CRF\(_2\) receptor activation prevents colorectal distension-induced visceral pain and spinal ERK1/2 phosphorylation in rats

Mulugeta Million\(^1\)*, Lixin Wang\(^1\), Yuhua Wang\(^1\), David W Adelson\(^1\), Pu-Qing Yuan\(^1\), Céline Maillot\(^3\), Santosh V Coutinho\(^1\), James A McRoberts\(^1\), Alfred Bayati\(^3\), Hillevi Mattsson\(^3\), Vincent Wu\(^1\), Jen-Yu Wei\(^1\), Jean Rivier\(^2\), Wylie Vale\(^2\), Emeran A Mayer\(^1\) and Yvette Taché\(^1\)

\(^1\)CURE/Digestive Diseases Research Center, and Center for Neurovisceral Sciences & Women’s Health, Department of Medicine, Division of Digestive Diseases, University of California Los Angeles, VA Greater Los Angeles Healthcare System, Los Angeles, California 90073, \(^2\)Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA 92186-5800, CA. \(^3\)AstraZeneca R&D, S-431 83 Mölndal, Sweden.

Running Title: CRF\(_2\) receptor activation and visceral pain

*Correspondence to: Dr. M. Million CURE Bldg. 115, Rm # 203; 11301 Wilshire Blvd., Los Angeles, CA 90073, USA. e-mail: mmuluget@ucla.edu
ABSTRACT

Background and aims: Activation of CRF₁ receptors is involved in stress-related responses and visceral pain, while activation of CRF₂ receptors dampens the endocrine and some behavioural stress responses. We hypothesized that CRF₂ receptor activation may influence visceral pain induced by colorectal distension (CRD) in conscious rats, and assessed the possible sites and mechanisms of action.

Methods: Male SD rats were exposed to CRDs (60 mm Hg, 10 min twice at a 10-min interval). Visceromotor responses (VMR) were measured by electromyography or visual observation. Spinal (L6-S1) extracellular signal-regulated kinase 1/2 (ERK 1/2) activation following in vivo CRD and the CRF₂ receptor gene expression in the T13-S1 dorsal root ganglia (DRG) and spinal cord were determined. The inferior splanchnic afferent (ISA) activity to CRD (0.4 ml, 20 s) was assessed by electrophysiological recording in an in vitro ISA nerve-inferior mesenteric artery (ia)-colorectal preparation.

Results: In controls, the VMR to the 2nd CRD was 31±4% higher than that of the 1st (P<0.05). The selective CRF₂ agonist, human urocortin 2 (hUcn 2, at 10 and 20 µg/kg) injected iv after the 1st distension, prevented the sensitisation and reduced the 2nd response by 8±1% and 30±5% (P<0.05) compared to the 1st response, respectively. RT-PCR detected CRF₂ receptor gene expression in the DRG and spinal cord. CRD (60 mm Hg for 10 min)-induced phosphorylation of ERK 1/2 in neurons of lumbosacral laminae I and IIo and the response was dampened by iv hUcn 2. CRD, in vitro, induced robust ISA spike activity that was dose dependently blunted by hUcn 2 (1-3 µg, ia). The CRF₂ receptor antagonist, astressin₂-B (200 µg/kg, sc or 20 µg, ia) blocked the hUcn 2 inhibitory effects in vivo and in vitro.

Conclusions: Peripheral injection of hUcn 2 blunts CRD-induced visceral pain, colonic afferent and spinal L6-S1 ERK 1/2 activity through CRF₂ receptor activation in rats.

Keywords: CRF₂ receptors, urocortin 2, astressin₂-B, visceral pain, colon, ERK 1/2; inferior splanchnic afferents, colorectal distension.

Abbreviations: AUC; area under the curve; CC, central canal; CRD, colorectal distension; CRF, corticotropin-releasing factor; DRG, dorsal root ganglia; EMG, electromyogram; hUcn 2, human urocortin 2; ia, intra-arterial(ly); ip, intraperitoneal(ly); iv, intravenous; IBS, irritable bowel syndrome; IML, intermediolateral column; ISA, inferior splanchnic afferent; MAPK, mitogen-activated protein kinase; pERK, phosphorylated extracellular signal-regulated kinase; RF: receptive field; RT-PCR, reverse transcriptase polymerase chain reaction; VMR, visceromotor response; IIo, laminae II outer layer.
INTRODUCTION

Activation of corticotropin releasing factor (CRF) signalling pathways plays a key role in the body’s response to stress [1-3]. The CRF family encompasses, in addition to CRF, three novel CRF-related mammalian ligands, urocortin 1 (Ucn 1), Ucn 2 and Ucn 3 [4,5]. The CRF ligands display distinct affinities to CRF receptor subtype 1 (CRF1) and/or CRF2. CRF has preferential affinity to CRF1, Ucn 1 binds with equal high affinity to both CRF receptors, while Ucn 2 and Ucn 3 exhibit high selectivity towards CRF2 receptors [4,5]. The availability of CRF1 antagonists that cross the blood brain barrier and recent development of selective peptide CRF2 receptor antagonists, such as antisauvagine-30 and astressin2-B [6,7], have spurred interest in defining the role of CRF receptor subtypes in the stress responses [2,3,8].

In experimental animals, CRF1 is the main receptor implicated in stress-related activation of colonic motility, induction of watery diarrhoea and hypersensitivity to colorectal distension (CRD) [9]. Likewise in healthy subjects, intravenous (iv) administration of ovine CRF, a preferential CRF1 receptor agonist [5], mimicked stress-related visceral responses [10,11]. The peptide lowered the threshold for sensation of the urge to defecate and of discomfort to CRD [10], and increased colonic motility index [11]. A recent study also indicates that the iv injection of the non-selective CRF receptor antagonist, α-helical CRF12-41 [5], improved colonic motility and visceral pain induced by rectal electrical stimulation in irritable bowel syndrome (IBS) patients [12]. These findings suggest possible relevance of CRF1 receptor antagonists for the treatment of IBS [9,13].

Regarding the role that CRF2 receptors play in mediating or modulating stress-induced responses, recent reports put forward the concept that the activation of CRF2 signalling pathways may be important to dampen stress sensitivity [8]. CRF2 ligands reduce the CRF1-mediated activation of the hypothalamic-pituitary-adrenal axis, energy expenditure and some behavioural responses to stress [8,14]. Activation of central CRF1 and peripheral CRF2 receptors also result in opposite changes in blood pressure [15-17].

In the current study, we investigated whether the activation of CRF2 receptor, induced by peripheral administration of Ucn 2, modulates CRD-induced visceral pain in vivo and in vitro and its possible sites and mechanisms of action. Visceromotor responses (VMR) were monitored in conscious rats by measuring external oblique abdominal muscle contractions to repeated isobaric CRD. The CRF2 receptor-mediated action of hUcn 2 was investigated using the selective CRF2 antagonist, astressin2-B [7], and assessing the CRF2 receptor gene expression in dorsal root ganglia (DRG) and spinal cord (T13-S1). Recent studies showed that spinal activation of extracellular signal-regulated kinase (ERK) is involved in the sensitisation process induced by applying irritants into the mice colon or rat hind paw [18,19]. We therefore investigated whether such a spinal signalling pathway is recruited in CRD-induced visceral pain and is modulated by hUcn 2, using immunohistochemical assessment of ERK phosphorylation in the lumbosacral segments of the spinal cord known to be activated by CRD [20]. Lastly, using a novel in vitro rat distal colon-inferior splanchnic nerve/inferior mesenteric artery preparation and electrophysiological recording of inferior splanchnic afferent (ISA) nerve activity, we examined whether close intra-arterial (ia) injection of Ucn 2 modulates CRD-induced activation of ISA discharges.

MATERIALS AND METHODS

Animals and compounds
Adult male Sprague-Dawley rats (SD, Harlan, San Diego, CA) weighing 280-320 g were housed in group-cages with free access to rat chow and tap water. Animals were quarantined under controlled conditions of temperature and humidity for at least one week. Experiments started between 9 AM and 10 AM in non-fasted rats unless otherwise mentioned. Protocols were approved by the Animal Care Committee of the Veteran Affairs Greater Los Angeles Healthcare System and the UCLA Animal Research Committee.

Human Ucn 2 and astressin2-B (Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA) were synthesized as described previously [7,21] and stored in powder form at –80°C. hUcn 2 was weighed and dissolved in saline, and astressin2-B in distilled water just before use. Bradykinin (Sigma Chemical Co, St Louis, MI) was dissolved in saline.

Colorectal distension and monitoring of visceral motor response

CRD in awake rats produces contractions of abdominal and hindlimb musculature. This VMR has been validated as a quantitative measure of visceral nociception [22].

For electromyogram (EMG) recording of abdominal muscles, fasted rats under sodium pentobarbital anaesthesia (45 mg/kg, intraperitoneal, ip, Nembutal, Abbott Labs, North Chicago, IL) were chronically implanted with electrodes (Teflon coated stainless steel, AstraZeneca, Mölndal, Sweden) by stitching them onto the external oblique musculature immediately superior to the inguinal ligament as previously described [23]. The cannula, housing the electrode leads, was externalised on the left side of the abdominal wall. Following surgery, rats were housed in pairs and allowed to recuperate for at least 10 days during which they were trained to the experimental conditions by placing them singly in Bollmann cages for 3 h per day for 3 days before the study. On the day of experiment, rats were briefly anaesthetized with isoflurane, and a 6-cm long plastic balloon tied around an Intramedic PE-100 tubing (Becton Dickinson, Franklin Lakes, NJ) was inserted intra-anally with the distal end positioned 1 cm proximal to the anus. An iv catheter (Insyte Autoguard, Becton Dickinson) was also inserted into the tail vein. Then rats were placed in Bollmann cages, and after a 60-min stabilization period were submitted to isobaric CRD (60 mm Hg, 10 min twice with a 10 min rest interval) using a pressure control device (AstraZeneca). Tonic CRD applied for 3-10 min at noxious and non noxious ranges has been used previously to investigate mediators involved in colonic sensitisation [24,25].

For VMR measurements, the externalised cannula was connected to a custom made EMG-amplifier assembled to a Pentium class computer running the LabView (National Instruments, Dallas, TX) based proprietary software program for data acquisition (AstraZeneca) [26]. EMG signals were amplified, filtered (×10000, 300-5000 Hz), digitised and rectified as detailed previously [23,26]. Basal area under the curve (AUC) of contraction of the average peak EMG amplitude (V*seconds) was calculated as the area under the rectified EMG signal trace for the 10 min period immediately preceding each 10-min CRD. The AUC of the EMG during the 1st and 2nd distensions were computed and the basal AUC subtracted to get the net AUC in response to CRD.

In vitro ISA nerve-colorectal preparation and recording

Fasted rats were exsanguinated by decapitation and the in vitro colorectal-ISA nerve preparation was isolated. This includes the proximal rectal segment (about 2 cm) and distal colon (about 3 cm proximal portion of the isolated segment) with the attached inferior mesenteric artery, the lumbar colonic nerve, inferior mesenteric ganglion and ISA nerves along with the intermesenteric nerve. The pelvic and hypogastric nerves were not included in the preparation.
The isolated segment was transferred to the main chamber of a Sylgard-coated (Dow Corning, Midland, MI) organ bath that was perfused continuously with oxygenated Ringers solution at a 2.0–2.5 ml/min flow rate as in previous studies [27]. A 20 cm-long PE-10 catheter (Becton Dickinson) was inserted into the inferior mesenteric artery for ia injections and secured in place by ligation. Subsequently, ISA nerves merging into the intermesenteric trunk were located and the proximal cut end of a thin nerve strand was placed on one electrode of a bipolar platinum wire electrode (diameter: 30 µm) and a strand of connective tissue was attached to the other electrode. Before each experiment, the most sensitive mechanoreceptive region of colorectal preparation was searched by gentle stimulation of the receptive field (RF) on the serosal side with a small paint-brush. Then a balloon (~9 mm diameter when distended with 0.4 ml air) attached to a PE-10 catheter was inserted through the oral end of the colorectal segment. The balloon was placed in the centre of the most mechanoreceptive region and a test CRD (20 s) for the nerve fibre spike activity conducted. The rationale for selecting short duration distension is based on the fact that the isolated colorectal preparation is devoid of the abdominal cavity pressure to counteract the intra-luminal distension pressure and lacks the in vivo reflex mechanisms to adjust its compliance to tonic isobaric distensions.

The action potential of ISA fibres were pre-amplified (DAM-6, ×100, 100-10 kHz band-pass filter; World Precision Instruments, Sarasota, FL), and further amplified 300-750 times to give an action potential with a peak-peak amplitude of 1.5 V, which was displayed on a digital storage oscilloscope (model 2211; Tektronix Inc., Beaverton, OR). Based on the amplitude and waveform, a particular unit can be traced off-line to match the waveform of the unit by the use of the analysis module of the WAVEFORM software as previously described [28]. The response pattern of different units was analysed for the 20 s-period before and during CRD.

Test substances were injected into the catheter positioned into the inferior mesenteric artery. The volumes of the dead space in the catheter (41.0±1.5 µl; n=10) and dead space plus vascular bed of the colorectal preparation (80.0±1.7 µl, n=3) were measured by injecting 0.02% Toluidine Blue ia. Based on this information, 0.1 ml was selected for ia injections that were performed over 20 s. About 40 s later, the system was flushed with 0.1 ml saline over 20 s. Because the organ bath was continuously perfused with oxygenated perfusate, compounds tested were rapidly flushed out of the system. The response magnitude after ia injection of vehicle or peptides per se was analysed by comparing the actual number of spikes/100 s before and after ia injections.

RT-PCR analysis of CRF2 receptor gene expression
After decapitation of naïve rats (n=6), both sides of DRGs and spinal cord tissue at T13-S1 levels of the spinal cord were collected in 2 separate experiments and stored at −80°C. Total RNAs were isolated with QIAGEN mini RNasy kit (QIAGEN Inc., Valencia, CA). The first-strand oligo-dT primed cDNA was synthesized from total RNA (500 ng of each pair of DRG and spinal cord [from T13–S1] segments) by ThermoScript RNase H-minus reverse transcriptase at 55°C for 1 h (Invitrogen, Carlsbad, CA). Oligonucleotide primers were designed to amplify a core region of CRF2 (sense: 5’-TGCAACACGACCTTGGACCAG; antisense: ATCACACGGCAGCTGTCTGCT PCR; 1120 bp). RT-PCR was performed using a Red-Taq DNA Polymerase System (Sigma). The house keeping gene, acetic ribosomal protein (ARP; sense: GTTGAACATCTCCCCCTTTCTC; antisense: ATGTCTCCATCGGATTCTCC; 402 bp) was used as an internal control. The amplified PCR products were fractionated by electrophoresis in 1% agarose gel with ethidium bromide and detected under UV light. The gel
images were recorded using a Kodak imaging system (1D Image Analysis, Eastman Kodak, Rochester, NY). The identity of CRF2 fragment (1120 bp) was confirmed by DNA sequencing (Cycle sequencing, Applied Biosystems, Foster City, CA).

**pERK 1/2 immunohistochemistry**

Rats were deeply anaesthetized with sodium pentobarbital (Nembutal®, Abbott, 70 mg/kg, ip), and perfused through a cardiac-aorta cannula with 500 ml/rat of 4% paraformaldehyde and 14% saturated picric acid in 0.1 M phosphate buffer solution (pH 7.2). The lumbosacral spinal cord (L6-S1) was dissected out and post-fixed in the same fixative overnight and cryoprotected by immersion in 10% sucrose for 1 day. The L6-S1 segment was transversally sectioned at 30 µm with a cryostat. Free-floating sections were processed with ABC techniques with slight modifications. Sections were incubated with rabbit anti-pERK (Phospho-pERK 44/42 Map Kinase antibody from Cell Signaling Technology, Inc., Beverly, MA) at 1:2,000 dilution for 2 nights at 4°C, and in biotinylated secondary goat anti-rabbit IgG at 1:1,000 for 1.5 h and lastly with avidin-biotin peroxidase complex at 1:400 for 1 h at room temperature. The chromogen was 3,3-diaminobenzedine tetrahydrochloride deposited as dark brown labelling in the presence of H₂O₂. The number of pERK 1/2 immunoreactive cells in laminae I and IIo was counted unilaterally in 15 sections randomly selected and expressed as cells/unilateral section. All samples were handled in the same experimental conditions.

**Colonic tissue damage assessment after in vivo CRD**

Distal colonic tissues were visually observed for macroscopic damage and scored based on colonic wall thickness and presence of lesions such as ulcerations as described previously [29]. For histological analysis, tissues were fixed by overnight immersion in neutral buffered formalin (4%), embedded in paraffin wax, sectioned (6 µm), stained with haematoxylin and eosin and examined by light microscopy. Crypt architecture, vascular and colonic wall damage, and the presence of inflammatory cells were assessed. In rare cases (<5%), when there were signs of colonic damage caused by balloon placement as evidenced by the presence of blood on the balloon, data were disregarded.

**Experimental protocols**

*Effect of iv hUcn 2 on VMR to two CRDs in conscious rats.* In non-fasted rats, chronically implanted with abdominal electrodes, 60 min after balloon and iv catheter placement, a 10-min basal EMG recording was made, then the CRD protocol (60 mm Hg for 10 min twice, with a 10-min rest interval) was initiated. In the first set of experiments, hUcn 2 (10 or 20 µg/kg) or saline was injected iv at the end of the first distension. In a second set, astressin2-B (200 µg/kg) or its vehicle (distilled water) was injected subcutaneously (sc, 0.5 ml) 20 min before the onset of the first distension, and hUcn 2 (20 µg/kg) or saline (0.1 ml) was injected iv at the end of the first distension. In another set of experiments, saline or hUcn 2 was given iv (20 µg/kg), 20 min before the beginning of the distension protocol. The VMR to the second distension was compared to that of the first distension response. Percent changes between the two CRDs and the different treatment groups were compared. Doses of peptides and routes of administration were based on our previous studies, where under similar conditions hUcn 2 exerted biological actions on the upper gut motor function that were blockade by astressin2-B [30].

In separate studies, three groups of rats, either naïve (no treatment), sham (balloon inserted into the colorectum without CRD) and CRD (60 mm Hg twice with a 10-min rest
interval) were euthanized at the time corresponding to the end of the two CRDs. Colons were processed for macroscopic and histological examinations.

**Effect of peripheral Ucn 2 on locomotor activity.** Rats were injected ip with saline or hUcn 2 (20 µg/kg) and 10 min later, placed in an open field white box (50 cm × 50 cm × 22 cm) with the floor divided into nine squares. The number of crossed squares (locomotor activity score) was recorded for every 10 min during 30 min. Between each trial, the floor was cleaned with alcohol to avoid scent.

**Effect of peripheral Ucn 2 on distal colonic transit in conscious rats.** Rats fasted for 24 h, had free access to a pre-weighed chow for a 2-h period, then a plastic bead (5 mm diameter) was inserted into the distal colon (4 cm proximal to the anus) as in previous studies [30], and saline or hUcn 2 (20 µg/kg) was injected iv through the tail vein. The time required to expel the bead (min) was monitored over 4 h.

**Effect of CRD and iv hUcn 2 on lumbosacral neuronal ERK 1/2 activation and VMR.** Sixty min after balloon and iv catheter placements, rats were injected iv with either saline (0.1 ml) or hUcn 2 (20 µg/kg), and 10 min later, a single 10-min CRD (60 mm Hg) or no distension (sham) was applied. Abdominal contractions were identified visually as a snaky contraction of the abdomen with inward turning of the hind limb, hump-backed position, or squashing of the lower abdomen against the floor as previously described [31]. Counting of contractions was done for 10 min after iv treatment and during the 10-min of CRD or no CRD. Such visual VMR assessment avoids the possible confounding effect of the chronic surgery and electrodes on spinal neuronal activation monitored in the study. At the end of CRD, all groups were euthanized and the lumbosacral spinal cord (L6-S1) processed for pERK 1/2 immunohistochemistry.

**In vitro colorectal preparation**

Each experiment included a 30-min stabilization period, followed by 5 min of initial basal ISA recording. At the end of each experiment, bradykinin (1 µg/0.1 ml = 4.9 X 10⁻⁶ M) was injected ia to assess the afferent nerve fibre responsiveness. This was followed, 10 min later, by the search of RF and injection of Toluidine Blue (0.02%, ia) to verify arterial patency. All ia injections were performed in 0.1 ml over 20 s.

In the first study, the ISA response to graded intensities of CRD was achieved by inflating the balloon with increasing volumes of air (0.1, 0.2, 0.3, 0.4 and 0.5 ml). Each CRD was maintained for 20 s and occurred within a ~5-10 min interval. Based on the results, 0.4 ml CRD for 20 s was used as a standard distension protocol. In the second study, ia vehicle was injected 2 min before CRD and this paradigm was repeated 4 times at ~6 min intervals. In the third study, sequential treatments were performed: vehicle ia, CRD, hUcn 2 (1 µg/0.1 ml = 1.87 X 10⁻⁶ M, ia), CRD, hUcn 2 (2 µg/0.1 ml = 3.75 X 10⁻⁶ M ia), CRD, Ucn 3 (3 µg/0.1 ml = 5.62 X 10⁻⁶ M, ia), CRD, Ucn 2 (3 µg ia) and CRD. There was a 2-min interval between ia injection and CRD, and a 10 min between the CRD and the next ia injection.

**Data analysis**

All values, unless otherwise indicated, are Mean ± SEM. Repeated measures paired t-test was used to compare the 1st vs. 2nd AUC, and the 1st vs. 2nd number of abdominal contractions as well as for in vitro ISA spike activity before and after injection or before and during CRD. AUC percent differences between the two CRD responses among treatment groups, the distal colonic bead expulsion time and the in vitro ISA activity comparison between groups was analysed using.
one way ANOVA followed by Newman-Keuls multiple-comparison test. P<0.05 was considered statistically significant.

RESULTS

Ucn 2 injected iv decreases repeated CRD-induced VMR in conscious rats chronically implanted with intramuscular abdominal electrodes. In rats injected iv with saline just after the 1st CRD, there was a 30.6±4.0% increase (P<0.05) in AUC during the 2nd CRD when compared to its 1st response (Figs. 1A,B, 2A), indicating sensitisation. hUcn 2 (10 and 20 µg/kg), injected iv after the 1st CRD, prevented the increase of the AUC to the 2nd CRD (Fig. 1B, 2A). In addition, at 20 µg/kg, hUcn 2 decreased the AUC response from 33.1±4.5 V*seconds (1st response) to 23.0±3.0 V*seconds (2nd response, P<0.05, vs 1st response) resulting in a 30.5±4.8% reduction of the visceral pain response proper (Fig. 2A). Prior administration of the CRF2 antagonist, astressin2-B (200 µg/kg, sc) blocked the inhibitory effect of hUcn 2 (20 µg/kg) on the 2nd response, compared to the 1st response (Fig. 2B). Astressin2-B (200 µg/kg, sc) alone did not influence the sensitisation response (Fig. 2B).

When hUcn 2 (20 µg/kg, iv) was given 20 min before the 1st CRD distension, the responses to the two CRD distensions were similar (20.6±3.3 vs 23.5±4.6 V*seconds, P>0.05, n=6), whereas after iv vehicle the 2nd response was significantly higher than the 1st (29.8±4.2 vs 38.3±6.4 V*seconds, P<0.05, n=6).

Tonic repeated CRD did not cause colonic tissue damage in rats. The macroscopic score of colons from naïve, sham and CRD (60 mm Hg for 10 min twice, with a 10-min rest interval) groups were not different (1.8±0.3, 2.0±0.2 and 2.2±0.4, respectively P>0.05). Similarly, histological analysis did not reveal differences in crypt and colonic wall structure or presence of inflammatory cells (Fig. 3). However, in 2 out of the 4 CRD rats, the submucosal space was enlarged compared with the other rats.

hUcn 2 injected ip did not alter locomotor activity in naïve rats. Rats injected with ip saline (n=7), crossed 65.3±7.9, 11.7±2.5 and 9.1±2.2 squares during the first, second and third 10-min period of observations, respectively. hUcn 2 (20 µg/kg, ip, n=7) did not affect (P>0.05) the locomotor activity score at any of the time points (squares crossed/10 min: 47.7±7.3; 16.4±4.9 and 14.7±3.1, respectively). Similarly, the number of squares crossed during the total 30-min period was not different between saline and hUcn 2 (20 µg/kg) injected rats (saline = 86.1±10.4 vs hUcn 2 = 78.8±10.1, P>0.05).

hUcn 2 injected iv did not alter distal colonic transit in naïve rats. In fasted rats (n=6), the distal colonic transit, assessed by the bead expulsion time, was 155.6±27.4 min. Neither iv saline nor hUcn 2 (20 µg/kg) altered distal colonic transit time (148.0±32.2 min, n=10 and 169.5±33.4 min; n=8 respectively).

CRF2 receptor gene expression. CRF2 receptor expression was detected by RT-PCR in the heart and T13-S1 DRGs, and spinal cord tissues in naïve rats (Fig. 4). Primers were designed to target the core regions of rat CRF2 gene in the area corresponding to the transmembrane domains and cytoplasmic tail of the receptor protein common to functional splice variants, CRF2a and CRF2b.
CRD-induced spinal ERK 1/2 phosphorylation and abdominal contractions were attenuated by iv hUcn 2 in conscious rats. A single CRD (60 mm Hg), induced a robust activation of ERK1/2 bilaterally in the lumbosacral spinal cord as monitored at the end of the 10-min CRD in the iv saline treated group. The pERK1/2 immunoreactivity was observed in the superficial dorsal horn (laminae I and IIo), along the lateral collateral visceral afferent pathway and the intermediolateral column (IML; Fig. 5A, B). A few pERK positive cells were observed in the non-distended sham group (Fig. 5A,C). CRD, under these conditions, induced abdominal contractions (number/10 min: 42.3±11.5 vs sham: 2.3±0.8, P<0.05). hUcn 2 (20 µg/kg, iv 10 min before the CRD) blunted the phosphorylation of ERK1/2 in the above spinal regions (Fig. 5A,C). The number of pERK1/2 positive cells in the laminae I and IIo induced by CRD was significantly reduced from 10.4±2.9 in saline to 5.4±1.0/unilateral section after iv hUcn 2 (Fig. 5C). hUcn 2 (20 µg/kg, iv) also decreased the number of contractions/10 min to 21.0±3.2 vs saline: 42.3±11.5 (P<0.05).

In rats injected with iv saline, the 1st and 2nd CRD induced 30.5±6.6 and 43.2±5.8 contractions/10 min respectively (P<0.05; a 42% increase in response to the 2nd distension). In rats injected iv with hUcn 2 (20 µg/kg, iv) before any distension, both the 1st and the 2nd responses were significantly lowered compared with iv saline (21.2±3.5 vs 30.5±5.6 contractions/10 min for the 1st VMR and 22.3±3.6 vs 43.2±5.8 contractions/10 min for the 2nd VMR, P<0.05).

Ucn 2 injected ia reduces CRD-induced ISA fibre activity in vitro. Incremental balloon distensions at ~ 6 min intervals induced a volume dependent increases in ISA activity (Fig. 6A, B). In 23 units from 11 in vitro rat preparations, the basal impulse/20 s was significantly increased from pre-distension values of 17±4 to 47±10 at 0.3 ml, 16±3 to 67±12 at 0.4 ml and 21±4 to 94±16 at 0.5 ml distension volumes, while CRD at 0.1 ml had no effect and the increase observed at 0.2 ml did not reach significance (basal 15±4 to 29±8) (Fig. 6B). Fifteen min after the last CRD, the 23 units responsive to CRD, were also responsive to the ia injection of bradykinin (1 µg/0.1 ml) suggesting the units to be multi-modal that respond to physical and chemical stimuli. Bradykinin resulted in a significant increase in basal ISA activity from 103±25 to 465±68 spikes/100 s while the ia injection of vehicle had no effect (pre injection 69±13; vehicle: 77±14 spikes/100 s; 23 units analysed from the same 11 preparations). Based on these data, CRD at 0.4 ml for 20 s was selected for further studies. Repeated vehicle ia injections and CRD (4 times at ~ 6-min intervals) induced a similar increase in ISA impulses/20 s as shown in 6 single units analysed from 3 experiments (Fig. 6C).

The ia injections of vehicle, hUcn 2 at 1, 2 and 3 µg, and astressin2-B (20 µg) per se did not alter basal ISA activity (138±23/147±29, 166±33/206±50; 147±30/180±31; 142±28/195±27 and 125±28/142±32 per 100 s respectively; n=8 single units analysed from 4 experiments). In the same preparations, consecutive ia injections of Ucn 2 (1, 2 or 3 µg) 2 min before CRD dose-dependently lowered the CRD-induced ISA response to 62±14, 54±14, and 44±10 spikes/20 s, respectively compared to ia vehicle + CRD (78±15 spikes/20 s), with significant inhibition at 3 µg hUcn 2 (Fig. 7B). Astressin2-B, which did not influence CRD response by itself, prevented the Ucn 2 (3 µg) inhibitory effect (Fig. 7A,B). Fifteen minutes after the last CRD, ia injection of bradykinin (1 µg) resulted in a significant increase in basal impulse activity of ISA to 544±91 spikes/100 s P<0.05 vs pre-injection 119±35 (same 8 units from 4 preparations) (Fig. 7B).
DISCUSSION
The present study shows that two repeated tonic noxious CRDs in awake rats produce visceral sensitisation and phosphorylation of ERK 1/2 in laminae I and IIo of the lumbosacral spinal cord known to receive visceral afferent input from the pelvic organs [20]. Activation of peripheral CRF₂ receptors by iv hUcn 2 [4] dampened the tonic CRD-induced visceral pain and spinal ERK1/2 phosphorylation, and blocked the sensitisation without affecting locomotor activity or distal colon transit. Injection of hUcn 2 also blunts CRD-induced ISA activation in an in vitro isolated colorectal preparation. The in vivo and in vitro actions of hUcn 2 were prevented by the CRF₂ receptor antagonist, astressin2-B, that by itself did not influence visceromotor and ISA responses to CRD. CRF₂ receptor gene expression was detected by RT-PCR in the DRG and spinal cord within the T13-S1 segments from naïve rats. These findings provide evidence that iv Ucn 2-induced CRF₂ receptor mediated blunting of visceral pain and inhibition of sensitisation to repeated CRD may involve modulation of colonic afferent fibre activity and ERK1/2 phosphorylation in spinal neurons receiving visceral input.

1. Development of visceral sensitisation by two repeated tonic CRDs
IBS patients subjected to successive incremental distensions of the rectum displayed a lowered rectal pain threshold compared with normal subjects [32]. Other studies showed that rectal hyperalgesia to repeated CRD is specific to IBS [33]. Several experimental models inducing visceral hyperalgesia used intracolonic application of chemical irritants [24], exposure to acute or chronic stress [34-36], or early life visceral pain in rats [37]. Our data provide evidence that two 10 min on/off noxious CRDs (60 mm Hg) induced visceral pain/sensitisation in awake rats either naïve or fitted with chronic abdominal electrodes. Similarly, other CRD models involving 10 repetitive phasic CRDs (30 or 50 mm Hg, 60 s on/30 s off) [38] or two 10-min CRD (60 mm Hg) separated by 10 phasic CRDs (80 mm Hg, 15 s on/30 s off) [39] resulted in visceral sensitisation in awake rats. It has been reported that repeated CRD (80 mm Hg, 60 times, 30 s on/90 s off for 2 h) induced signs of inflammation [20]. However, under our conditions, except for an enlarged submucosal space, the colonic wall, examined macroscopically and histologically, revealed no sign of inflammation and damage at the end of the two 10 min CRDs applied with a 10-min rest interval. Since enhanced colonic mechanosensation in the absence of colonic injury is the hallmark of IBS [32,33], the present CRD paradigm, which does not produce tissue damage, is a relevant experimental model to study modulation of visceral sensitisation.

2. hUcn 2 inhibition of CRD-induced visceral pain/sensitisation and splanchnic afferent activity
Activation of CRF₂ receptors blunted visceral pain and prevented sensitisation induced by noxious CRD. This is supported first by the demonstration that the selective CRF₂ agonist, hUcn 2 [4] injected iv reduced visceral pain and inhibited the sensitisation to CRD in awake rats. Second, the effects of iv hUcn 2 were completely blocked by the selective CRF₂ antagonist, astressin2-B [7]. By contrast, we previously reported that the activation of CRF₁ receptor using the preferential CRF₁ ligand, ovine CRF [5], lowered the sensation of discomfort to the colon in normal human subjects [10]. It is unlikely that the inhibition of VMR to iv hUcn 2 may have been confounded by behaviour changes since we showed that peripheral injection of hUcn 2 did not affect the locomotor activity response to an open field environment. The analgesic effect of hUcn 2 to CRD is also not secondary to alterations in colonic motility because hUcn 2, injected
at a dose modulating the visceral pain response to CRD, did not affect basal propulsive motor function of the distal colon as reported previously [30]. The absence of basal modulation of distal colonic transit by peripheral activation of CRF₂ receptors contrasts with the potent stimulation of colonic motility by the activation of peripheral CRF₁ receptors in rats and mice [9]. Taken together these observations are consistent with CRF₂, unlike CRF₁, receptors mediating iv Ucn 2 inhibitory effects on pain/sensitisation and provide the first evidence of visceral pain modulation by CRF₂ receptor activation.

Sensory information from the distal colon/rectum is conveyed through the inferior (lumbar) splanchnic nerves which project to the thoracolumbar spinal cord and through pelvic nerves that enter the lumbosacral cord [40]. In vivo and in vitro electrophysiological studies in rodents have shown that CRD activates mechanosensitive afferents located in both splanchnic and pelvic fibres [41-43]. Although CRF₂ receptors have been detected within the rat and human colons [44,45], there is evidence that peripherally administered Ucn 2 can also reach the brain parenchyma at a moderate rate [46]. Therefore, we used novel in vitro approach to assess whether Ucn 2 acts peripherally to modulate CRD-induced activation of ISA activity. In an isolated rat colorectal ISA-inferior mesenteric artery preparation, phasic CRD increased ISA impulses, and this response was dose-dependently reduced by ia injection of hUcn 2. In addition, hUcn 2 inhibitory action was blocked by ia astressin2-B, which by itself did not influence significantly the ISA response to CRD. The intensity-encoding properties of ISA was demonstrated by the linear increase in their activity with 0.1 ml ascending volume of balloon inflation from 0.1 to 0.5 ml and the reproducible ISA firing activity in response to repeated CRD of similar intensity (0.4 ml). In addition, the single unit responsive to phasic CRD had a robust response to ia injection of bradykinin, which consistently excites visceral nociceptors [41]. Similar patterns were reported in rat pelvic afferent fibres in response to graded or similar phasic CRD and bradykinin injection in vivo [41]. These data indicate that iv hUcn 2-induced dampening of visceral pain response to the noxious CRD may occur through peripheral activation of CRF₂ receptors that blunts ISA activation to CRD. Although the lack of sensitisation of the in vitro ISA fibre activity in response to repeated distensions argues for central sensitisation as a major mechanism for the observed visceral hyperalgesia in awake rats, the in vitro ISA fibre activity study was done using short phasic (20 s) distension and intensity (0.4 ml). These may not induce the necessary temporal and intensity components to cause increased excitability of ISA fibres.

3. hUcn 2 modulates ERK 1/2 activation in lumbosacral dorsal horn by CRD

A number of recent studies have demonstrated that the activation of Aδ- or C-fibres by noxious thermal, mechanical, chemical or electrical stimuli of somatic origin induce a rapid activation (phosphorylation) of two mitogen activated protein kinases (MAP kinases), p42 and p44 referred to as ERK 1 and 2 (ERK 1/2) in lamina I and IIo neurons of the spinal cord and that ERK 1/2 activation is involved in the pain response [18]. Colorectal afferents projecting to the spinal cord are mostly composed of C (unmyelinated) or Aδ (small myelinated) fibres [40]. While much attention has been focused on ERK signal transduction mechanisms responsible for the modulation of somatic pain/hyperalgesia [18,47,48], little is known as to whether such pathways are part of the visceral pain/sensitisation process. One study in awake mice indicates that intracolonic instillation of capsaicin or mustard oil resulted in a rapid increase in pERK 1/2 selectively in lumbosacral spinal cord as measured by Western blot analysis [19]. In the present study, we showed that noxious CRD applied for 10 min in naïve rats induced phosphorylation of
ERK1/2 in lumbosacral spinal cord neurons. The pERK labelling was observed within 10 min and topographically located in discrete area of the dorsal horn (lamina I, IIo and the lateral collateral visceral afferent nerve bundles). These data indicate that visceral pain induced by noxious CRD can rapidly induce an activation of the MAPK (ERK) pathways in individual neurons of the spinal cord at laminar location where primary afferent C-fibres terminate [20,40]. Therefore, examination of the presence of phosphorylated ERK 1/2 in the superficial dorsal horn may be a useful marker of postsynaptic neurons actively responsive to visceral pain.

Several studies also established that rapid ERK phosphorylation plays an important role in hypersensitivity after noxious somatic stimuli and inflammation by modulating neuronal excitability [49]. In a parallel study to the ERK 1/2 activation, rats subjected to a 2nd CRD displayed a 42% enhanced abdominal contraction suggesting a similar sensitisation occurring in naïve rats of the pERK 1/2 study to those fitted with chronic abdominal electrodes. Peripheral administration of hUcn 2 blunted the CRD-induced ERK 1/2 activation, as well as the VMR indicating a correlation between the VMR response and pERK 1/2 immunostaining occurring in spinal cord laminae I and IIo. Although to be further confirmed, these data suggest that the inhibitory action of hUcn 2 against repeated CRD-induced visceral sensitisation may involve the reduction of spinal ERK 1/2 activation. Given, the rapid time frame of hypersensitivity induced by CRD (<1 h), ERK activation may mainly induce post-translational changes in ion channels or receptors modulating neuronal excitability [49]. hUcn 2 may modulate pERK 1/2 induction through inhibition of CRD-induced primary afferent activation that conveys nociceptive messages to the spinal cord. Gene expression of CRF2 receptors in the lumbosacral DRG of rats demonstrated by RT-PCR, supports the involvement of CRF2 in the hUcn 2 action and the possible post translational change induced by ERK 1/2 activation. However, of note is that the afferents innervating the colon constitute the minority of DRG cells that were harvested. In addition, systemic injection of Ucn 2 has been reported to have some accesses to the brain parenchyma [46], and we showed the expression of CRF2 receptors in the spinal cord by RT-PCR. Whether iv hUcn 2 has access to the spinal cord to act directly on spinal neurons leading to reduce ERK 1/2 phosphorylation needs to be further investigated.

The present study adds another paradigm, visceral pain/sensitisation, in which CRF2 receptor activation exerts an opposite effect than that of the CRF1 receptor [50,51]. These data lend support to the concept that susceptibility to stress or noxious stimuli may be viewed not only in the context of an exaggerated activation of the CRF1 pathways, but may also result from an inadequate mounting of CRF2 signalling mechanisms that curtail the CRF1-mediated stress response. The present demonstration of the analgesic effect of CRF2 receptor activation in a model of visceral pain/sensitization would also be relevant to the increased interest on the potential use of CRF receptor modulation in the treatment of IBS [9,13,52].
ACKNOWLEDGEMENTS
This work was supported by NIH grants, R01 DK-33061 (YT), RO1 DK-57238 (YT), DK-57238-01 A1S1 (MM), P-50 DK64539 (EAM, YT, MM,) and DK26741 (WV, JR). We thank AstraZeneca, Timothy Maryanov, Marciano Sablad, Jing Fang Zhao and Honghui Liang for the material and technical support provided. We would like also to thank Teresa Olivas for editing the manuscript.

LISCENCE FOR PUBLICATION
The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its licensees, to permit this article (if accepted) to be published in Gut and any other BMJPG products and to exploit all subsidiary rights, as set out in our licence (http://gut.bmjjournals.com/misc/ifora/licenceform.shtml).

COMPETING INTEREST: None declared
REFERENCES
FIGURE LEGENDS

Figure 1. A representative illustration of abdominal contraction traces (A) and individual AUC response to the 1st and 2nd CRD in saline and hUcn 2 treated rats (B). Rats were chronically implanted with abdominal electrodes and ~10 days later were briefly anaesthetized with isoflurane and fitted with an intracolic balloon. After 60 min recovery and habituation to Bollmann cages, they were submitted to two 10-min CRDs at 60 mm Hg with a 10-min rest interval. Saline or hUcn 2 was injected iv (0.25 ml) just at the end of the first CRD. Note that the abdominal contraction response to the same distension during the second set is higher than the first set. Values in 1A are EMG voltage, whereas in 1B values are AUC units of the response to the 1st and 2nd CRD.

Figure 2. Effect of hUcn 2 (A) and astressin2-B on tonic repeated CRD-induced visceral pain/hypersensitivity (B) in conscious rats. Rats were chronically implanted with abdominal electrodes and ~10 days later were injected sc with vehicle (water) or astressin2-B. After 10 min, rats were submitted to a 1st CRD (10-min at 60 mm Hg) followed by iv injection of saline or hUcn 2. Rats were allowed to rest for 10 min and received the 2nd distension (60 mm Hg, 10 min). Values are Mean±SEM of total percent differences between the 1st and 2nd responses to CRDs. #P<0.05 vs all other groups; * P<0.05 vs saline or hUcn 2 (20 µg/kg) (n=5-6 rats/group, ANOVA).

Figure 3. Photomicrographs of distal colon tissues from naïve (A = no balloon placement and no distension), sham (B = balloon placed into the colorectum but no CRD) and distension (C = rats fitted with balloon and submitted to the two 10-min, 60 mm Hg CRD). Colonic tissues were processed for haematoxylin and eosin staining at the end of the 2nd CRD. Magnification, ×100. Note that there are no architectural or inflammatory response differences between the different groups except the enlarged submucosal space in the CRD group.

Figure 4. CRF2 gene expression in rat DRGs and spinal cord at T13-S1 segments detected by RT-PCR. Images were from one rat representative of 3-6 rats studied. Each band of the DRG RT-PCR products was pooled from the ganglia of both left and right sides. Lanes 1-7: DRG T13-L4, L6, S1. Lanes 8-9: spinal cord T13- L1, L3-4, L6-S1. ARP: acetic ribosomal protein (house keeping gene), ht: heart (CRF2 gene expression control), cont: negative control (without RT).

Figure 5. Modulation of ERK 1/2 activation in the spinal cord L6-S1 by hUcn 2 in rats. A = Photomicrograph of pERK 1/2 immunoreactivity. Under brief isoflurane anaesthesia, rats were fitted with a rectocolic distension balloon and tail vein catheter and kept in Bollmann cages for 60 min. Conscious rats were then injected iv with saline or hUcn 2 (20 µg/kg) and 10-min later received either a single 10-min CRD at 60 mm Hg or no distension (sham). At the end of the distension period, rats were euthanized for pERK 1/2 immunohistochemistry. I-II: laminae I-II, IML: intermediolateral column; CC: central canal. Scale bar 100 µm. B = Higher magnifications from saline+CRD group showing the presence of pERK 1/2 in the larger neurons of laminae I and smaller neurons of outer laminae II (i), as well as in neurons and fibres (arrows) along the lateral collateral visceral afferent fibres (ii). Scale bar 100 µm. C = Values are Mean±SEM of number of pERK 1/2 positive cells in the laminae I and IIo of the lumbosacral spinal cord of rats. *P<0.05 vs all other groups (n=4 rats/group, ANOVA). Note that in this experiment hUcn 2 or saline was given prior to the first distension.
**Figure 6.** Effects of repeated CRDs on ISA spike activity in an *in vitro* colorectal preparation from fasted rats. A = Representative spike counts vs time histogram showing the response of one unit to graded volume CRD (0.1-0.5 ml, 20 s each). The bin width is 14 s. The top two insets in A show, in a faster time scale, the stimulus-response function for 0.1 and 0.4 ml CRDs. B = Twenty-three ISA fibre units analysed from 11 rat *in vitro* preparations (1-4 units per preparation). CRD (0.3-0.5 ml) induces a volume-dependent significant increase in ISA. C = Similar ISA response to repeated CRDs (0.4 ml, 20 s each). Vehicle was injected ia 2 min before each CRD. Values for B and C are Mean±SEM of 20-s spike counts pre and during CRD in 6 ISA fibre units analyzed from 3 rats *in vitro* preparation (1-3 units/preparation). *, ***, P<0.05 and 0.001 vs pre-distension.

**Figure 7.** Effects of close ia (inferior mesenteric artery) injection of hUcn 2 on CRD-induced ISA spike activity in an isolated colorectal preparation from fasted rats. A = Representative single unit response to CRD (0.4 ml for 20 s) after vehicle (saline, 0.1 ml, ia), hUcn 2 (3 µg, 0.1 ml) or astressin2-B (20 µg, 0.1 ml plus hUcn 2, 3 µg, 0.1 ml). The lower panels show raw data, at faster time scale, of the single unit response in the astressin2-B + hUcn2 trace (left panel, time starts at 111 s whereas the right panel time starts on the first second of CRD). Raw data were output using Spike 2 (CED Ltd, Cambridge). B = hUcn 2 dose-dependently inhibits the distension-induced rise in ISA spike frequency. The inhibitory effect of hUcn 2 is prevented by astressin2-B (Ast2-B). Bradykinin (BK, black bar, 1 µg, 0.1 ml) injected at the end of the experiment caused robust ISA fibre activity. Values are Mean±SEM of 20-s pre and during CRD in the hUcn 2 study or 100-s pre and after bradykinin injection in 8 single units from 4 rat *in vitro* preparation, 1-3 units/preparation). *P<0.05 vs pre-CRD; # P<0.05 vs vehicle + CRD; ***, P<0.05 vs pre-injection of BK. F = Flush; ia = intra-arterial.
A

Saline (iv)

60 mmHg

10 min.

60 mmHg

10 min.

EMG Amplitude (μV)

0 600 1200 1800 2400 3000 sec

B

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>hUcn 2 10 μg/kg</th>
<th>hUcn 2 20 μg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area under the curve of contraction (AUC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>