**) mucosal stroking of graded intensity (10-1000mg vfh) is blocked in the presence of the selective μ-opioid receptor antagonist CTOP (10μM) (N=3, n=4) (Stretch: unpaired t-test; Stroke 2-way ANOVA with Bonferroni post hoc). Also shown is a representative example of the response to (**i**) 5g circular stretch and (**ii**) 500mg vfh mucosal stroking in the presence of CTOP before and after incubation with IBS-C supernatant. (**B**) (**i**) Gel electrophoresis of quantitative RT-PCR products indicates colonic DRG neurons express mRNA for OPRM-1. (**ii**) Expression of OPRM-1 (μ-opioid receptor) is increased in L6-S1 DRG from chronic visceral hypersensitivity mice compared to healthy mice (N=3 pooled each healthy and CVH mice) (unpaired t-test). Results expressed as fold difference in CVH mice compared to healthy mice. (**C**) The μ-opioid receptor agonist DAMGO inhibits muscular / mucosal afferent responses to (**i**) 5g circular stretch and (**ii**) mucosal stroking of graded intensity (N=5, n=6) in CVH mice (Stretch: paired t-test; Stroke 2-way ANOVA with Bonferroni post-hoc). Shown is a representative example of the response to (**i**) 5g circular stretch and (**ii**) 500mg vfh mucosal stroking before and after DAMGO incubation. (**D**) The inhibitory effect of DAMGO on response to (**i**) 5g circular stretch and (**ii**) graded mucosal stroking is not increased in CVH mice

(N=5, n=6) compared to healthy mice (N=6, n=7) (unpaired t-test). Results expressed as % change in response to 5g circular stretch and 500mg vfh mucosal stroking before and after incubation with DAMGO. Healthy mice data has been previously published in a different form of analysis (Hughes et al., 2013a). N=number of animals, n=number of observations. All data expressed as Mean±SEM. *n.s.* not significant *$P<0.05$.

Figure 4: Characterization of β-endorphin antibody

Mass spectrometry analysis of (**A**) immunoprecipitants of β-endorphin antibody / magnetic bead conjugates from β-endorphin spiked samples and (**B**) native β-endorphin reveal peaks with a mass of 3463, the expected mass of β-endorphin. This peak was not present in immunoprecipitants of (**C**) conjugated beads with β-endorphin antibody without incubation of β-endorphin or (**D**) unconjugated beads with incubation of β-endorphin.

Figure 5: β-endorphin expression in PBMC from healthy subjects.

β-endorphin is located in unstimulated monocytes and LPS stimulated macrophages, but not unstimulated and PMA/ionomycin stimulated T cells and B cells. (**A**) (**i**) trace of forward *vs.* side scatter on PBMC. Red circle highlights lymphocyte gate, black circle highlights monocyte / macrophage gate. Representative traces of (**ii**) CD14 and (iii) CD33 cells gated on monocyte / macrophage gate and (**iv**) CD4, (**v**) CD8, (**vi**) CD19 gated lymphocyte gate. (**B**) The mean fluorescence intensity (MFI) of β-endorphin is greater that isotype control in (**i**) unstimulated CD14 monocytes, but not in unstimulated (**ii**) CD4 T cells, (**iii**) CD8 T cells or (**iv**) CD19 B cells. (**C**) The MFI of

TNF-α is greater than isotype control in (**i**) LPS stimulated CD33 monocyte / macrophages and PMA/ionomycin stimulated (**ii**) CD4 and (**iii**) CD8 T cells and (**iv**) CD19 T cells, indicating these stimulations worked. (**D**) The MFI of β-endorphin is greater than isotype control in (**i**) LPS stimulated CD33 monocyte / macrophages, but not in PMA/ionomycin stimulated (**ii**) CD4 or (**iii**) CD8 T cells or (**iv**) CD19 B cells.

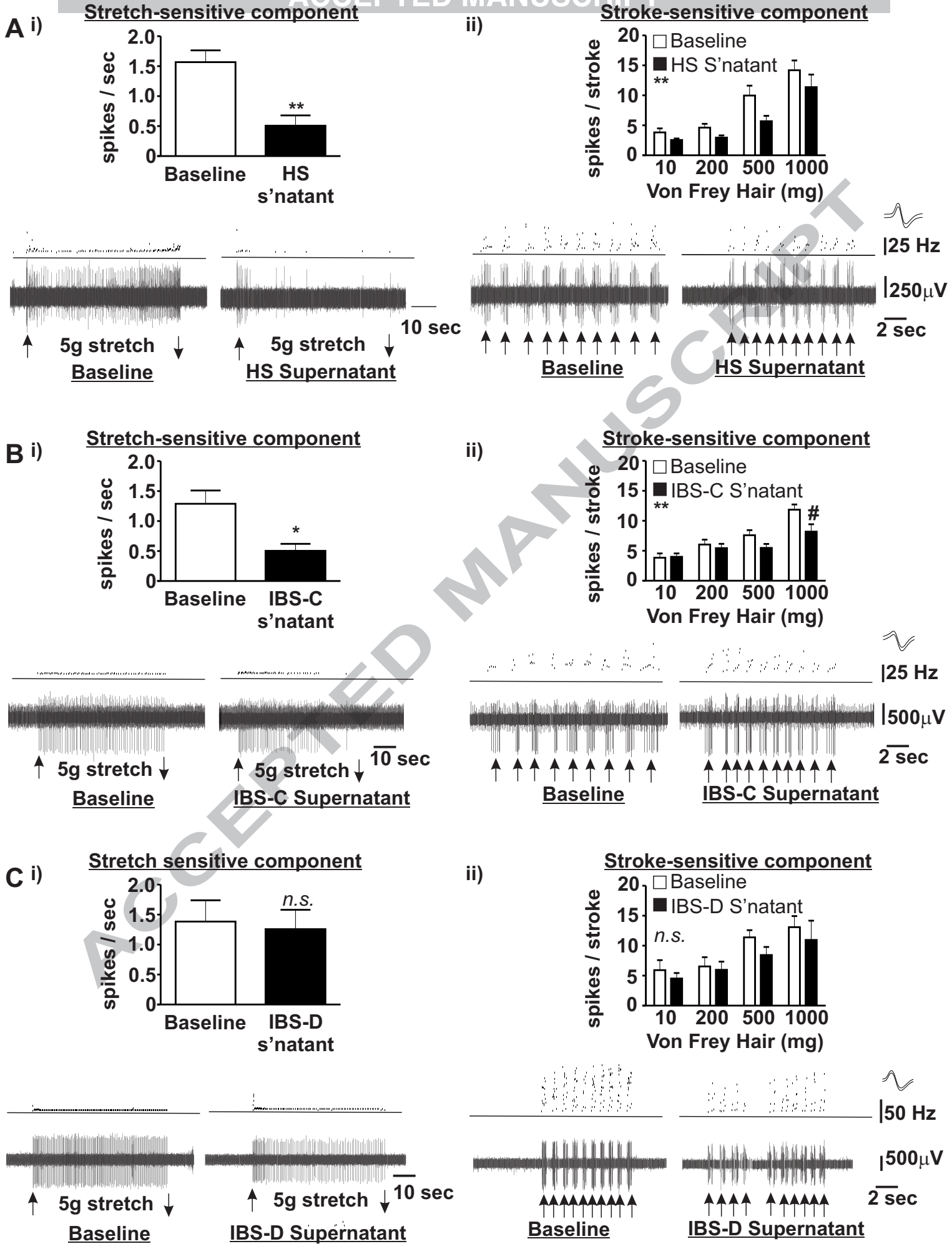
Figure 6: β-endorphin expression in PBMC from IBS patients.

In unstimulated monocytes β-endorphin levels are lower in IBS patients compared to HS. However in LPS stimulated macrophages β-endorphin levels are higher in IBS patients compared to HS. (**A**) (**i**) The MFI of β-endorphin in unstimulated CD14 monocytes is lower in IBS patients (N=27) compared to HS (N=18) (unpaired t-test), but (**ii**) the proportion of CD14 cells in the monocyte gate does not differ between HS and IBS patients. (unpaired t-test). (**B**) The MFI of β-endorphin in LPS stimulated CD33 monocyte / macrophages is increased in IBS patients (N=18) compared to HS (N=12) (unpaired t-test) but (**ii**) the proportion of CD33 cells in the monocyte / macrophage gate does not differ between HS and IBS patients. (unpaired t-test). All data expressed as Mean±SEM. *n.s.* not significant **P*<0.05, ***P*<0.01. (**C**) Table summarizing the lack of correlation between β-endorphin MFI in unstimulated CD14 PBMC and LPS stimulated CD33 PBMC with self-reported ranked symptoms of pain intensity and pain frequency in IBS patients.

Figure 7: β-endorphin co-localizes with CD68 macrophages in human colonic mucosal biopsies.

(**A**) Immunofluorescence staining of human colonic mucosal tissue sections stained with antibodies against CD68 (upper panel) or CD3 (middle panel) in red and β-endorphin (green). Merge of images shows β-endorphin co-expression in a sub-population of CD68 or CD3 cells (yellow). –ve control images are obtained by omitting primary antibody (lower panel). Open arrows: β-endorphin co-localization with CD68 or CD3. Closed arrows: β-endorphin cells that do not co-localize with either CD68 or CD3. Arrowheads: CD68 or CD3 cells that do not co-localize with β-endorphin. Scale bars in all panels equals 50μm. (**B**) β-endorphin containing cells are more likely to be CD68 (N=6) than CD3 (N=11) in the lamina propria of colonic biopsies (unpaired t-test) (**C**) There are less CD68 cells in the colonic lamina propria from IBS patients (N=14) compared to HS (N=9). (unpaired t-test). Results expressed as Mean±SEM. ** $P<0.01$, *** $P<0.001$.

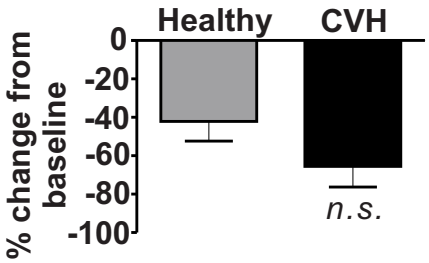
Figure 1 Hughes et. al. "Immune derived opioidergic inhibition....."



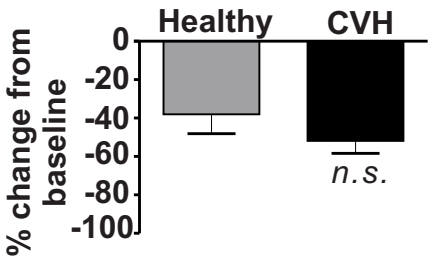
A

PBMC supernatants from Healthy Subjects

i) **Stretch-sensitive component**



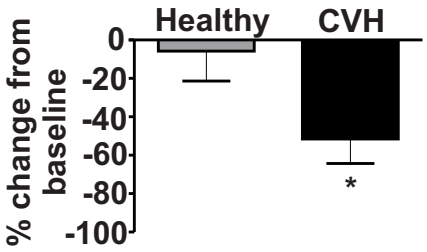
ii) **Stroke-sensitive component**



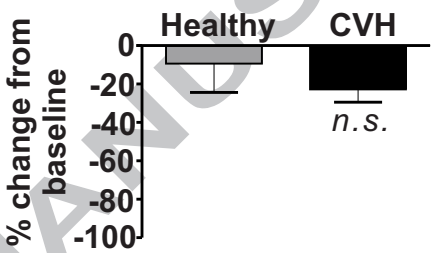
B

PBMC supernatants from IBS-C Subjects

i) **Stretch sensitive component**



ii) **Stroke sensitive component**

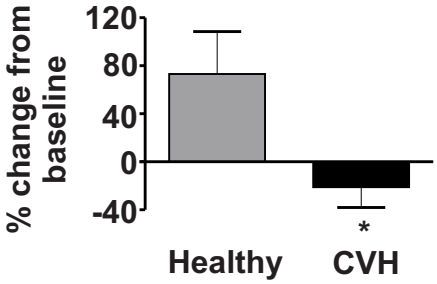


C

PBMC supernatants from IBS-D Subjects

i) **Stretch sensitive component**

ii) **Stroke sensitive component**



D

	Healthy Subjects		IBS-C subjects		IBS-D subjects	
	Stretch	Stroke	Stretch	Stroke	Stretch	Stroke
Healthy Mice	↔	↓	↔	↔	↑	↑
CVH mice	↓	↓	↓	↓	↔	↔

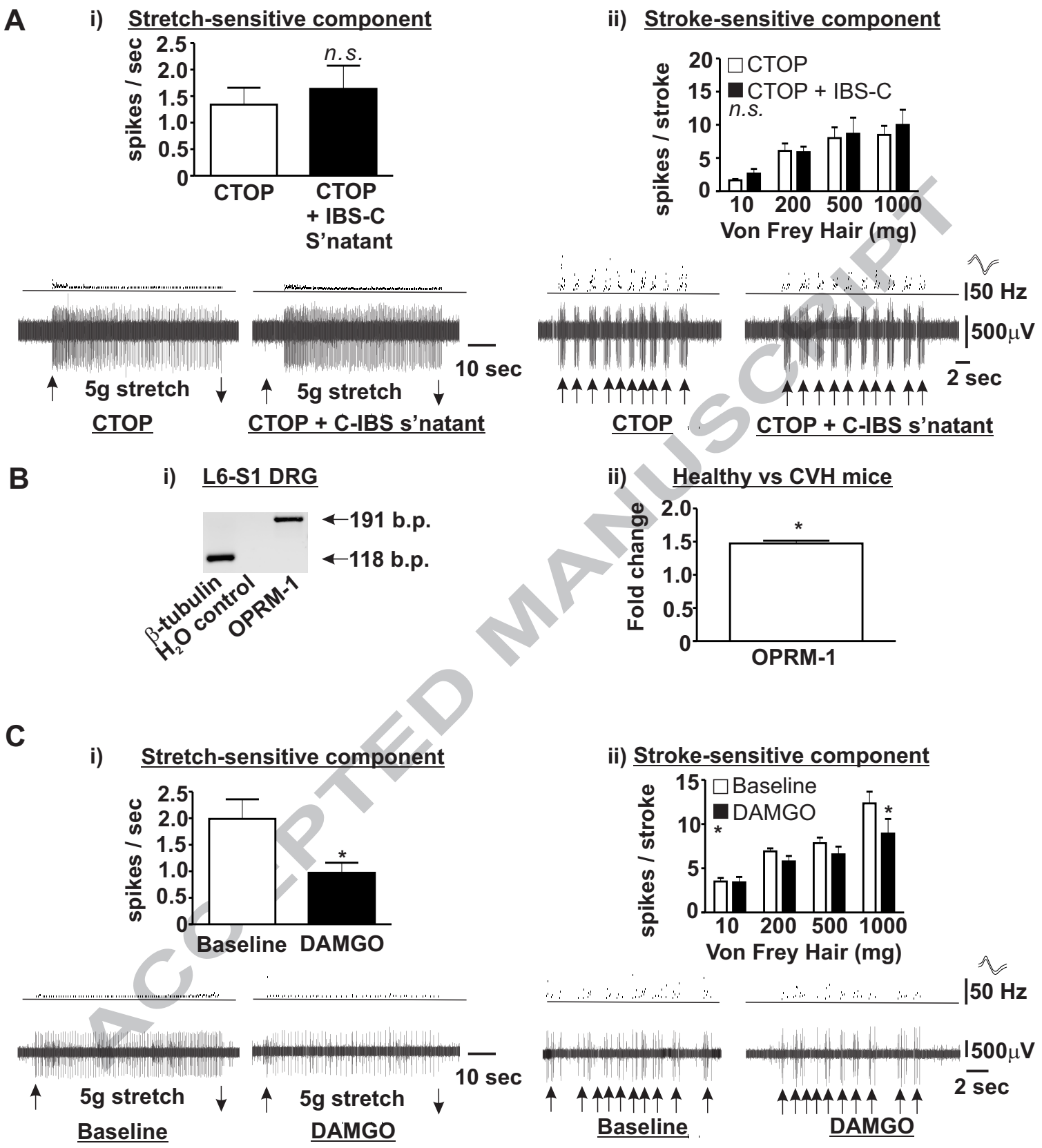
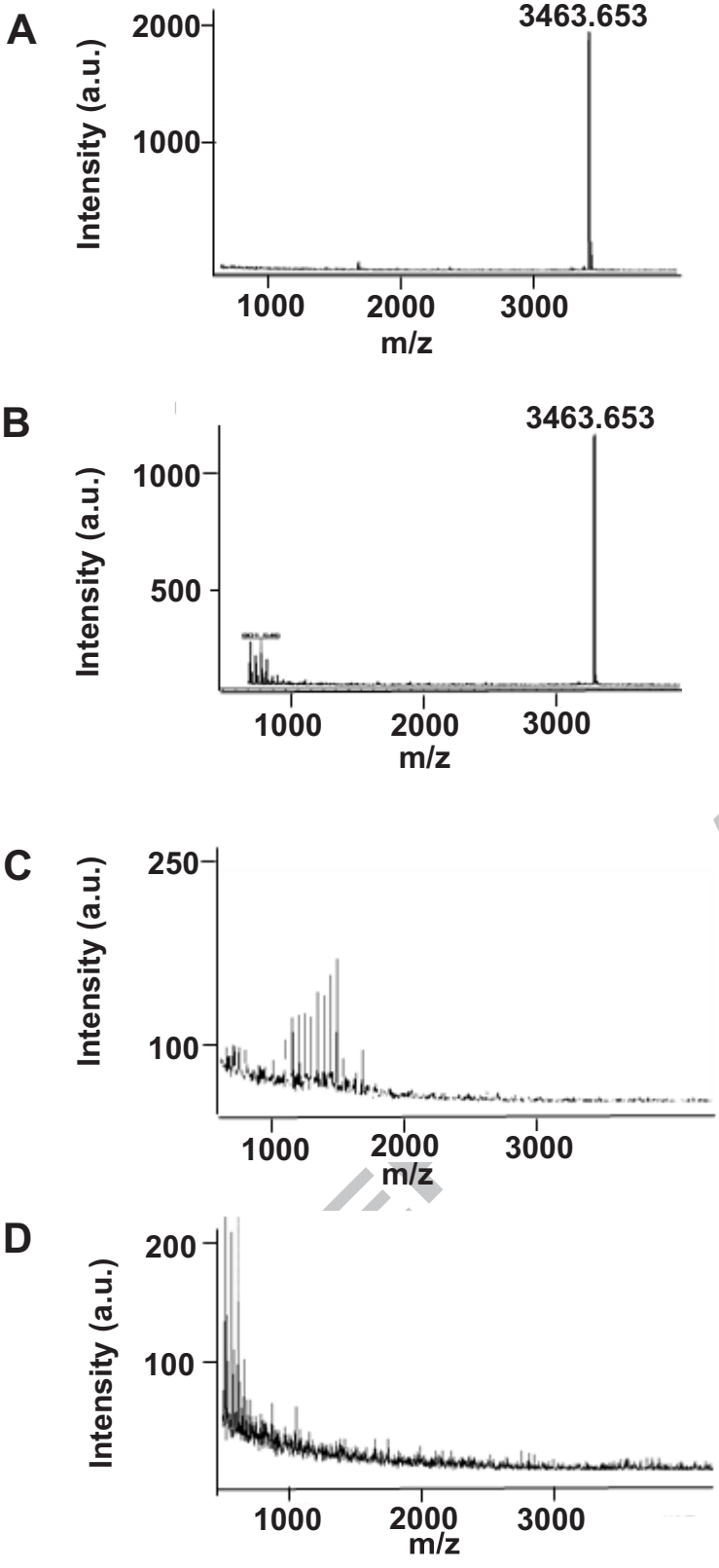


Figure 4



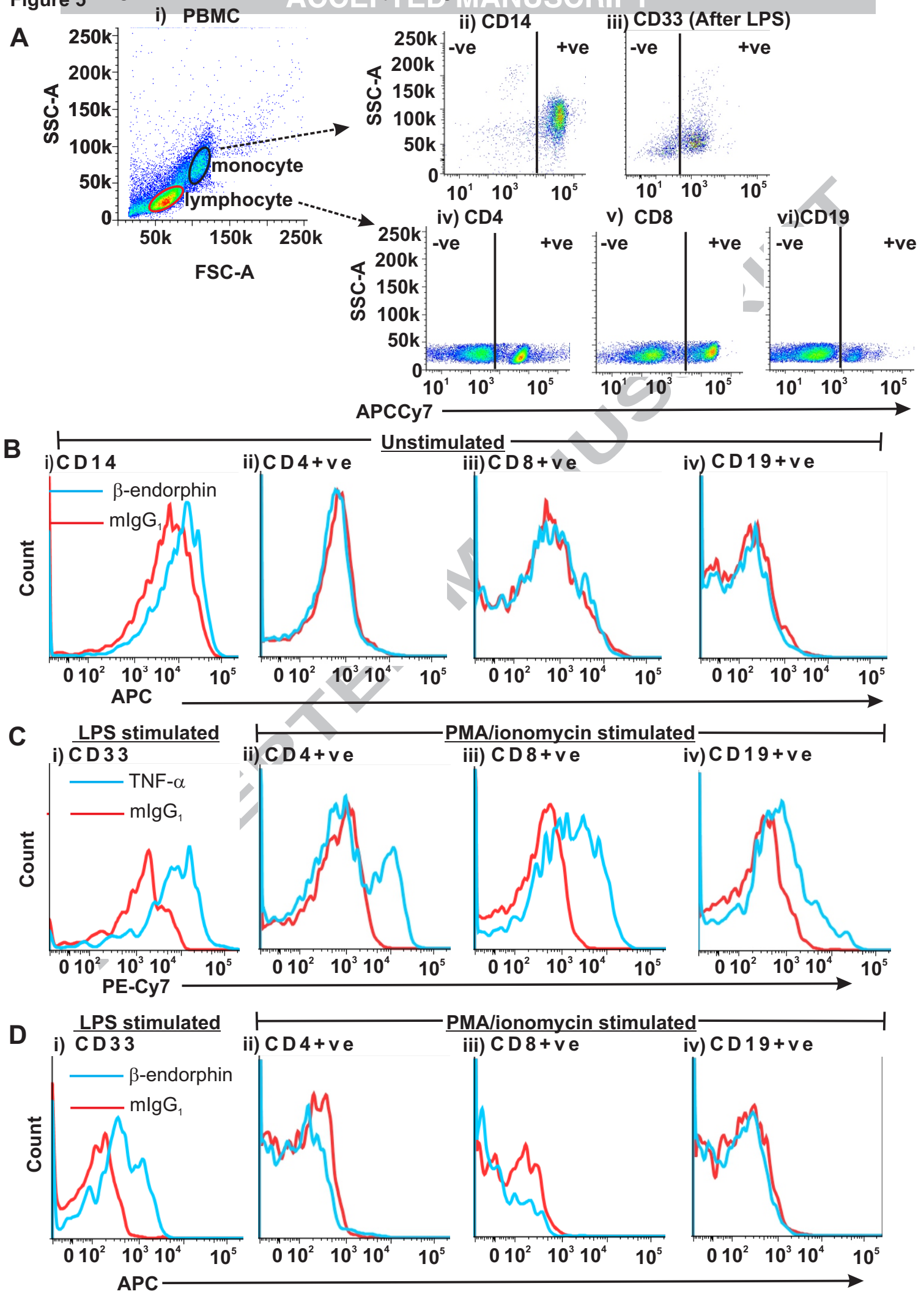
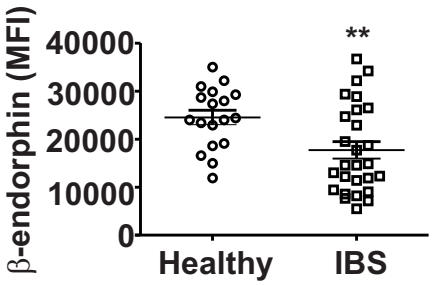
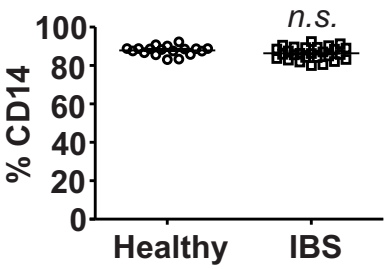


Figure 6 Hughes et. al. "Immune derived opioidergic inhibition....."

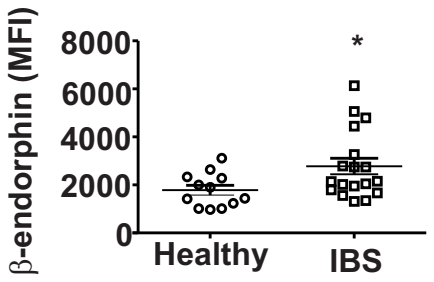
A i) **β-endorphin MFI in unstimulated CD14 monocytes**



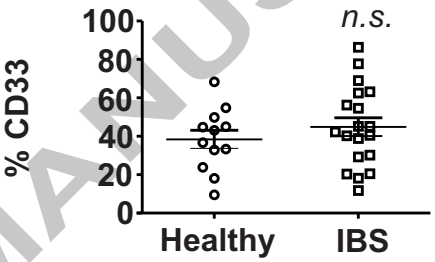
ii) **Proportion of CD14 monocytes**



B i) **β-endorphin MFI in LPS stimulated CD33 macrophages**



ii) **Proportion of CD33 monocyte/macrophages**



C **Correlation of β-endorphin MFI with IBS symptoms**

	Unstimulated CD14 PBMC		LPS stimulated CD33 PBMC	
	r	**P**	**r**	**P**
Pain Intensity	0.1198	0.61 (*n.s.*)	0.1235	0.65 (*n.s.*)
Pain Frequency	0.1349	0.56 (*n.s.*)	0.094	0.73 (*n.s.*)

Figure 7 Hughes et. al. "Immune derived opioidergic inhibition……"