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Activation of Corticotropin-Releasing Factor Receptor 2 Mediates the Colonic Motor Coping Response to Acute Stress in Rodents

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Abstract

Background & Aims—Corticotropin-releasing factor receptor-1 (CRF1) mediates the stress-induced colonic motor activity. Less is known about the role of CRF2 in the colonic response to stress.

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Methods—We studied colonic contractile activity (CCA) in rats and *CRF2*-/-, CRF-overexpressing, and wild-type mice using still manometry; we analyzed defecation induced by acute, partial-restraint stress (PRS), and/or intraperitoneal (IP) injection of CRF ligands. In rats, we monitored activation of the colonic longitudinal muscle myenteric plexus (LMMP) neurons and localization of CRF1 and CRF2 using immunohistochemical and immunoblot analyses. We measured phosphorylation of ERK1/2 by CRF ligands in primary cultures of LMMP-neurons (PC-LMMPn) and cAMP production in HEK-293 cells transfected with CRF1 and/or CRF2.

Results—In rats, a selective agonist of CRF2 (urocortin 2) reduced CRF-induced defecation (>50%), CCA, and Fos expression in the colonic LMMP. A selective antagonist of CRF2 (astressin2-B) increased these responses. Urocortin 2 reduced PRS-induced CCA in wild-type and CRF-overexpressing mice, whereas disruption of *CRF2* increased PRS-induced CCA and CRF-induced defecation. CRF2 co-localized with CRF1 and neuronal nitric oxide synthase in the rat colon, LMMP, and PC-LMMPn. CRF-induced phosphorylation of ERK in PC-LMMPn; this was inhibited or increased by a selective antagonist of CRF1 (NBI35965) or astressin2-B, respectively. The EC_{50} for the CRF-induced cAMP response was 8.6 nM in HEK-293 cells that express only CRF1; this response was suppressed 10-fold in cells that express CRF1 and CRF2.

Conclusions—In colon tissues of rodents, CRF2 activation inhibits CRF1 signaling in myenteric neurons and the stress-induced colonic motor responses. Disruption of CRF2 function impairs colonic coping responses to stress.

Keywords

colonic contraction; myenteric neurons; nNOS; stress response

Introduction

Clinical and experimental studies show that chronic or uncontrolled stress triggers or exacerbates a number of pathologies including gastrointestinal diseases (1-4). Corticotropin-releasing factor (CRF) is the primary hypothalamic mediator of the mammalian neuroendocrine and behavioral responses to stress (5). The CRF signaling system, in addition to CRF, encompasses three CRF related peptides, urocortins (Ucns), Ucn 1, Ucn 2 and Ucn 3 and two receptor subtypes, CRF_1 and CRF_2 (6; 7). CRF and Ucn 1 bind to both CRF_1 and CRF_2 receptors, although with different affinities (5-7). On the other hand, Ucn 2 and Ucn 3 bind selectively to CRF_2 receptors (7). CRF_1 is found abundantly in the central nervous system with limited expression in peripheral tissues, whereas CRF_2 is widespread in the periphery and confined in discrete brain nuclei (8-12). Multiple alternatively spliced transcripts of CRF_1 have been identified (CRF_{1a}-CRF_{1i}), with only a few of them being functional. CRF_2 is expressed in three major functional isoforms in humans (CRF_{2a}, CRF_{2b}, CRF_{2c}), two in rodents (CRF_{2a}, CRF_{2b}) and 5 additional non-coding CRF_{2a} variants (7; 11; 13-15). Rodent CRF_{2a} is primarily expressed in the brain, whereas CRF_{2b} is mainly found in the periphery, including the gastrointestinal tract (11; 14; 16; 17). CRF_{2a} and CRF_{2b} isoforms display similar pharmacological profile (18). The dominant mode of signaling for both CRF_1 and CRF_2 is the Gs-coupled adenylate cyclase-phosphokinase A cascade, although PLC-PKC and ERK-MAPK cascades are reported in different cell types (13; 19).

In rodents stress or CRF injected into the brain or periphery induces colonic secretomotor and pain sensation alterations that are blocked by CRF_1 antagonists (22-26). These findings have led to the consensus that CRF_1 receptor is the primary receptor involved in the stress-induced alteration of lower gut secretomotor and pain sensation. However except the preliminary data on the inhibitory actions of Ucn 2 in mice defecation (27) and rat visceral pain (48) responses, the mechanisms of action and role of peripheral CRF_2 in the colonic response to CRF or stress are largely unknown.

In the present study, we investigated whether peripheral CRF_2 activation or blockade modulates the colonic motor activity to peripheral injection of CRF or stress in rats and mice and assessed the underlying mechanisms involved in the CRF_2-mediated inhibitory actions. We show that CRF_2 activation plays a critical role in harnessing the CRF_1-mediated stimulation of colonic motor function induced by acute partial restraint stress (PRS) or peripheral injection of CRF by modulating CRF_1 signaling and/or recruiting inhibitory pathways such as nitric oxide (NO). Such modulation is essential to establish homeostasis and it is likely that alteration of CRF_2-signaling impairs the normal stress-coping mechanisms and may contribute to the development of stress-related gut diseases.

Methods

Animals

Adult male Sprague-Dawley rats (SD, 280-300g, Harlan, Indianapolis, ID), male and female CRF_2-/- (32.7±3.8g) and their wild-type littermates (WTL, C57BL/6J, 31.7 ± 0.3g), CRF-overexpressing (CRF-OE, 30.9±2.1g) and their WTL (C57BL/6, 28.8±0.6g), from the Oregon Health and Science University, Portland, OR (28), and the University of California Los Angeles, CA were used. Animals were maintained under temperature (20–24°C) and light-(12-h light-12^{-}h dark) controlled environment and fed *ad-libitum* with standard rodent chow (Prolab RMH 2500-5P14; Purina LabDiet, St. Louis, MO) and tap water. Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Protocols (9906-820; 08047-05 and 06014-08) were approved by the VA Institutional Animal Care and Use Committee.

Substances

Human/rat/mice urocortin 1 (Ucn 1), human Ucn 2 (hUcn 2), mouse Ucn 2 (mUcn 2), h/rCRF and astressin$_2$-B (Clayton Foundation Laboratories, The Salk Institute, La Jolla, CA) were synthesized and purified as previously described (29). NBI-35965 was obtained from Neurocrine Bisosciences Inc. (San Diego, CA).

Stress models

Water avoidance stress (WAS) in rats and partial-restraint stress (PRS) in rats and mice for 1h were used as acute-stressors (30; 31) and CRF-OE mice as a genetic model of chronic stress (27; 28; 31).

Measurements of colonic motor function in rats and mice

Colonic contractions—Contraction was recorded in conscious non-fasted rats and mice using newly developed minimally-invasive solid-state manometry catheter (23; 31). Pressure sensors were positioned at 8 and 4-cm (rats) and at 2-cm (mice) past the anus. The 8-cm site (rats) corresponds to proximal-transverse whereas the 4-cm (rats) and 2-cm (mice) correspond to the distal colon. Colonic contractions were quantified by measuring for every minute the area under the curve (AUC) of the phasic component of the intraluminal pressure change that was extracted from the original trace (32). Because acute PRS-induced activation of colonic contractions primarily occur during the first 20-min (31), the frequency, amplitude, duration and propagation of contractions were determined for the 0-20-min and 20-60 min time periods. See supplement for additional information.

Fecal pellet output (defecation) and diarrhea—In non-fasted conscious rats and mice, defecation was monitored as number of fecal pellets output (FPO) for 1 or 2-h (30; 31). The % of rats with diarrhea was calculated.

Immunohistochemistry: rat colon longitudinal muscle myenteric plexus (LMMP)-wholemount preparation

Neuronal Fos—Proximal and distal colonic LMMP wholemount preparations were dissected and Fos-immunohistochemistry assessed as in our previous studies (33; 34). The mean number of Fos-IR nuclei/myenteric-ganglion from each rat was used to generate a mean number.. See supplement for additional information.

Double labeling of CRF_2 with CRF_1 or neuronal nitric oxide synthase (nNOS)—The proximal and distal segments of colon collected from 2 naïve adult male SD rats were processed for LMMP wholemount preparations as above (34) and processed for CRF_1, CRF_2 and nNOS immunostaining as described (11; 34). See supplement for additional information.

Immunohistochemistry: rat colon primary culture LMMP neurons (PC-LMMPn)

LMMP neurons were cultured using slightly modified method (35). CRF_1 and CRF_2 receptors expression was determined using RT-PCR on 0.1 μg of poly A^+ RNA isolated from non-fixed 5 days old primary culture cells by oligo-dT cellulose spin column (FastTrack 2.0 Kit, Invitrogen). In separate preparation, 4′,6-diamidino-2-phenylindole, anti-Hu, CRF_1 and CRF_2 receptor immunoreactivity (IR) was performed in 5 days old fixed cultured neurons. CRF_1 and CRF_2 presence in the PC-LMMPn was further confirmed by Western blot. See supplement for additional information.

pERK1/2 in rat colon PC-LMMPn

Phosphorylation of ERK1/2 in response to CRF, Ucn 1 or Ucn 2 and the CRF or Ucn 1 effects in the presence or absence of Ucn 2 or selective CRF_1 or CRF_2 antagonists, NBI35965 or astressin$_2$-B respectively, was determined in 5-day cultured PC-LMMPn of rats by Western blot. Ucn 1 is used in this experiment because of its higher affinity to CRF_1 and CRF_2 than CRF (5-7). Selective inhibition of CRF_1 or CRF_2 receptors, in the presence of Ucn 1, would allow better detection of the respective role of CRF_1 or CRF_2 in the neuronal response. Band intensities were normalized to control (basal) for comparison. See supplement for additional information.

DNA transfection in HEK-293 cell lines and cAMP measurement

Stable CRF_1 and CRF_2-expression—Rat CRF_1 and CRF_{2b} cDNA encoding full-length CRF_1 and CRF_{2b} protein, respectively, was cloned into pcDNA3.1 expression vector (Invitrogen) as in our previous study (11). Confirmed plasmid DNA was then transfected into human embryonic kidney (HEK)-293 cells (1 μg/10^6 cells) using Lipofectamine 2000 as a carrier. Representative HEK-293 cell lines from at least three positive clones were used as controls to characterize CRF_1-CRF_2 interaction in subsequent functional experiments.

cAMP measurement—Similar protocol as in our previous study was used (11). See supplement for detailed information.

Data analysis

Values are presented as means±SEM. Colonic contractile or defecation differences between groups were analyzed by One Way ANOVA whereas time course data were compared using One Way ANOVA for repeated measures followed by a Student-Newman-Keuls post hoc test. Fos IR nuclei in the colon myenteric-ganglia and cAMP production difference between groups were tested by student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Urocortin 2 injected IP blunts IP CRF-induced defecation, diarrhea and myenteric neuron activation whereas IP astressin$_2$-B enhances the responses

Defecation and diarrhea—Control rats injected IP with vehicle (water) followed 5-min later by saline or rats injected with hUcn 2 (10 μg/kg)+saline exhibited similar defecation over the 60 min period (0.4±0.2 vs 0±0/h) (Fig 1A). CRF (3 μg/kg, IP) in vehicle pretreated rats, compared with vehicle+saline group, increased significantly the 60-min defecation (5.3±0.8 vs 0.4±0.2/h) (Fig 1A). Pretreatment with hUcn 2 (10 μg/kg, IP) inhibited the CRF-induced defecation by 64% (Fig. 1A).

In a separate experiment, IP vehicle+CRF at 3 and 10 μg/kg, increased defecation to 5.7±0.3 and 8.0±1.2 pellets/h and induced diarrhea in 0% and 40% of rats, respectively compared to vehicle+saline (0±0 pellets/h and 0% diarrhea). Astressin$_2$-B (50 μg/kg, IP), compared to vehicle, enhanced the CRF-induced defecation at 10 μg/kg (13.5±1.3 vs 8.0±1.2 pellets/2h, $p<0.05$) and showed a trend to increase at 3 μg/kg (7.8±1.3 vs 5.7±0.3 pellets/2h, $p>0.05$, NS) (Fig. 1D). Likewise IP astressin$_2$-B (50 μg/kg) enhanced significantly the IP CRF-induced diarrhea both at 3 μg/kg (0% to 44%) and 10 μg/kg (40% to 80%) (Fig. 1E).

LMMP neuronal activation—CRF (3 μg/kg, IP), compared to saline, increased the number of Fos-positive myenteric neurons in the proximal (11.7±1.9 vs 1.0±0.9 cells/ganglion), distal (7.4±1.5 vs 0.5±0.3 cells/ganglion) and the combined proximal-distal (9.6±1.1 vs 0.7±0.4 cells/ganglion) LMMP (Fig. 1B-C), simultaneous with increased defecation (Fig. 1A). Pretreatment with hUcn 2 (10 μg/kg, IP) decreased the CRF-induced neuronal activation in both segments (Fig. 1B-C). In a separate experiment, astressin$_2$-B, compared to vehicle, enhanced the CRF-induced Fos-expression in the proximal colon LMMP neurons (13.2±0.5 vs 10.7±1.0 cells/ganglion, $p<0.05$) but not in the distal colon (11.2±0.4 vs 10.0±0.6 cells/ganglion, $p>0.05$).

Urocortin 2 injected IP prevents acute stress-induced stimulation of colonic motor function in rats

Partial-restrain stress—Rats injected IP with saline and exposed to 1h PRS exhibited robust contractile activities in the proximal-transverse and distal colon for the first 20-min followed by a period of relative quiescence (20-60 min) (Fig. 2A-C). High amplitude phasic contractions (>15 mmHg) occurred with a frequency of 22±7.1 and 40± 7.2/h in the proximal-transverse and distal colon respectively, with 33% propagating from proximal to the distal site (Fig. 2A-B). hUcn 2 (10 μg/kg, IP) prevented the enhanced initial 20-min colonic response as shown by the reduced AUC (Fig. 2C), frequency and propagation of contractions (Fig. 2B). However hUcn 2 did not affect the duration and amplitude (Fig 2B) of contractions during this period. During the 20-60 min period, Ucn 2 had no effect on frequency, duration and propagation while increasing amplitude at both 8 and 4-cm sites (Fig 2B).

Water avoidance stress—Rats injected IP with saline and exposed to WAS (1h) had higher defecation than non-stressed rats (3.5±0.3 vs 0.4±0.1 pellets/h). hUcn 2 (10 μg/kg, IP)-reduced significantly the WAS-induced defecation by 49% (3.5±0.3 vs 1.8±0.2 pellets/h).

Urocortin 2 injected IP prevents stress-induced colonic motor response in mice

WTL mice—mUcn 2 (10 μg/kg, IP) prevented acute PRS-induced initial increase in distal colonic high amplitude contractions termed as giant migrating contractions (GMCs) compared to IP saline (0.1 ml) (Fig. 3A-C). mUcn 2 decreased the mean AUC of the 0-20-

min period by 34.7% (Fig. 3B-C). This was mainly due to decreased frequency of all contractions (24.5±5.2 vs 54.9±6.1 contractions/h; $p<0.05$), and specifically in the short duration contractions (<40 sec) and GMCs (14.5±3.6 vs 27.9±3.0 contractions/h; $p<0.05$) (Fig. 3D-F). mUcn 2 decreased also the acute PRS-induced defecation compared with saline (5.3±0.6 vs 13.7±1.2; $p<0.05$), concomitant to the decrease in colonic contractions.

CRF-OE mice—The initial increase in distal colonic contractile activity in response to PRS was shortened to 10-min as reported before (31) but was significantly higher activity than in the remaining recording period (Fig. 4A-D). mUcn 2 (10 μg/kg, IP), compared to vehicle, reduced PRS-induced activation of colonic contractility, resulting in a significant 54.2% decrease of AUC in the first 20-min (Fig. 4A-D) together with a decrease in PRS-induced defecation (4.6±1.5 vs 11.0±2.3 pellets/h, $p<0.05$).

CRF_2 receptor deletion (CRF_2-/- mice) exaggerates colonic contraction and defecation response to acute PRS or IP CRF

CRF_2-/- mice, compared to WTL, displayed increased frequency of GMCs, AUC for the 0-60 min period ($p<0.05$) (Fig. 5A-D) and defecation (11.5±0.8 vs 4.2±1.1 pellets/h; $p<0.05$) when exposed to PRS. Injection of CRF (10 μg/kg,IP), compared to saline, in female WTL mice, placed in novel individual cages, had no effect on defecation (4.0±1.0 vs 4.6±0.9 pellets/h) but significantly increased defecation in female CRF_2-/- mice (8.5±0.7 vs 5.8±0.9 pellets/h).

Double-immunostaining of CRF_2/CRF_1 in the rat colon LMMP and PC-LMMPn

LMMP—Rat proximal and distal colon LMMP neurons exhibited CRF_2 (cell bodies and fibers) and CRF_1 (mainly cell membrane)-receptor antibody IR (Fig. 6A, supplement Fig. 1). The two receptors are co-localized in the majority of cells. Similarly CRF_2 are co-localized with nNOS (Fig. 6B, supplement Fig 1).

PC-LMMPn—5-day cultured primary neurons uniformly displayed IR to Anti-Hu D/C demonstrating the neuronal nature of these cells (Fig. 6C). CRF_1 and CRF_2 are co-localized in the neuronal cells (Fig 6C). The presence of CRF_1 and CRF_2 in the primary neurons was further confirmed by Western blot (Fig 6D) and RT-PCR (Fig. 6E).

CRF-induced ERK phosphorylation in PC-LMMPn is enhanced by CRF_2 blockade

CRF and Ucn 1 (10 and 100 nM)-induced phosphorylation of ERK1/2 in neuronal cells in a dose-dependent manner (Fig. 7A). Pre-incubation with the selective CRF_1 antagonist, NBI-35965 (1 μM), prevented CRF or Ucn 1 (100 nM)-induced phosphorylation (Fig. 7B) while $astressin_2$-B further enhanced phosphorylation stimulated by CRF and Ucn 1 (Fig. 7B). Ucn 2 by itself had no (10 nM) or moderate (100 nM) effect on pERK (Fig. 7A). However pre-incubation with Ucn 2 (10 or 100 nM) prevented the CRF (100 nM)-induced pERK (Fig. 7B).

CRF-induced cAMP production in CRF_1-only transfected cells is blunted in CRF_1/CRF_{2b} double-transfected cells

CRF (10^{-10}-10^{-6} M) induced a concentration dependent cAMP response (EC_{50}:8.6 nM) through CRF_1 in HEK-cells that expressed only CRF_1-receptors. CRF induced also cAMP production in CRF_2-only transfected cells with an EC_{50} value of 220 nM. However compared to the effect of CRF on CRF_1-only cells, the potency of CRF was decreased by 10-fold (EC_{50}: 8.6 nM→ 86 nM) in cells that co-expressed both CRF_1 and CRF_2-receptors (Fig. 7C).

Discussion

The bodily response to stress, including that of the colon, is primarily initiated by the activation of CRF_1 (5). However little is known about the role of CRF_2.in the colonic responses to stress. The present studies provide convergent evidence that activation of peripheral CRF_2 plays a physiological role to counteract CRF_1-mediated stimulation of colonic motor response to acute-stress and IP CRF in rodents. In addition, we show that CRF_1-CRF_2 receptors are colocalized and interact in the rat colon wholemount as well as in primary myenteric neurons and in CRF receptor transfected cell line where CRF_2 activation curtails a CRF_1-mediated phosphorylation of ERK1/2 and cAMP production.

Peripheral activation of CRF_2 by IP Ucn 2 consistently blunted acute-stress or CRF-induced defecation and colonic contractile responses in rodents. Colonic contraction in rats and mice submitted to PRS and monitored by still manometry is characterized by the occurrence of an immediate (first 20-min) high amplitude (>15 mmHg) and propagative contractile activity. Comparable propagative contractions termed giant migrating contractions (GMC) in the colon of non-fasted freely moving (36; 37), or fasted and anesthetized rats (37; 38) are reported, albeit with different frequencies. The frequency differences are probably due to the acute-stress (present) versus the freely moving (36) and anesthetized state (37; 38) as well as the use of still manometry (present) versus strain-gauge (36; 37) or perfused manometry (38). Increased and coordinated colonic high amplitude contractions or GMCs are associated with defecation in several species including humans (36; 37; 39). We previously established in mice that the initial 20-min colonic activity in response to acute-PRS is strongly correlated with defecation (31). The present CRF_2-mediated reduction of defecation thus is attributable to the suppression of the overall AUC and specifically the frequency and propagation of the high amplitude contractions.

Given that acute-stress and IP CRF-induced colonic stimulation is primarily mediated by CRF_1-activation (22; 33) and that CRF also binds to CRF_2, although with less affinity compared to CRF_1 (5), we set out to determine whether simultaneous activation of both receptors, leads to the modulation of CRF_1 mediated colonic propulsive motor function. The data indicate that blockade of CRF_2, in rats, exacerbates IP CRF-induced defecation and diarrhea. Similarly, the defecation response to a sub-threshold dose of CRF and the colonic contractile response to acute-PRS are enhanced by CRF_2 deletion in mice. These data clearly show that CRF_2 indeed has a physiological role in the colonic secretomotor-response to acute-stress or IP CRF and point to the existence of a possible CRF_1-CRF_2 receptor interaction in the responses. In addition, the data that mUcn 2 not only blunts the acute stress-induced colonic contractions in WTL but also does so in mice under chronic stress setting, i.e. the CRF-OE mice, is of significance because chronic stress has more relevance in several diseases including colonic sensorimotor-responses in humans and animals (1-4; 40).

A potential target for the CRF_1-CRF_2 interaction in the colonic responses includes colonic myenteric neurons. This is supported first by the dense colocalization of CRF_1-CRF_2 and CRF_2-nNOS in the rat colonic myenteric wholemount and primary neurons. Second CRF_2 activation blunted IP CRF-induced myeneteric Fos-expression, a marker for neuronal activation (41), while blockade of CRF_2 receptor enhanced the Fos-response. We previously established that IP CRF activates colonic myenteric neuron through peripheral CRF_1 (33; 34) and that nearly all (96-98%) Fos-expressing cells are CRF_1-IR (34). The study showed also that Fos-activation by IP CRF is correlated with increased defecation (34). Although Fos-expression is a general marker for neuronal activation (41), the fact that CRF_1-CRF_2 are colocalized on myenteric neurons and that CRF_2 inhibits in tandem the CRF-induced myenteric neuron Fos-expression and defecation suggest that enteric neurons are direct or

indirect targets of CRF ligands. In support for a direct action on enteric neurons, studies in guinea pigs have shown that CRF or Ucn 1 increases colon myenteric neurons firing through a direct action on neuronal CRF_1 (25; 42). Collectively these findings point to a possible direct inhibitory action of CRF_2 on CRF_1-containing neurons and/or indirectly through the release of inhibitory neurotransmitters such as NO

Further evidence in support of a CRF_1-CRF_2 interaction in the observed CRF_2 mediated inhibition of the colon to CRF and acute-stress come from data in the primary myenteric neuron and HEK-293 cell. CRF- and Ucn 1-activated myenteric neuron ERK1/2 is prevented by selective CRF_2 agonist and selective CRF_1 antagonist but enhanced by selective CRF_2 antagonist. Since, CRF and Ucn 1 bind to both receptors, although with different affinities (5), the data clearly show that when both receptors are simultaneously activated, CRF_2 modulates a CRF_1 mediated event. The physiological significance of CRF_2 dependent inhibition of a CRF_1 mediated ERK activation in primary LMMP neurons can not be fully explained in the present study because we only assessed pERK levels at one time point and that events that precede or post ERK1/2 phosphorylation following CRF_1 and/or CRF_2 activation are not yet characterized in this system. However, it is shown that Fos-expression in enteric neuron is associated with defecation (34) and that c-fos transactivate transcription of several neurotransmitter biosynthetic enzymes that have AP-1 responsive elements including choline acetyltransferase (ChAT), a key enzyme in the synthesis of acetylcholine (43). Blockade of this cascade thus could interfere with the CRF_1 mediated, at least neuronal activation, and possibly release and/or synthesis of neurotansmitters.

Similarly, CRF's potency to induce cAMP production in CRF_1/CRF_{2b} transfected cells was 10-fold lower than that in cells transfected with CRF_1 only. This potency loss in the double-transfected cells is unlikely to be due to differential availability of CRF. First HEK-293 cells do not constitutively express the CRF_1 and CRF_2 (11) and the density of transfected receptors is about the same. Second, the concentration of CRF used in the current study is 100 times in excess to the EC_{50} (8.6 nM) required to activate CRF_1 receptors in the transfected cells. Third, although CRF_1 has higher affinity (>4-20X) to CRF than CRF_2 (5), CRF in low (nM) concentrations can induce a CRF_2 mediated cAMP production in HEK-293 cells (11; 44). Thus, the blunted CRF-effect on cAMP production is likely due to a receptor-to-receptor interaction at coupling and/or signaling level. Such opposite effects of CRF may be explained through a dual mechanism where CRF-peptides, which bind to both receptors, activate cAMP primarily through Gs-coupled CRF_1 and inhibit it through Gi-coupled CRF_2 variant, as has been recently reported (44). It is conceivable also that CRF or Ucn 1 would activate both receptors, but that CRF_1 remains desensitized longer than CRF_2 as shown for NK_1 and NK_3 receptors (45) and/or that CRF_2 activation may share intracellular signaling targets of CRF_1.

Acute-stress triggers integrated responses to maintain homeostasis and ensure survival of an organism. In the absence of proper counter-regulation, the stress-response runs in an overdrive state that can become maladaptive and could predispose to diseases (46) The altered colonic motor response to acute or chronic stress following blockade or deletion of CRF_2 is consistent with an adaptive counter regulatory role of CRF_2 in the stress-response. Interestingly, in mice heart CRF_{2b} confers cardioprotection that is lost following chronic stress-induced down-regulation of the CRF_{2b} variant (47). Protective effects mediated by CRF_2 are also reported in experimental visceral pain (48) and colitis (49). In line with this, the present data provide a basis for the concept that stress-related gut functional alterations should not be solely viewed through a CRF_1-mediated pathway but also through a dampened CRF_2 signaling that overstrains the colonic motor response to stress The notion is particularly relevant in view of the recent data on the lack of effect of a CRF_1 antagonist to improve intestinal transit symptoms in IBS patients (50). Understanding the role of CRF_2

and its regulation in the gut response to stress may open new pharmacological targets in the treatment of stress related disorders of the gut, such as IBS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cAMP	cyclic adenosine monophosphate
CCA	colonic contractile response
CRF	corticotropin releasing factor
CRF_1	corticotropin releasing factor receptor 1
CRF_2	corticotropin releasing factor receptor 2
CRF_2 -/- or CRF_2-KO	CRF_2 deficient mice
CRF-OE	corticotropin releasing factor overexpressing
EIA	Enzyme Immuno Assay
ERK1/2	extracellular signal-regulated kinase1/2
FPO	Fecal pellet output
GMCs	Giant migrating contractions
HEK	human embryonic kidney cells
hUcn 2	human urocortin 2
IR	immunoreactivity
LMMP	longitudinal muscle myenteric plexus
mUcn 2	mouse urocortin 2
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
PC-LMMPn	primary culture longitudinal muscle myenteric plexus neurons
PRS	partial-restraint stress
Ucn 1	urocortin 1
WAS	water avoidance stress
WLT	wild type littermate

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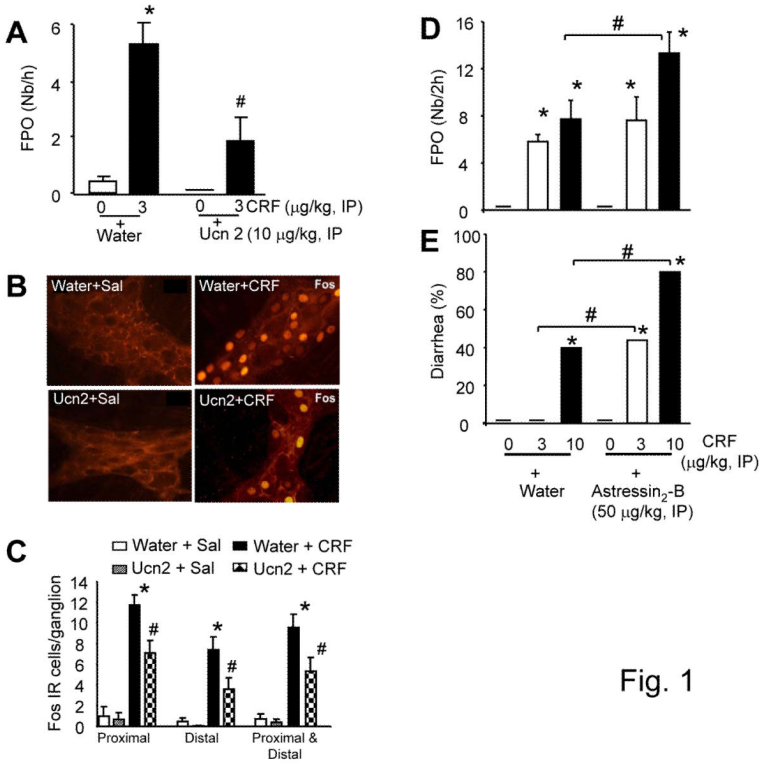

Fig. 1

Figure1.

Peripheral pretreatment (−5 min) with hUcn 2 blunts IP CRF-induced defecation and colonic myenteric Fos-IR whereas astressin2B enhances the response in rats. (A): Defecation response, expressed as fecal pellet output (FPO) to IP CRF in the presence or not of IP hUcn 2, *p<0.05 vs controls; #p<0.05 vs CRF; n=8-11/group. (B): Confocal photomicrograph of proximal colon LMMP neurons Fos expression. Colons are from rats in A above (8/group) that received IP water+saline (top-left), water+CRF (top-right), hUcn 2+saline (bottom-left) and hUcn 2+CRF (bottom-right). (C): Fos-IR (cell/ganglion) in the proximal, distal and proximal-distal colon in response to CRF (3 μg/kg, IP) in the presence or not of hUcn 2 (10 μg/kg, IP), *p<0.05 vs all others; #p<0.05 vs their respective water+sal or Ucn 2+sal; n=8/group (D-E): Selective blockade of CRF_2 receptor by IP $astressin_2$-B exacerbated IP CRF-induced defecation (D) and diarrhea (E). Bar, mean±SEM. n=8/group, *p<0.05 vs water+saline or $astressin_2$-B+saline; #p<0.05 vs the corresponding water+CRF 10 μg/kg (D,E) or water+CRF 3 or 10 μg/kg (E).

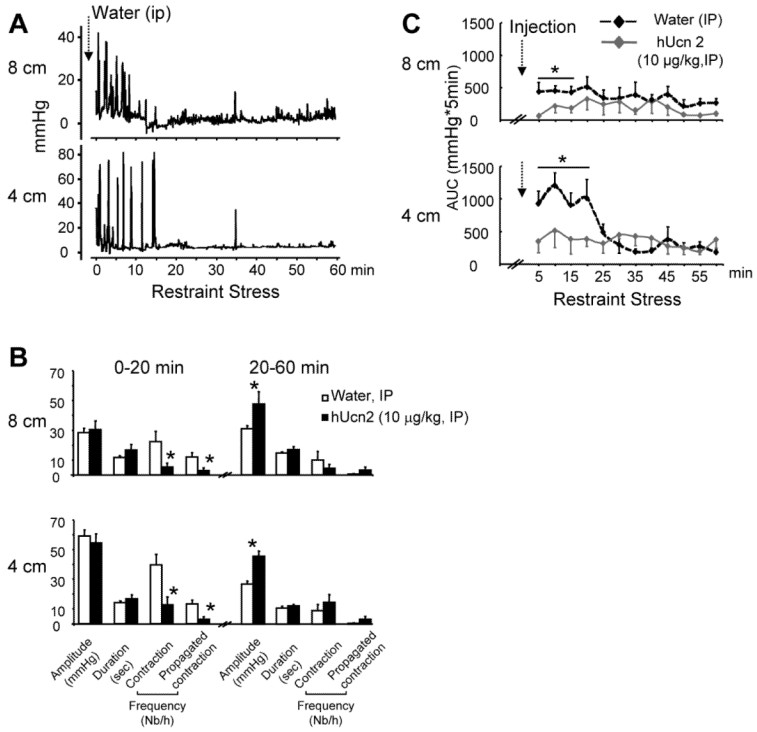

Figure 2.

Acute partial-restraint stress-induced colonic contractions in rats is prevented by hUcn 2 (10 µg/kg, IP). (A): Representative solid state manometry trace with pressure sensor catheter probes placed in the colon at 8 and 4 cm past the anus. (B): Graphs showing amplitude, duration, total frequency (>15 mmHg) and propagated contraction frequency. Bar, mean ±SEM of n=5/group*: $p<0.05$ versus water (C): Time course of AUC pressure changes. Dotted arrows show IP injection just before the onset of restraint stress. Values are rolling averages (mean±SEM) of AUC computed for every 5 min, *$p<0.05$ vs contractile responses on all other time points. n=8/group.

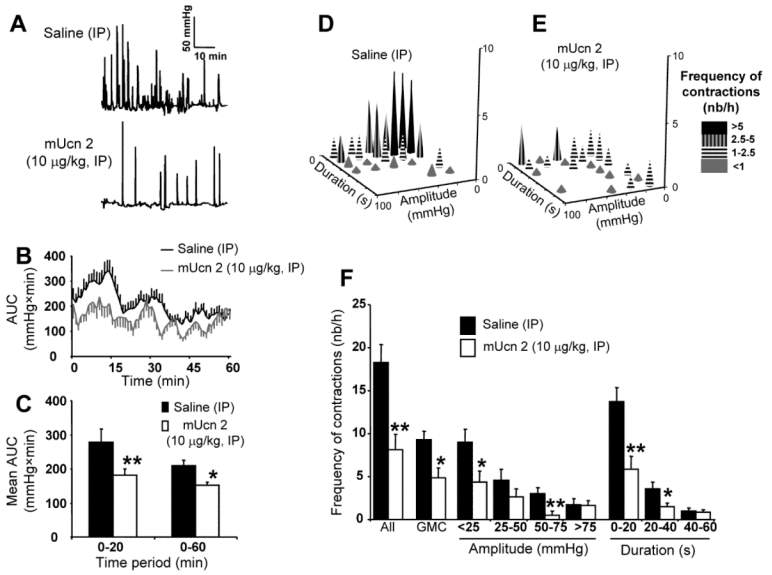

Figure 3.

mUcn 2 (10μg/kg) injected IP reduced distal colonic contraction to acute partial-restraint stress in wild-type mice. Representative distal CCA traces following saline or mUcn 2 treatment (A). Time course of AUC pressure changes over 1 h (B) and in blocks of 0-20 and 20-60 min period (C). Bar, mean ± SEM of n = 8-10/group. *p<0.05; **: p<0.01 vs saline. (D-E): Plots show distal colonic contractions pattern of WTL mice during the first 20-min of PRS following injection with saline (D) or mUcn 2 (E). Plots show the frequency of contractions as a function of amplitude or duration of contractions. (F): Distal colonic contractions profile (frequency, amplitude and duration of contractions), including giant migrating contractions (GMCs) over 1-h period. Bar, mean±SEM of n=6 mice/group*: p<0.05 versus saline; **: p<0.01 vs saline.

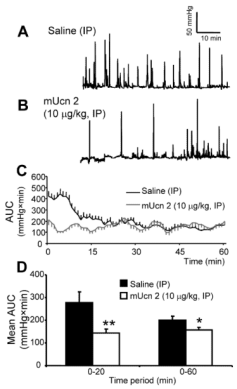

Figure 4.

mUcn 2 (10 μg/kg, IP) reduces distal colonic contractions to impartial-restraint stress in CRF overexpressing (CRF-OE) mice. (A-B): Representative distal CCA traces of CRF-OE mice injected with saline (A) or mUcn 2 (B). (C-D): Time course of the AUC of intracolonic pressure changes over 1-h (C) and in blocks of 0-20, 0-60-min (D). Bar, mean±SEM of n=6-7/group. *: $p<0.05$ **: $p<0.01$ vs saline.

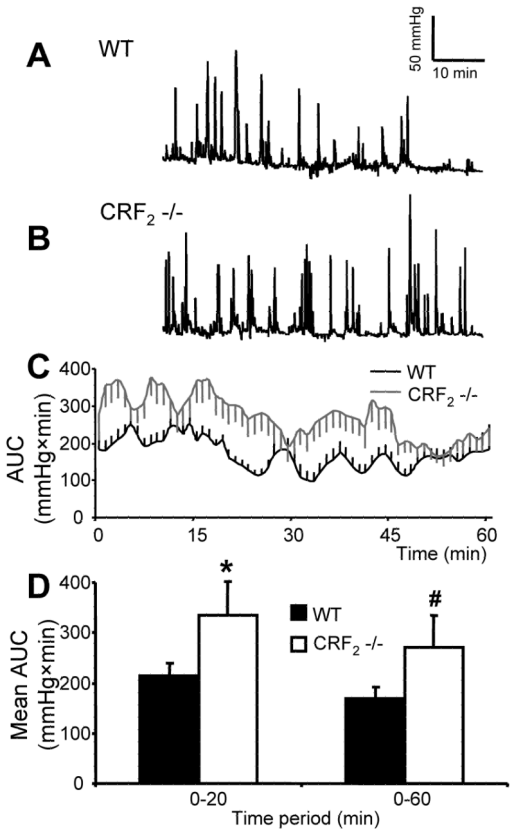

Figure 5.

Distal colonic contractions to partial-restraint are enhanced in CRF_2-/- mice compared to wild-type. (A-B): Representative trace in WTL mouse (A) and CRF_2-/- mouse (B). (C): Time course of AUC of intracolonic pressure changes over 1h (D) and in blocks of 0-20 and 0-60-min period (D). Bar, mean±SEM of n=7/group.*: $p<0.05$ vs WTL 0-20-min time period; #: $p<0.05$ vs WTL 0-60 min.

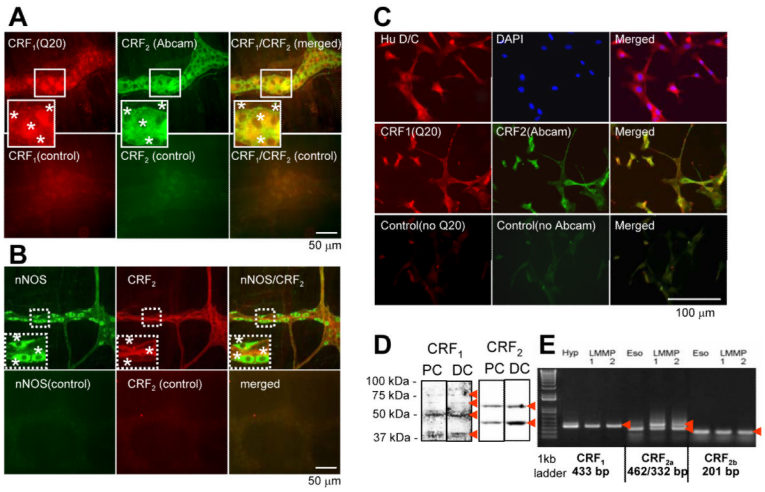

Figure 6.

CRF_1 and CRF_2 are coexpressed in rat colonic myenteric neurons. (A-B): Confocal microscope images showing double labeling of CRF_2 with CRF_1 (A) and CRF_2 with nNOS (B) in rat distal colon wholemount LMMP preparation. (C): double labeling of nuclear marker, 4′,6-diamidino-2-phenylindole (DAPI) and anti-Hu IR in rat colon myenteric ganglion primary culture neurons (top panel) and double labeling of CRF_2 with CRF_1 (middle panel). Lower panels in A,B,C show negative control, stained with normal goat IgG. (D): Western blot analysis of CRF_1 (bands at 39, 50, 60, 75 kDa, arrows) and CRF_2 (at 42, 55 kDa, arrows) in proximal colon (PC) and distal colon (DC) PC-LMMPn. (E): RT-PCR analysis for expression of CRF_1, CRF_{2b} and CRF_{2a} splice variants in the rat proximal colon PC-LMMPn. Hypothalamus (Hyp) was used as positive control for CRF_1 and Esophagus (Eso) for CRF_{2b} and CRF_{2a} to generate predicted PCR products (*arrows)*.

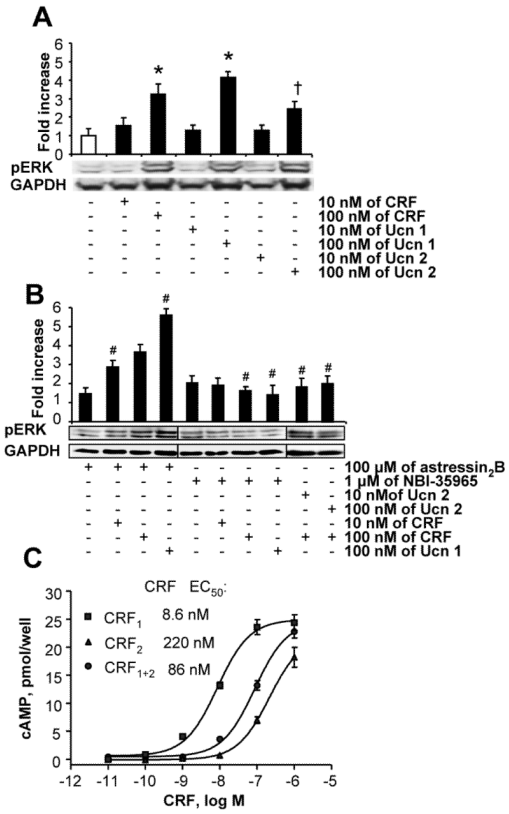

Figure 7.

Activation of CRF_2 suppresses phosphorylation of pERK in PC-LMMPn and cAMP production in HEK-293 cells. (A-B) Western blot analysis of pERK1/2 in response to CRF, Ucn 1 or Ucn 2 (A). Blockade of the CRF-induced pERK by Ucn 2 or by a selective CRF_1 antagonist, NBI-3965 and enhancement of the response by selective CRF_2 antagonist $astressin_2$-B (B). Bar, mean±SEM, n=3/group, * $p<0.05$ vs no-treatment and the respective10 nM dose in A, †$p<0.05$ vs all other groups in A, #$p<0.05$ vs the no-treatment and the respective CRF or Ucn 1 alone dose in A. (C): Dose-dependent increases in intracellular cAMP production in $CRF_{1\alpha}$, $CRF_{2\beta}$ and $CRF_{1\alpha}$+$CRF_{2\beta}$ transfected HEK-293 cells stimulated with CRF for 30 min. Note the 10-fold decrease in potency of CRF in CRF_1 and CRF_2 coexpressing cells vs. in CRF_1 only expressing cells.