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A Balance Theory of Peripheral Corticotropin-Releasing Factor Receptor Type 1 and Type 2 Signaling to Induce Colonic Contractions and Visceral Hyperalgesia in Rats

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29

Abstract

Several recent studies suggest that peripheral corticotropin-releasing factor receptor type 1 (CRF1) and type 2 (CRF2) have a counter regulatory action on gastrointestinal functions. We hypothesized that the activity balance of each CRF subtype signaling may determine the changes in colonic motility and visceral sensation. Colonic contractions were assessed by the perfused manometry and contractions of colonic muscle strips were measured in vitro in rats. Visceromotor response (VMR) was determined by measuring contractions of abdominal muscle in response to colorectal distensions (CRD, 60 mmHg for 10 min twice with a 30 min rest). All drugs were administered through intraperitoneal route in in vivo studies. CRF increased colonic contractions. Pretreatment with astressin, a non-selective CRF antagonist, blocked the CRF-induced response, but astressin$_2$-B, a selective CRF2 antagonist, enhanced the response by CRF. Cortagine, a selective CRF1 agonist, increased colonic contractions. In in vitro study, CRF increased contractions of muscle strips. Urocortin 2, a selective CRF2 agonist, itself did not alter the contractions but blocked this increased response by CRF. VMR to the second CRD was significantly higher than that of the first. Astressin blocked this CRD-induced sensitization, but astressin$_2$-B or CRF did not affect it. Meanwhile, astressin$_2$-B together with CRF significantly enhanced the sensitization. Urocortin 2 blocked, but cortagine significantly enhanced the sensitization. These results indicated that peripheral CRF1 signaling enhanced colonic contractility and induced visceral sensitization, and these responses were modulated by peripheral CRF2 signaling. The activity balance of each subtype signaling may determine the colonic functions in response to stress.

Introduction

Stress alters gastrointestinal (GI) motility and visceral sensation, and both central and peripheral corticotropin-releasing factor (CRF) receptors are involved in these changes (1,2). In addition to CRF, CRF-related peptides, urocotins (Ucns; Ucn1, Ucn2 and Ucn3) also bind to CRF receptors, and they are prominently expressed in peripheral tissues where they mediate visceral stress responses (3,4). CRF and Ucns exert its action through the activation of two receptors, CRF receptor type 1 (CRF1) and type 2 (CRF2) (5,6). Activation of each CRF receptor induces distinct responses in GI tract, i.e., stimulation of colonic motility and inducing visceral hypersensitivity to colorectal distension (CRD) by CRF1 (7), and delayed gastric emptying (GE) by CRF2 exclusively (8). However, since these peptides bind both CRF receptor subtypes with their distinct affinity (9-11), we may be allowed to think that both receptors signaling may be activated simultaneously and contribute to stress and CRF-induced altered GI functions.

We have very recently demonstrated that peripherally administered CRF enhanced gastric contractions through CRF1, even though it delayed GE and this action was inhibited by activation of peripheral CRF2 in rats (12). Other researchers also showed that activation of peripheral CRF2 inhibited intraperitoneal (ip) CRF-induced, CRF1 dependent stimulation of defecation (13). Moreover, CRD induces visceral hypersensitivity through CRF1 and it is prevented by peripheral CRF2 stimulation in rats (14,15). These lines of evidence suggest that each peripheral CRF receptor subtype may have a counter action in regulating GI functions. With regard to this point, we made a hypothesis in our previous paper regarding gastric contractions (12). Briefly, CRF1 signaling may be the direct force to stimulate gastric contractions. On the other hand, CRF2 signaling may inhibit the CRF1 signaling, thereby modulating gastric contractions. In other words, both peripheral CRF receptor subtypes are simultaneously activated during stress or when CRF is injected, and the activity balance of each subtype signaling may determine the functional changes in gastric

contractions. This model may also explain the findings demonstrated in the above mentioned studies regarding fecal pellet output and visceral sensation by others (13-15).

In the present study, we tried to clarify whether CRF or stress-induced altered colonic functions such as stimulated colonic motility and visceral hypersensitivity are also regulated by the same mechanism. We assessed colonic contractions using the perfused manometric method in freely moving conscious rats, and CRF, selective CRF receptor agonist or antagonist was alone or were simultaneously administered to clear the role of activation balance of CRF1 and 2 signaling. We also assessed contractions of colonic muscle strips in vitro. Moreover, visceromotor response (VMR) induced by CRD was evaluated by measuring abdominal muscle contractions electrophysiologically to test the hypothesis.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing between 200 and 250 g were housed under controlled light/dark conditions (lights on 07:00−19:00). The room temperature was regulated to 23−25 °C. Rats were allowed free access to standard rat chow (Solid rat chow, Oriental Yeast, Tokyo, Japan) and tap water. Experiments started between 8 AM−2 PM and finished no later than 4 PM.

Chemicals

A rat/human CRF and human Ucn2 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in normal saline. Astressin, astressin$_2$-B (Sigma-Aldrich) and cortagine (PolyPeptide Laboratories,

Torrance, CA, USA) were dissolved in double-distilled water. The dose of the chemicals were determined according to the previous reports (12,16,17).

Implantation of catheter for manometric recordings

In non-fasted rats, small incision about 1.5 cm in length was made in the abdominal wall, and cecum and proximal colon were taken out through the incision under ether anesthesia. The small hole was made at the 3 cm from the ileocecal junction (proximal colon) by 18 G needle. An open-tipped catheter (3-Fr, 1 mm internal diameter, Atom, Tokyo, Japan) for manometric measurement was inserted through the hole and pushed 2 cm into the colonic lumen toward the mouth, and was fixed by purse-string sutures at the point of exit from colonic wall. Then it passed through the abdominal wall musculature and a subcutaneous (sc) tunnel to exit at the back of the neck, and was secured to the skin. The rats were allowed to recover in individual cages for 5−7 days before the experiments.

Manometric recordings

Colonic contractions were measured in non-fasted rats by the perfused manometric method described in previous studies (18,19). At the experiments, these prepared animals were put in wire-bottom and non-restraint polycarbonate cages. The manometric catheter was threaded through a flexible metal sheath to protect it from biting and connected to an infusion swivel (Instech Laboratories, Plymouth Meeting, PA, USA) to allow free movement. The catheter was infused continuously with degassed distilled water at a rate of 1.5 ml/h using a heavy-duty pump (CVF-3100, Nihon Kohden, Tokyo, Japan) and was connected to a pressure transducer (TP-400T, Nihon Kohden). Pressure signals from the transducer were digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and stored by computer software (LabChart 7, AD

Instruments). First, after 1 h of stabilization, the basal state of the colonic pressure waves was measured for 1 h. Then, the catheter was disconnected and the animal was taken out from the cage. Drug or vehicle was injected intraperitoneally in a 0.2-ml volume under brief ether anesthesia. In some experiments, drug or vehicle was injected twice with 10 min interval. After injection(s), the rat was put in the cage again and the catheter was re-connected to a pressure transducer. The pressure waves were monitored for 1 h after injection. Using the recordings, we evaluated the motor index (MI) to assess colonic motor activity as described below.

Evaluation of the MI

The MI was determined by the area under the manometric trace (AUT). AUT was calculated using software (LabChart 7, AD Instruments). Basal MI was defined as AUT for the 1 h period before drug or vehicle injection. The %MI was calculated by the following formula**:** (AUT for the 1 h period after injection)/(basal MI) × 100. In this experiment, pressure signals were recorded continuously, but the measurements were stopped briefly in order to perform ip injection(s) as stated above. In relation to injection, time for recovery from the anesthesia and re-stabilization of baseline of manometric trace was required in order to obtain adequate recordings for the analysis. Therefore, the manometric data during the recovery period for approximately 5－7 min were excluded from the later analysis.

Measurement of contractions of colonic muscle strips

This experiment was conducted following procedures as described previously (17) with minor modification. Briefly, the rat was anesthetized with ether and killed by cervical dislocation immediately before the measurement. The proximal colon was removed and opened along the mesenteric border. Colonic muscle strips approximately 2 × 10 mm were cut circumferentially. The

muscle strips were suspended in an organ bath containing 2.7 ml of Krebs solution (NaCl 118.07 mM, KCl 4.69 mM, NaH_2PO_4 1.01 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.52 mM, $MgSO_4 7H_2O$ 0.57 mM, glucose 11.1 mM), aerated with 95 % O_2 and 5 % CO_2 at 37 ± 0.5 °C. One end of the strip was fixed to the bottom of chamber using tissue clip and the other end was connected to an isometric force transducer (ORIENTEC, Tokyo, Japan) by the clip and silk thread. Muscle strips were equilibrated at an applied tension of 1 g for 1 h. Mechanical activity was recorded on a polygraph recorder. Previous study (17) reported that muscle strips of rat distal colon showed spontaneous phasic contractions and CRF increased the amplitude of contractions significantly with maximal response obtained at a dose of 3×10^{-6} M. The response started several min after application of CRF and became stable within 10 min.

According to the above evidence, we assessed the mean amplitude of contractions before (for 10 min) and after (for 15 min) drug administration to estimate the contractile response induced by CRF. The % change of amplitude was determined by calculating following the formula: (mean amplitude of contractions after administration)/(mean amplitude of contractions before administration) × 100. We also tested the effect of Ucn2.

Measurement of visceral sensation

Visceral pain in response to CRD was assessed by abdominal muscle contractions in conscious rats, which was validated as quantitative measure of visceral nociception (20). In the present study, electrodes for measuring abdominal muscle contractions electrophysiologically were acutely implanted on the day of the experiment.

Implantation of electrodes

Under ether anesthesia, skin incision about 5 mm in length was made in non-fasted rats.

The electrodes (Teflon coated stainless steel, 0.05 mm diameter, MT Giken, Tokyo, Japan) were inserted approximately 2 mm into left side external oblique musculature through the incision and fixed by cyanoacrylate instant adhesive (Aron Alpha, TOAGOSEI, Tokyo, Japan) together with the incised skin. The electrode leads were externalized through this closed incision and threaded through a urethane tube. The distension balloon (a 6 cm long plastic balloon tied around a 4-Fr polyvinyl chloride catheter, Atom, Tokyo, Japan) was inserted intra-anally with the distal end positioned 1 cm proximal to the anus. The balloon was secured in place by taping the catheter to the tail. They were trained to the experimental conditions by placing them singly in Bollmann cages for 3 h per day for 3 consecutive days before the study.

CRD and monitoring VMR

After completing the surgery for electrodes implantation and balloon placement, the animals were put in Bollmann cages. Then electrode leads were connected to a custom made electromyogram (EMG) amplifier. EMG signals were amplified, filtered (3000 Hz) and digitized by a PowerLab system, and stored by computer software (LabChart 7). After a 60 min stabilization period of recovery and stabilization in the cages, they were submitted to isobaric CRD (60 mmHg, 10 min twice with a 30 min rest). Such an acute preparation was previously validated to study visceral hyperalgesia induced by CRD in rats (14,21). Basal area under the curve (AUC) was determined by calculating the AUC of EMG signal trace for the 10 min period immediately preceding each CRD using LabChart 7 software. The VMR (μV×min) was calculated by subtracting the basal AUC from the one during distension period. The % change of VMR between the first and second distensions was determined by calculating following the formula**:** (VMR of the second distension)/(VMR of the first distension) × 100.

Since repeated tonic noxious CRD was reported to induce visceral sensitization (15), first

we determined whether VMR to the second CRD is increased as compared with one to the first CRD in our experimental setting. Then in order to test the effect of CRF-related drugs on VMR, vehicle or drug was administered by ip injection at the end of the first CRD and 30 min later, the second CRD was submitted.

Statistical analysis

Data were expressed as means ± S.E. Multiple comparison was performed by one-way analysis of variance followed by Tukey's Honestly-Significant-Difference Test. Comparison between two groups was performed using the Student's t or paired t test. SYSTAT 13 software (Systat Software, Chicago, IL, USA) was used throughout the study.

Ethical considerations

Approval by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#11042, approved on March 7, 2011) was obtained for all studies.

Results

Colonic contractions

First, we examined the effect of ip CRF on colonic contractions. Although a dose of 15 μg/kg of CRF did not increase the MI (100.3 ± 13.0 % for CRF, n = 5, vs. 98.5 ± 11.7 % for vehicle, n = 7, $p > 0.05$), 30 and 60 μg/kg of CRF significantly increased it ($F = 3.53$, $p < 0.05$, 127.6 ± 19.6 % for 30 μg/kg, n = 7 and 146.6 ± 7.4 % for 60 μg/kg, n = 10, vs. vehicle, $p < 0.05$, Fig. 1A). Demonstrable recordings are shown in Figure 1B and this stimulatory effect of CRF was observed

immediately after the administration.

Next, we examined the effect of astressin, a non-selective CRF antagonist, on ip CRF-induced enhanced colonic contractions to clarify whether this response is mediated through peripheral CRF receptors. As demonstrated in Figures 2A and B, astressin (100 μg/kg) itself did not change the MI (99.3 ± 9.5 % for astressin + vehicle, n = 6, vs. 98.6 ± 10.1 % for vehicle + vehicle, n = 8, $p > 0.05$). However, the antagonist 10 min prior to ip CRF blocked the response induced by CRF at a dose of 60 μg/kg ($F = 3.53$, $p < 0.05$, 105.1 ± 13.1 % for astressin + CRF, n = 6, vs. 144.5 ± 12.9 % for vehicle + CRF, n = 6, $p < 0.05$), suggesting that the stimulatory effect of CRF is mediated through activating peripheral CRF receptors.

In order to determine the CRF receptor subtype which mediates this action of CRF, the effect of a selective CRF2 antagonist, astressin$_2$-B was investigated. Astressin$_2$-B (100 μg/kg) itself did not modify the MI (100.5 ± 9.4 % for astressin$_2$-B + vehicle, n = 5, vs. 101.4 ± 10.5 % for vehicle + vehicle, n = 8, $p > 0.05$), but it further enhanced the CRF-induced stimulation of colonic contractions significantly ($F = 7.5$, $p < 0.05$, 188.0 ± 19.6 % for astressin$_2$-B + CRF, n = 7, vs. 138.5 ± 16.4 % for vehicle + CRF, n = 7, $p < 0.05$, Fig. 3A and B).

Next, to further investigate the role of peripheral CRF2 signaling on colonic contractility, the effect of a selective CRF2 agonist, Ucn2 was tested. Ucn2 (60 μg/kg) neither modified the basal colonic contractility nor the enhanced colonic contraction induced by CRF (Table 1).

Finally, the effect of cortagine (60 μg/kg), a selective CRF1 agonist was determined. It significantly increased the MI (Table 1).

Contractions of colonic muscle strips

Muscle strips demonstrated spontaneous phasic contractions (Fig. 4A). CRF (3×10^{-6} M)

increased the amplitude of phasic contractions (% change in amplitude of contractions, $F = 12.0$, $p < 0.05$, 143.8 ± 9.5 % for CRF, $n = 7$, vs. 99.5 ± 2.6 % for vehicle, $n = 4$, $p < 0.05$), but Ucn2 (10^{-6} M) did not alter the contractions (105.7 ± 3.5 % for Ucn2, $n = 7$, vs. vehicle, $p > 0.05$, Fig. 4A and B). Next, in order to test the effect of Ucn2 on CRF-induced stimulation of contractions, Ucn2 or vehicle was added to the organ bath, 10 min prior to application of CRF or vehicle (Fig. 4C). Ucn2 itself did not modify but CRF increased the contractions ($F = 11.0$, $p < 0.05$, 96.1 ± 3.5 % for Ucn2 + vehicle, $n = 6$, vs. 100.0 ± 7.1 % for vehicle + vehicle, $n = 5$, $p > 0.05$, 138.9 ± 8.3 % for vehicle + CRF, $n = 9$, vs. vehicle + vehicle, $p < 0.05$). Whereas, Ucn2 blocked the CRF-induced stimulation (99.1 ± 4.4 % for Ucn2 + CRF, $n = 13$, vs. vehicle + CRF, $p < 0.05$).

VMR in response to CRD

Apparent abdominal muscle contractions were detected by EMG in response to CRD (Fig. 5A). VMR during the second CRD was significantly enhanced as compared with that of the first CRD (75.8 ± 7.8 μV×min for the first CRD, vs. 86.6 ± 9.3 μV×min for the second CRD, $n = 25$, $p < 0.05$, Fig. 5B), which is consistent with the previous reports (14,15,21).

Next, in order to determine whether the CRD-induced visceral sensitization is mediated through peripheral CRF receptors, the effect of astressin was tested. Ip astressin at a dose of 200 μg/kg immediately after the first CRD blocked this enhanced VMR. On the other hand, astressin$_2$-B (200 μg/kg) did not modify it (Table 2).

Next, we tested the effect of CRF on this enhanced VMR. Ip CRF (60 μg/kg) did not display any significant effect, but Ucn2 (60 μg/kg) or cortagine (60 μg/kg) blocked or further enhanced this response, respectively (Table 2).

Finally, we tested the effect of CRF under the condition with blocking CRF2 signaling by astressin$_2$-B. Vehicle or astressin$_2$-B (200 μg/kg) was injected at the end of the first CRD and 10

min later, vehicle or CRF (60 μg/kg) was administered. The second CRD was submitted 30 min later from the second injection. Astressin$_2$-B or CRF itself did not any significant effect (% change in VMR, 123.2 ± 2.3 % for astressin$_2$-B + vehicle, n = 5, 112.8 ± 6.1 % for vehicle + CRF, n = 6, vs. 127.1 ± 4.3 % for vehicle + vehicle, n = 12, $p > 0.05$), but CRF together with astressin$_2$-B induced significantly higher VMR change as compared with that of vehicle + vehicle or vehicle + CRF-treated group ($F = 4.7$, $p < 0.05$, 150.7 ± 12.2 % for astressin$_2$-B + CRF, n = 6, vs. vehicle + vehicle, vehicle + CRF, $p < 0.05$, Fig. 5C).

Discussion

The present study clearly showed that the actions of peripheral CRF receptors and provides the new insight regarding the signaling balance of each CRF receptor subtype on the regulation of functional colonic changes induced by ip CRF or CRD. Briefly, CRF1 signaling is the main force to activate the colonic functions, such as motility and sensation. CRF2 signaling plays a modulatory role in the intensity of the CRF1 signaling, therefore contributing to the regulation of colonic functions (Fig. 6).

In the colonic motility study, there has been one report suggesting the validity of our proposed hypothesis as follows. Gourcerol et al. showed that ip Ucn2 inhibited and astressin$_2$-B further enhanced ip CRF-induced stimulation of defecation in rats (13). However, the acceleration of colonic transit in response to restraint stress and central administration of CRF was reported to not always correlate with an increase in fecal pellet output (22), suggesting that fecal output study may not be adequate for testing the effect of CRF on colonic motility. Therefore, we manometrically measured intraluminal colonic pressure waves in the present study, which seems

292 more directly to reflect the colonic motor activity.

293 Previous studies using EMG or strain gauge demonstrated that peripheral administration of
294 CRF stimulated colonic motor activity in rats (23,24). The present study reconfirmed this
295 stimulatory action of peripheral CRF by the perfused manometry and it was completely blocked by
296 ip astressin, which has poor penetrance into brain (25). Moreover, we also demonstrated that CRF
297 stimulated colonic muscle strips contractions in vitro. These results suggest that ip CRF stimulates
298 colonic contractions through peripheral CRF receptors.

299 Our manometric study showed that astressin itself did not change colonic contractions,
300 indicating that peripheral CRF signaling does not contribute to the basal colonic contractility. Ip
301 cortagine significantly stimulated the contractions, which reconfirmed the known fact that
302 peripheral CRF stimulates colonic motility through CRF1 (2). Since Ucn2 or astressin$_2$-B itself did
303 not change the basal contractions, CRF2 signaling alone does not regulate the colonic contractility.
304 Meanwhile, astressin$_2$-B further enhanced ip CRF-induced stimulation of contractions. These
305 results may support our proposed hypothesis because of the following explanations. Colonic
306 contractility may be determined by the state of the intensity of CRF1 signaling. CRF2 signaling
307 may be involved in the CRF1-triggered colonic contractility by modulation of CRF1 activity. In
308 basal condition, both CRF signaling are not activated, and CRF2 agonist/antagonist by itself does
309 not change colonic contractility because of a lack of activation of CRF1 signaling. CRF activates
310 both CRF1 and CRF2, and it has been reported that CRF has a much higher affinity for CRF1
311 compared to that for CRF2 (9-11). Therefore, CRF induces strong activation of CRF1 signaling
312 prevailing over the inhibition by CRF2 signaling, leading to stimulation of colonic contractility.
313 CRF2 antagonist blocks the inhibition of CRF1 signaling by CRF through CRF2, thereby further
314 enhancing the stimulatory action of CRF.

315 Several recent studies indicated that peripheral CRF1 signaling displays a significant

316 contribution to stress-related altered visceral sensation. It was shown that water avoidance stress
317 (WAS)-induced visceral hyperalgesia was prevented by sc astressin (26). In the present study, CRD
318 induced visceral hypersensitivity, which is consistent with the previous studies (15,27), and it was
319 prevented by ip astressin, suggesting that this response is mediated through peripheral CRF
320 receptors. Moreover, ip cortagine further enhanced but Ucn2 suppressed this CRD-induced
321 sensitization. These results imply that CRD may activate peripheral CRF1 inducing visceral
322 sensitization, and activation of CRF2 may inhibit the CRF1-triggered sensitization.

323 Next, we evaluated the effect of CRF and astressin$_2$-B. Interestingly, neither CRF nor
324 astressin$_2$-B itself induced significant effect on VMR, but astressin$_2$-B together with CRF
325 significantly enhanced the sensitization. These results may support the validity of our proposed
326 hypothesis by following explanations. CRD may activate peripheral CRF1 and induce CRF1-
327 dependent visceral sensitization. When exogenous CRF is administered in this condition, both
328 signaling of CRF receptor subtypes are activated simultaneously and increases the signal intensity
329 in addition to the one induced by CRD. Although CRF has higher affinity for CRF1 (9-11),
330 activating CRF2 by ip CRF may be enough to suppress the intensity of CRF1 signaling in
331 modulation of visceral sensation, resulting that an overall response by exogenous CRF is not
332 remarkable. Meanwhile, blocking CRF2 by astressin$_2$-B disinhibits CRF1 signaling, consequently,
333 CRF1-dependent pure stimulatory action induced by CRF can be observed.

334 Our theory may be supported by the several results from the studies using CRF1 or 2
335 deletion mice. There is evidence that VMR to CRD is prevented in CRF1 deletion mice (28), and
336 exaggerated colonic contractions and defecation response to acute partial restraint stress or ip CRF
337 are observed in CRF2 deletion mice (13).

338 Whereas, we demonstrated several inconsistent results with the hypothesis. The first, Ucn2
339 did not inhibit the CRF-induced stimulation of colonic contractions. CRF2 signaling would inhibit

the CRF1-triggered stimulation and this response was observed in our previous study with gastric contractions indeed (12), which is conflict result between gastric and colonic contractility. The reason of this discrepancy may be explained by the difference of dominant CRF receptor subtype signaling. In the rat stomach, CRF1 is less abundantly expressed as compared to CRF2 (29), suggesting CRF2 signaling is the dominant. On the other hand, the fact of the predominant expression of functional CRF1 relative to CRF2 in colonic myenteric neurons in guinea-pig suggests that CRF1 is the dominant signaling in colon (30). The dominant CRF1 signaling in colon may lead to induce strong activation of CRF1 by CRF, and consequently, Ucn2 could not suppress it in contrast to stomach. Meanwhile, our in vitro study showed that Ucn2 blocked CRF-induced enhanced contractions of colonic muscle strips, which is in conflict with the result by manometry. The discrepancy may come from the difference of experimental conditions, such as denervated or innervated organs. In anyway, the in vitro results may further support our proposed theory.

The next, astressin$_2$-B did not modify CRD-induced sensitization, which is consistent with the previous report (15) but in conflict with the hypothesis. The blocking CRF2 would further enhance CRF1 signaling activated by CRD and augment the sensitization. Stress activates CRF signaling (31), but the activation balance of CRF1 and 2 signaling may vary depending on the nature of loaded stress. WAS stimulates defecation, which is mediated through activating CRF1 in rats (2). Moreover, we previously demonstrated that this stress enhanced gastric contractions without altering GE, possibly mediated through peripheral CRF1 (32), suggesting that WAS may exclusively stimulate CRF1 signaling. Meanwhile, restraint stress stimulates defecation and delays GE through simultaneously activating CRF1 and CRF2 (16,33,34). Judging from these above results, it seems reasonable to think that CRD may activate exclusively CRF1 signaling, which may explain the discrepancy. The activity balance of each CRF receptor subtype signaling during stress may depend on the released peptides such as CRF and Ucns, and their relative affinity for CRF

receptors. It was also reported that CRF receptors were recruited or eliminated by acute stress such as open field stress and CRD in rat colon, and the expression profile of CRF1 and 2 was dependent on the stress sensitivity of the animals and the nature of loaded stress (35). On the basis of this evidence, it is quite likely that altered expression profile of CRF receptors induced by stress may also contribute to determine the activity balance of the signaling.

Several studies demonstrated the possible action sites of peripheral CRF on colonic motility. Ip CRF induces colonic myenteric Fos expression through peripheral CRF1 and the nearly all Fos expressing cells are CRF1 immunoreactive (36). Moreover, Fos activation by ip CRF is correlated with increased defecation (36). Whereas, CRF2 stimulation inhibits ip CRF-induced Fos activation and blockade of CRF2 enhances Fos response (13). These results strongly suggest that the site of action of peripheral CRF and possible target for CRF1 and 2 interaction on colonic motility are myenteric neurons. Our in vitro results, i.e., blockade of CRF-induced enhanced contractions of muscle strips by Ucn2 is also consistent with the above speculation.

Stress-induced altered colonic motility is mediated through peripheral serotonin pathway (37), and serotonin signal is thought to contribute to the pathogenesis of irritable bowel syndrome (IBS) (38). Whereas, activating central or peripheral CRF receptors stimulates peripheral serotonin signaling resulting in altered colonic motility (17,37). Kimura et al. demonstrated that Ucn1/CRF stimulated contractions of colonic muscle strips through stimulation of CRF1 in myenteric plexus and this response was mediated through enhancing serotoninergic neurotransmission (17). Therefore, CRF1 and 2 interaction occurred in colonic myenteric neurons may modulate the serotoninergic neuron activity of colonic enteric nervous system, thereby altering colonic motility.

The mechanisms of this modulatory action by CRF2 in colonic motility have not been determined definitely. Liu et al. (30) demonstrated in myenteric plexus of guinea pig colon that CRF1 was mainly expressed in ganglion cell somas and CRF2 was expressed in varicose nerve

388 fibers. CRF1 and 2 evoked depolarization of different types of myenteric neurons. In addition, only
389 small population of CRF1 positive neurons expressed CRF2. Meanwhile, they also suggested
390 immunohistochmically that CRF2 might be expressed at pre-synaptic transmitter release sites.
391 Therefore, it is possible to think that CRF2 might regulate a neurotransmitter release, thereby
392 modulating the neuronal activity induced by CRF1.

393 Suggestive evidence demonstrating the target for CRF1 and 2 interaction on modulating
394 visceral sensation is poor. However, CRF2 is proved to be expressed in dorsal root ganglia,
395 and CRD induces activation of splanchnic afferents in in vitro experiment using colorectal
396 preparation with the attached mesenteric artery and splanchnic afferent nerve, which is blunted by
397 intra-arterial injection of Ucn2 (15). In this context, CRF may modulate visceral sensation
398 through CRF receptors on spinal afferents directly.

399 Recent studies suggest that enterochromaffin (EC) cells are target of peripheral CRF. BON
400 cells which are EC-like cell line, express CRF1 and 2 (39), and release serotonin through activating
401 CRF1 (40). Luminally released serotonin from EC cells activates mucosal 5-HT_3 receptors located
402 on the vagal afferents, which stimulates colonic motility via the vagovagal reflex (41). Whereas,
403 serotonin from EC cells is also thought to contribute to visceral hypersensitivity through activating
404 spinal afferents (42). In this context, CRF1 and 2 interaction may also occur at EC cells in
405 modulating both colonic motility and sensation.

406 It became certain that mast cells of GI tract also play an important role in stress-induced
407 visceral sensitization (43). Mast cells contain and release a large variety of mediators such as
408 serotonin, prostaglandins and cytokines in response to various stimuli, and these mediators may
409 contribute to stress-induced visceral hypersensitivity (44,45). Mast cells have both CRF1 and 2 at
410 their surface (46,47) and their degranulation is triggered by peripheral CRF in GI tract (48).
411 Therefore, it seemed reasonable to think that both CRF receptor subtypes signaling may also

412 interact at mast cells level and modulate visceral sensation.

413 In EC and mast cells, interaction of CRF1 and 2 signaling might occur in cellular level.
414 Gourcerol et al. speculated that CRF2 activation may share intracellular signaling targets of CRF1,
415 leading to inhibit CRF1 signaling (13).

416 Neurokinin A (NKA) and NKB bind the three NK receptors (NKR) such as NK1R, NK2R
417 and NK3R with different affinity. These receptors are G protein-coupled receptors, which are
418 coexpressed in enteric neurons (49), similar to CRF receptors. Activation of one receptor could
419 trigger processes that regulate the same or a different receptor, which is known phenomenon as
420 homologous or heterologous desensitization, respectively (50). Activation of the NK1R causes
421 heterologous desensitization of the NK3R but not *vice versa* in enteric neurons (51). These lines of
422 evidence also raise the possibility that CRF2 activation might desensitize CRF1, thereby reducing
423 CRF1 signal intensity.

424 Our study has several limitations. CRF is thought not to penetrate to the brain because of
425 blood-brain barrier (52). Whereas, there is a study revealing that peripherally administered Ucn2
426 reaches brain parenchyma at a moderate rate which is not similar to CRF (53). Therefore, we could
427 not completely deny the possibility that effect of ip Ucn2 is mediated through not only peripheral
428 but central CRF receptors. Since we only examined the proximal colonic contractility, it is not clear
429 that our theory is also applicable in distal colonic motility. There is a report suggesting the
430 difference of CRF1 and 2 profile between proximal and distal colon in rats (35), therefore,
431 responsiveness to CRF-related peptides may be different between proximal and distal colon. Further
432 studies are needed.

433 Abnormal colonic motility and visceral hypersensitivity play an important role in the
434 pathogenesis of IBS, particularly diarrhea-predominant type (54). The CRF1 signaling possibly
435 contributes to IBS symptoms (54), but according to our results, this issue may be interpreted that the

436 CRF1 and 2 signaling balance is abnormally shifted to CRF1 in IBS. In this context, in addition to
437 CRF1 antagonist, CRF2 agonist is thought to be promising tool in treating IBS by resetting CRF1
438 and 2 signaling balance.

439 In summary, we demonstrated that peripheral CRF1 signaling enhanced colonic
440 contractility and induced visceral sensitization, and these responses were modulated by peripheral
441 CRF2 signaling. Both CRF receptor subtypes were activated simultaneously and the activity
442 balance of each subtype signaling may determine the functional changes in response to ip CRF or
443 CRD. These new findings contribute to further understanding the mechanisms of stress-related
444 alterations of colonic motor and sensory functions.

445

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451

References

1. **Taché Y, Brunnhuber S.** From Hans Selye's discovery of biological stress to the identification of corticotropin-releasing factor signaling pathways: implication in stress-related functional bowel diseases. *Ann N Y Acad Sci.* 2008;1148:29-41.
2. **Taché Y, Perdue MH.** Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. *Neurogastroenterol Motil.* 2004;16 Suppl 1:137-142.
3. **Martínez V, Wang L, Million M, Rivier J, Taché Y.** Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides.* 2004;25:1733-1744.
4. **Fekete EM, Zorrilla EP.** Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol.* 2007;28:1-27.
5. **Hillhouse EW, Grammatopoulos DK.** The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. *Endocr Rev.* 2006;27:260-286.
6. **Perrin MH, Vale WW.** Corticotropin releasing factor receptors and their ligand family. *Ann N Y Acad Sci.* 1999;885:312-328.
7. **Taché Y, Martínez V, Wang L, Million M.** CRF_1 receptor signaling pathways are involved in stress-related alterations of colonic function and viscerosensitivity: implications for irritable bowel syndrome. *Br J Pharmacol.* 2004;141:1321-1330.
8. **Nozu T, Martínez V, Rivier J, Taché Y.** Peripheral urocortin delays gastric emptying: role of CRF receptor 2. *Am J Physiol Gastrointest Liver Physiol.* 1999;276:G867-874.
9. **Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW.**

Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A.* 2001;98:7570-7575.

10. **Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE.** Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A.* 2001;98:2843-2848.

11. **Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W.** Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature.* 1995;378:287-292.

12. **Nozu T, Tsuchiya Y, Kumei S, Takakusaki K, Okumura T.** Peripheral corticotropin-releasing factor (CRF) induces stimulation of gastric contractions in freely moving conscious rats: role of CRF receptor types 1 and 2. *Neurogastroenterol Motil.* 2013;25:190-197.

13. **Gourcerol G, Wu SV, Yuan PQ, Pham H, Miampamba M, Larauche M, Sanders P, Amano T, Mulak A, Im E, Pothoulakis C, Rivier J, Taché Y, Million M.** Activation of corticotropin-releasing factor receptor 2 mediates the colonic motor coping response to acute stress in rodents. *Gastroenterology.* 2011;140:1586-1596 e1586.

14. **Million M, Maillot C, Adelson DA, Nozu T, Gauthier A, Rivier J, Chrousos GP, Bayati A, Mattsson H, Taché Y.** Peripheral injection of sauvagine prevents repeated colorectal distension-induced visceral pain in female rats. *Peptides.* 2005;26:1188-1195.

15. **Million M, Wang L, Wang Y, Adelson DW, Yuan PQ, Maillot C, Coutinho SV, McRoberts JA, Bayati A, Mattsson H, Wu V, Wei JY, Rivier J, Vale W, Mayer EA,**

Taché Y. CRF_2 receptor activation prevents colorectal distension induced visceral pain and spinal ERK1/2 phosphorylation in rats. *Gut.* 2006;55:172-181.

16. **Million M, Maillot C, Saunders P, Rivier J, Vale W, Taché Y.** Human urocortin II, a new CRF-related peptide, displays selective CRF_2-mediated action on gastric transit in rats. *Am J Physiol Gastrointest Liver Physiol.* 2002;282:G34-40.

17. **Kimura T, Amano T, Uehara H, Ariga H, Ishida T, Torii A, Tajiri H, Matsueda K, Yamato S.** Urocortin I is present in the enteric nervous system and exerts an excitatory effect via cholinergic and serotonergic pathways in the rat colon. *Am J Physiol Gastrointest Liver Physiol.* 2007;293:G903-910.

18. **Nozu T, Kumei S, Takakusaki K, Ataka K, Fujimiya M, Okumura T.** Central orexin-A increases colonic motility in conscious rats. *Neurosci Lett.* 2011;498:143-146.

19. **Ataka K, Kuge T, Fujino K, Takahashi T, Fujimiya M.** Wood creosote prevents CRF-induced motility via 5-HT_3 receptors in proximal and 5-HT_4 receptors in distal colon in rats. *Auton Neurosci.* 2007;133:136-145.

20. **Ness TJ, Gebhart GF.** Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat. *Brain Res.* 1988;450:153-169.

21. **Gaudreau GA, Plourde V.** Involvement of N-methyl-d-aspartate (NMDA) receptors in a rat model of visceral hypersensitivity. *Behav Brain Res.* 2004;150:185-189.

22. **Nakade Y, Mantyh C, Pappas TN, Takahashi T.** Fecal pellet output does not always correlate with colonic transit in response to restraint stress and corticotropin-releasing factor in rats. *J Gastroenterol.* 2007;42:279-282.

23. **Maillot C, Million M, Wei JY, Gauthier A, Taché Y.** Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats.

Gastroenterology. 2000;119:1569-1579.

24. **Tsukamoto K, Nakade Y, Mantyh C, Ludwig K, Pappas TN, Takahashi T.** Peripherally administered CRF stimulates colonic motility via central CRF receptors and vagal pathways in conscious rats. *Am J Physiol Regul Integr Comp Physiol.* 2006;290:R1537-1541.

25. **Stengel A, Taché Y.** Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. *Annu Rev Physiol.* 2009;71:219-239.

26. **Larauche M, Bradesi S, Million M, McLean P, Taché Y, Mayer EA, McRoberts JA.** Corticotropin-releasing factor type 1 receptors mediate the visceral hyperalgesia induced by repeated psychological stress in rats. *Am J Physiol Gastrointest Liver Physiol.* 2008;294:G1033-1040.

27. **Million M, Zhao JF, Luckey A, Czimmer J, Maynard GD, Kehne J, Hoffman DC, Taché Y.** The newly developed CRF1-receptor antagonists, NGD 98-2 and NGD 9002, suppress acute stress-induced stimulation of colonic motor function and visceral hypersensitivity in rats. *PLoS One.* 2013;8:e73749.

28. **Trimble N, Johnson AC, Foster A, Greenwood-van Meerveld B.** Corticotropin-releasing factor receptor 1-deficient mice show decreased anxiety and colonic sensitivity. *Neurogastroenterol Motil.* 2007;19:754-760.

29. **Yuan PQ, Wu SV, Taché Y.** Urocortins and CRF type 2 receptor isoforms expression in the rat stomach are regulated by endotoxin: role in the modulation of delayed gastric emptying. *Am J Physiol Gastrointest Liver Physiol.* 2012;303:G20-31.

30. **Liu S, Ren W, Qu MH, Bishop GA, Wang GD, Wang XY, Xia Y, Wood JD.** Differential actions of urocortins on neurons of the myenteric division of the enteric nervous system in guinea pig distal colon. *Br J Pharmacol.* 2010;159:222-236.

31. **Taché Y, Bonaz B.** Corticotropin-releasing factor receptors and stress-related alterations of

548 gut motor function. *J Clin Invest.* 2007;117:33-40.
549 32. **Nozu T, Kumei S, Takakusaki K, Okumura T.** Water-avoidance stress enhances gastric
550 contractions in freely moving conscious rats: role of peripheral CRF receptors. *J*
551 *Gastroenterol.* 2013;49:7.
552 33. **Nakade Y, Tsuchida D, Fukuda H, Iwa M, Pappas TN, Takahashi T.** Restraint stress
553 delays solid gastric emptying via a central CRF and peripheral sympathetic neuron in rats.
554 *Am J Physiol Regul Integr Comp Physiol.* 2005;288:R427-432.
555 34. **Martínez V, Wang L, Rivier J, Grigoriadis D, Taché Y.** Central CRF, urocortins and
556 stress increase colonic transit via CRF_1 receptors while activation of CRF_2 receptors delays
557 gastric transit in mice. *J Physiol.* 2004;556:221-234.
558 35. **O'malley D, Julio-Pieper M, Gibney SM, Gosselin RD, Dinan TG, Cryan JF.**
559 Differential stress-induced alterations of colonic corticotropin-releasing factor receptors in
560 the Wistar Kyoto rat. *Neurogastroenterol Motil.* 2010;22:301-311.
561 36. **Yuan PQ, Million M, Wu SV, Rivier J, Taché Y.** Peripheral corticotropin releasing factor
562 (CRF) and a novel CRF_1 receptor agonist, $stressin_1$-A activate CRF_1 receptor expressing
563 cholinergic and nitrergic myenteric neurons selectively in the colon of conscious rats.
564 *Neurogastroenterol Motil.* 2007;19:923-936.
565 37. **Nakade Y, Fukuda H, Iwa M, Tsukamoto K, Yanagi H, Yamamura T, Mantyh C,**
566 **Pappas TN, Takahashi T.** Restraint stress stimulates colonic motility via central
567 corticotropin-releasing factor and peripheral $5\text{-}HT_3$ receptors in conscious rats. *Am J*
568 *Physiol Gastrointest Liver Physiol.* 2007;292:G1037-1044.
569 38. **Stasi C, Bellini M, Bassotti G, Blandizzi C, Milani S.** Serotonin receptors and their role in
570 the pathophysiology and therapy of irritable bowel syndrome. *Tech Coloproctol.*
571 2014;18:613-621.

39. **von Mentzer B, Murata Y, Ahlstedt I, Lindstrom E, Martínez V.** Functional CRF receptors in BON cells stimulate serotonin release. *Biochem Pharmacol.* 2007;73:805-813.

40. **Wu SV, Yuan PQ, Lai J, Wong K, Chen MC, Ohning GV, Taché Y.** Activation of Type 1 CRH receptor isoforms induces serotonin release from human carcinoid BON-1N cells: an enterochromaffin cell model. *Endocrinology.* 2011;152:126-137.

41. **Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T.** Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *Am J Physiol Regul Integr Comp Physiol.* 2003;284:R1269-1276.

42. **Mawe GM, Coates MD, Moses PL.** Review article: intestinal serotonin signalling in irritable bowel syndrome. *Aliment Pharmacol Ther.* 2006;23:1067-1076.

43. **van den Wijngaard RM, Klooker TK, de Jonge WJ, Boeckxstaens GE.** Peripheral relays in stress-induced activation of visceral afferents in the gut. *Auton Neurosci.* 2010;153:99-105.

44. **Gue M, Del Rio-Lacheze C, Eutamene H, Theodorou V, Fioramonti J, Bueno L.** Stress-induced visceral hypersensitivity to rectal distension in rats: role of CRF and mast cells. *Neurogastroenterol Motil.* 1997;9:271-279.

45. **Larauche M.** Novel insights in the role of peripheral corticotropin-releasing factor and mast cells in stress-induced visceral hypersensitivity. *Neurogastroenterol Motil.* 2012;24:201-205.

46. **Theoharides TC, Donelan JM, Papadopoulou N, Cao J, Kempuraj D, Conti P.** Mast cells as targets of corticotropin-releasing factor and related peptides. *Trends Pharmacol Sci.* 2004;25:563-568.

47. **Wallon C, Yang PC, Keita AV, Ericson AC, McKay DM, Sherman PM, Perdue MH,**

Soderholm JD. Corticotropin-releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro. *Gut.* 2008;57:50-58.

48. **Overman EL, Rivier JE, Moeser AJ.** CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. *PLoS One.* 2012;7:e39935.

49. **Grady EF, Baluk P, Bohm S, Gamp PD, Wong H, Payan DG, Ansel J, Portbury AL, Furness JB, McDonald DM, Bunnett NW.** Characterization of antisera specific to NK1, NK2, and NK3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. *J Neurosci.* 1996;16:6975-6986.

50. **Bohm SK, Grady EF, Bunnett NW.** Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J.* 1997;322:1-18.

51. **Frieling T, Dobreva G, Weber E, Becker K, Rupprecht C, Neunlist M, Schemann M.** Different tachykinin receptors mediate chloride secretion in the distal colon through activation of submucosal neurones. *Naunyn Schmiedebergs Arch Pharmacol.* 1999;359:71-79.

52. **Nijsen M, Ongenae N, Meulemans A, Coulie B.** Divergent role for CRF_1 and CRF_2 receptors in the modulation of visceral pain. *Neurogastroenterol Motil.* 2005;17:423-432.

53. **Kastin AJ, Akerstrom V.** Differential interactions of urocortin/corticotropin-releasing hormone peptides with the blood-brain barrier. *Neuroendocrinology.* 2002;75:367-374.

54. **Taché Y, Kiank C, Stengel A.** A role for corticotropin-releasing factor in functional gastrointestinal disorders. *Curr Gastroenterol Rep.* 2009;11:270-277.

Table 1. Effect of CRF receptor agonists on colonic contractions in rats.

	N	Motor index change (%)
Vehicle + Vehicle	7	98.5 ± 11.7
Urocortin 2 (60 μg/kg) + Vehicle	9	98.9 ± 7.3
Vehicle + CRF (60 μg/kg)	5	146.8 ± 24.5 *
Urocortin 2 (60 μg/kg) + CRF (60 μg/kg)	6	144.2 ± 16.3 *
Vehicle	5	93.2 ± 7.9
Cortagine (60 μg/kg)	5	130.9 ± 9.7 #

The motor index change was the % differences of area under the manometric trace of the colon for 1 h before and after drug(s) administration. N; The number of animals. *$p < 0.05$ vs. vehicle + vehicle-treated group. #$p < 0.05$ vs. vehicle-treated group.

625 Table 2. Effect of CRF receptor agonists/antagonists on enhanced visceromotor response (VMR)
626 induced by colorectal distention (CRD) in rats.

	N	VMR change (%)
Vehicle	9	126.7 ± 6.1
Astressin (200 μg/kg)	9	89.2 ± 8.1*
Vehicle	8	122.3 ± 6.9
Astressin$_2$-B (200 μg/kg)	7	119.6 ± 3.5
Vehicle	8	124.7 ± 9.2
CRF (60 μg/kg)	9	111.3 ± 3.2
Vehicle	8	122.6 ± 3.4
Urocortin 2 (60 μg/kg)	7	91.4 ± 5.7*
Vehicle	8	121.4 ± 7.8
Cortagine (60 μg/kg)	6	153.4 ± 12.8*

627

628 VMR was determined by measuring abdominal muscle contractions electrophysiologically. VMR
629 change was the % differences of VMR during the first and the second CRD. N; The number of
630 animals. *$p < 0.05$ vs. vehicle-treated group.

631

Figure Legends

Figure 1.

The effect of intraperitoneal (ip) injection of CRF on colonic contractions. **A**. CRF (30 and 60 μg/kg) increased the motor index significantly. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle-treated group. **B**. Representative recordings.

Figure 2.

The effect of intraperitoneal (ip) astressin (100 μg/kg) on ip CRF (60 μg/kg)-induced stimulation of colonic contractions. **A**. Representative recordings. Pretreatment with ip astressin, 10 min prior to ip CRF, blocked the action of CRF. **B**. Ip astressin blocked the increased motor index induced by CRF. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle + vehicle-treated group. #p < 0.05 vs. vehicle + CRF-treated group.

Figure 3.

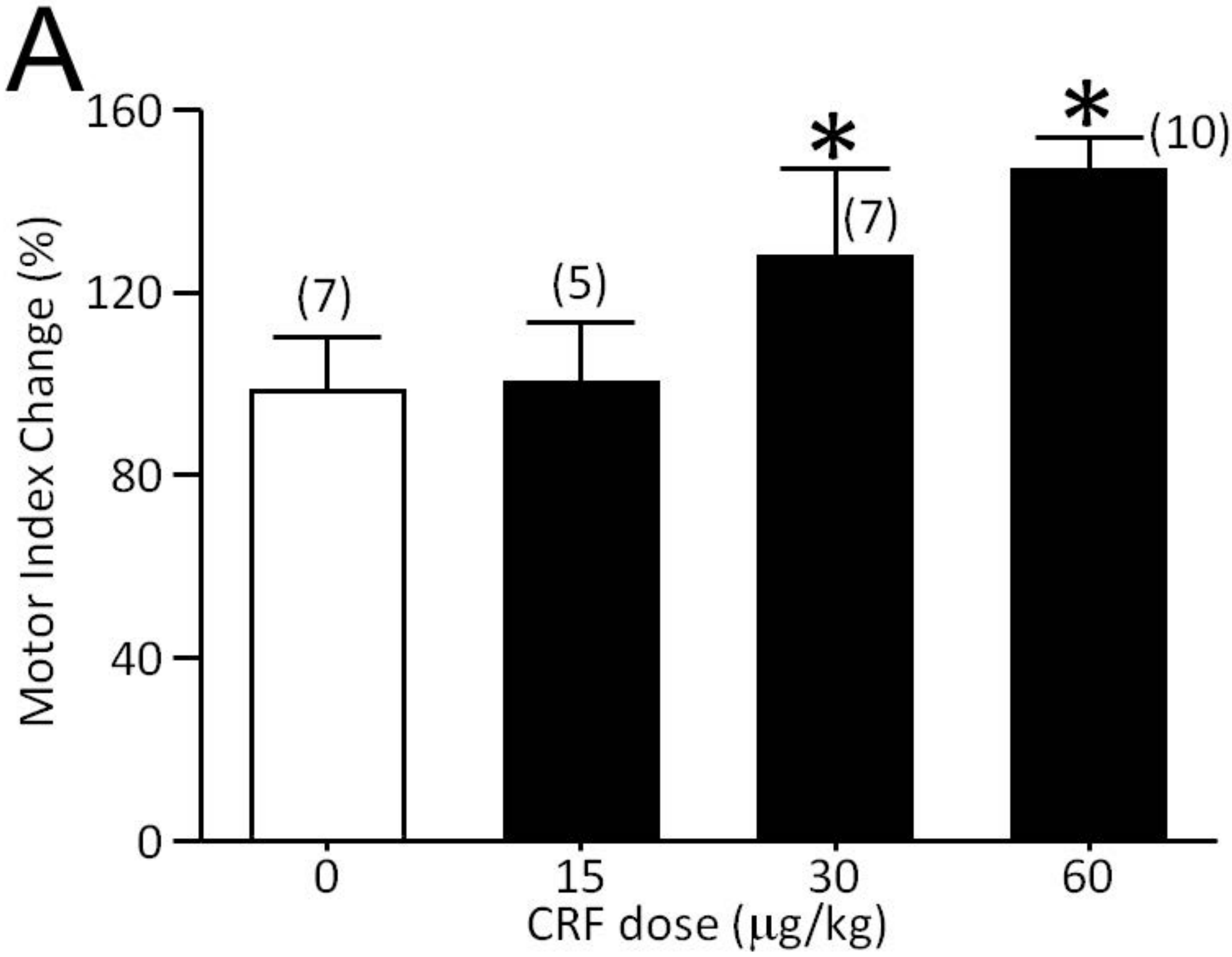
The effect of intraperitoneal (ip) injection of astressin$_2$-B (100 μg/kg) on ip CRF (60 μg/kg)-induced stimulation of colonic contractions. **A**. Representative recordings showing astressin$_2$-B, 10 min prior to ip CRF, significantly enhanced the CRF-induced stimulation. **B**. Astressin$_2$-B itself did not alter the motor index but significantly enhanced the increase induced by CRF. Each column represents the mean ± S.E. Number of rats examined is shown in the parenthesis. *p < 0.05 vs. vehicle + vehicle-treated group. #p < 0.05 vs. vehicle + CRF-treated group.

Figure 4.

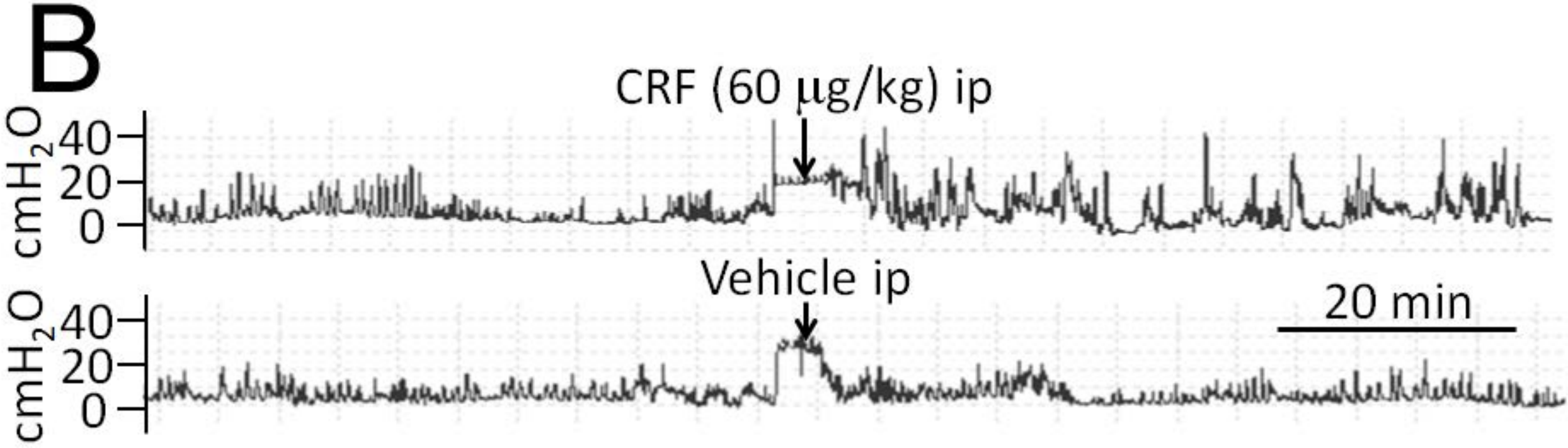
656 The effect of CRF and urocortin 2 on contractions of colonic muscle strips. **A**. Representative
657 recordings. **B**. % change in the amplitude of contractions before and after drug administration.
658 Muscle strips developed spontaneous phasic contractions. CRF (3×10^{-6} M) increased the amplitude
659 of contractions, but urocortin 2 (10^{-6} M) did not modify the contractions. *$p < 0.05$ vs. vehicle-
660 treated group. **C**. The effect of urocortin 2 on CRF-induced stimulation of contractions. Urocortin 2
661 (10^{-6} M), 10 min prior to application of CRF (3×10^{-6} M), abolished the stimulation by CRF. *$p <$
662 0.05 vs. vehicle + vehicle-treated group. #$p < 0.05$ vs. vehicle + CRF-treated group. Each column
663 represents the mean ± S.E. Number of muscle strips examined is shown in the parenthesis.
664
665 Figure 5.
666 The effect of CRF and astressin$_2$-B on visceromotor response (VMR) to colorectal distention
667 (CRD).
668 The rats were submitted to two CRDs at 60 mmHg for 10 min with a 30 min rest interval. The
669 abdominal contractions were electrophysiologically measured and VMR was determined by
670 calculating area under the curve of the trace of electromyogram (EMG). **A**. Apparent abdominal
671 muscle contractions were detected by EMG in response to CRD. **B**. VMR during the second CRD
672 was significantly enhanced as compared with that of the first CRD, indicating that CRD induced
673 visceral sensitization. *$p < 0.05$ vs. the first CRD. **C**. Intraperitoneal astressin$_2$-B (200 μg/kg) or
674 CRF (60 μg/kg) itself did not alter the CRD-induced sensitization, while astressin$_2$-B together with
675 CRF further enhanced the sensitization significantly. *$p < 0.05$ vs. vehicle + vehicle-treated group.
676 #$p < 0.05$ vs. vehicle + CRF-treated group. Each column represents the mean ± S.E. Number of rats
677 examined is shown in the parenthesis.
678
679 Figure 6.

Schematic illustration of our hypothesis on the mechanism of peripheral CRF-induced stimulation of colonic contractions and colorectal distention (CRD)-induced visceral sensitization. CRF1 signaling is the direct force to stimulate colonic motility and sensation. CRF2 plays a regulatory role and inhibits the CRF1 signaling. Both CRF1 and 2 are simultaneously activated during CRD or when CRF is injected, and the activity balance of each subtype signaling may determine the functional colonic changes, i.e., shifting the balance to CRF1 boosts the activity of colonic contractions and sensation. As in the left panel, strong CRF2 signaling is capable of inhibiting the CRF1 signaling at a strong power, leading to a weak stimulation of the CRF1 signaling, followed by a little enhancement of colonic functions. Whereas, as in the right panel, weak CRF2 signaling could not inhibit the CRF1 signaling well, thereby conserving the power of CRF1 signaling, and inducing a strong stimulation of colonic functions. The balance may be determined by the injected or released peptides during CRD such as CRF and urocortins, which display distinct affinity for each CRF receptor, and expression profile of colonic CRF1 and 2 may also contribute to the signal balance. CRF1 and 2; CRF receptor type 1 and 2. ○; CRF1 ligand. ●; CRF2 ligand.

A


160
120
80
40
0
Motor Index Change (%)
(7)
(5)
*
(7)
*
(10)
0
15
30
60
CRF dose (μg/kg)

B


CRF (60 μg/kg) ip
cmH2O
40
20
0
Vehicle ip
20 min

A

Astressin（100 μg/kg）ip

CRF (60 μg/kg) ip

Vehicle ip

CRF (60 μg/kg) ip

20 min

cmH2O

40

20

0

B

Motor Index Change (%)

160

120

80

40

0

(8)

(6)

*

(6)

#

(6)

Vehicle + Vehicle

Astressin + Vehicle

Vehicle + CRF

Astressin + CRF

A

Astressin$_2$-B (100 μg/kg) ip

CRF (60 μg/kg) ip

cmH$_2$O 40 20 0

20 min

Vehicle ip

CRF (60 μg/kg) ip

cmH$_2$O 40 20 0

B

Motor Index Change (%)

200 150 100 50 0

(8) (5) (7) (7)

* #

Vehicle + Vehicle

Astressin$_2$-B + Vehicle

Vehicle + CRF

Astressin$_2$-B + CRF

A

Vehicle

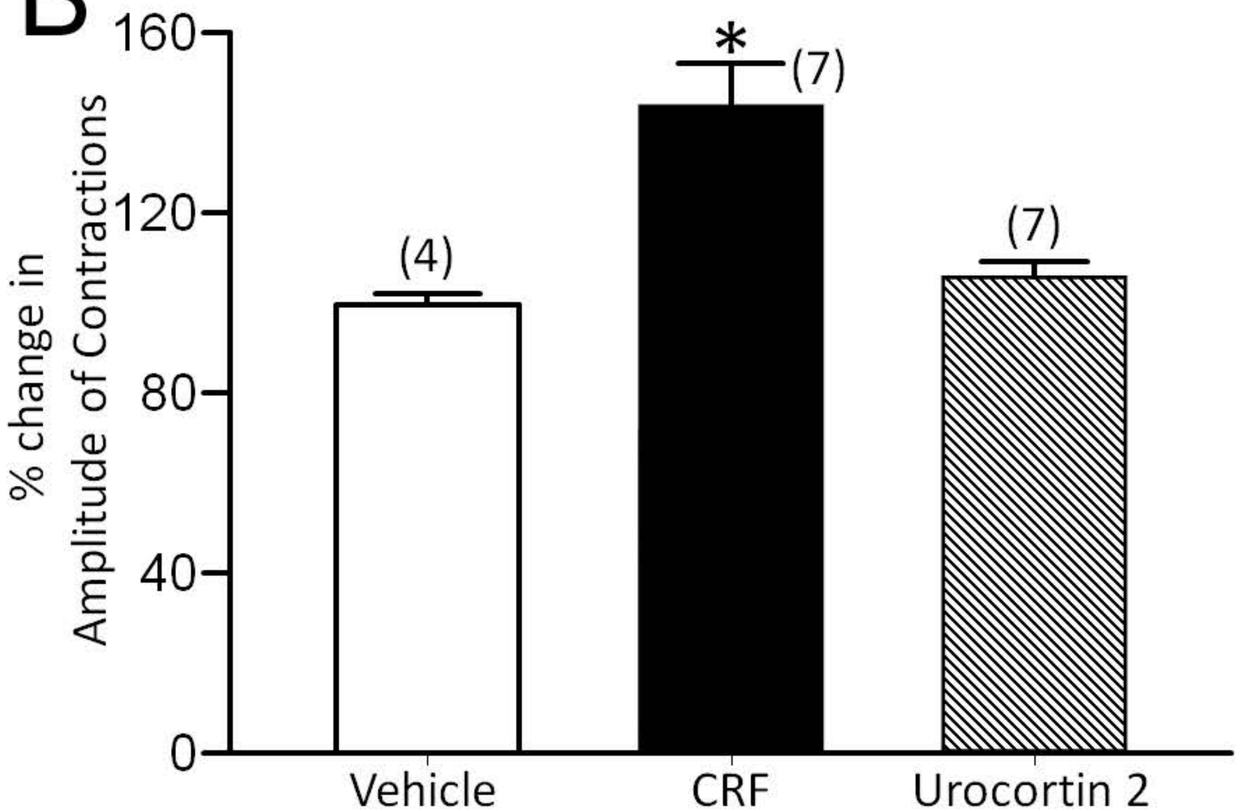
1 min

CRF 3 × 10^{-6} M

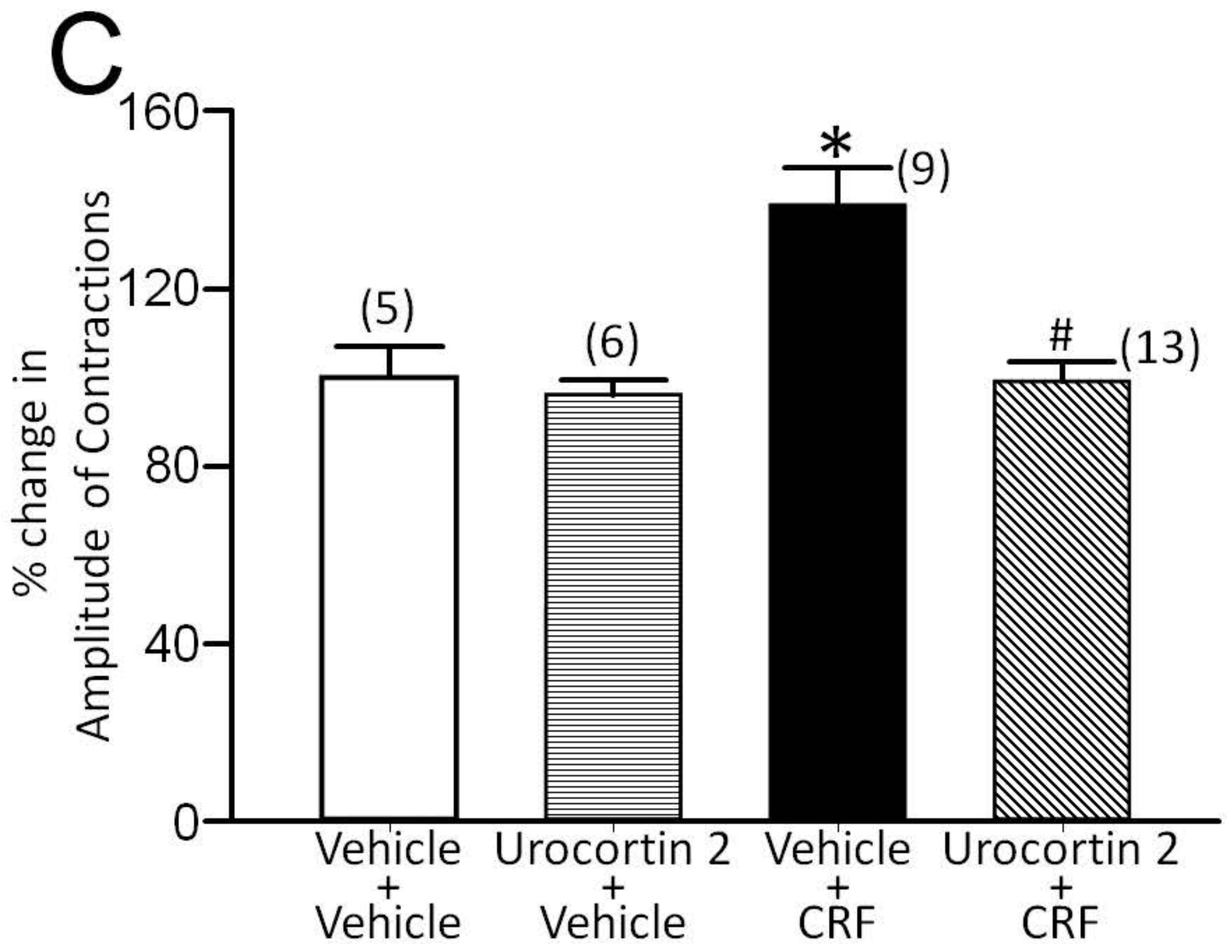
1 g

Urocortin 2 10^{-6} M

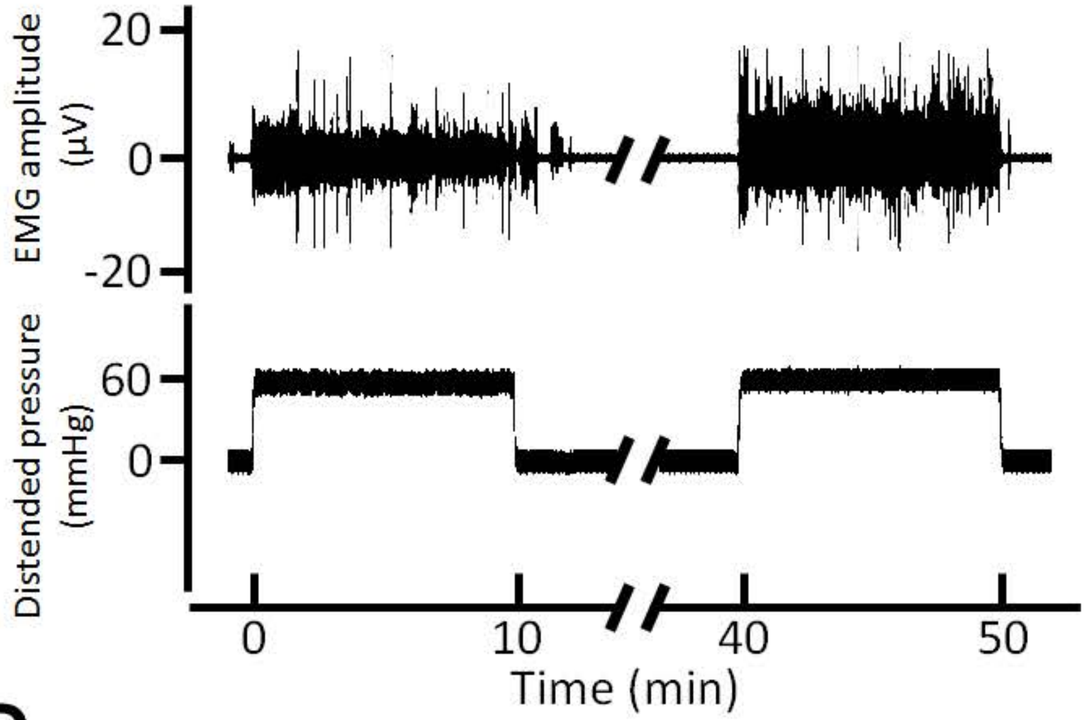
B



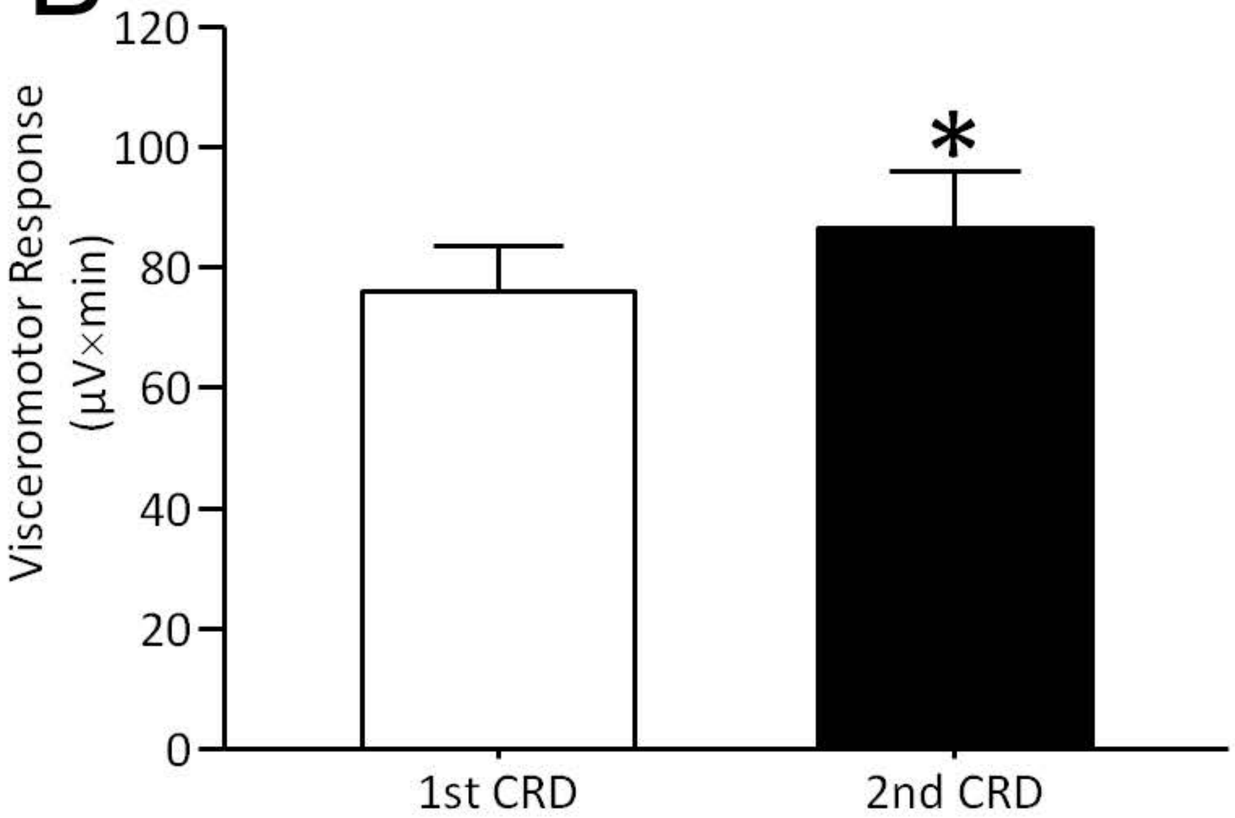
C



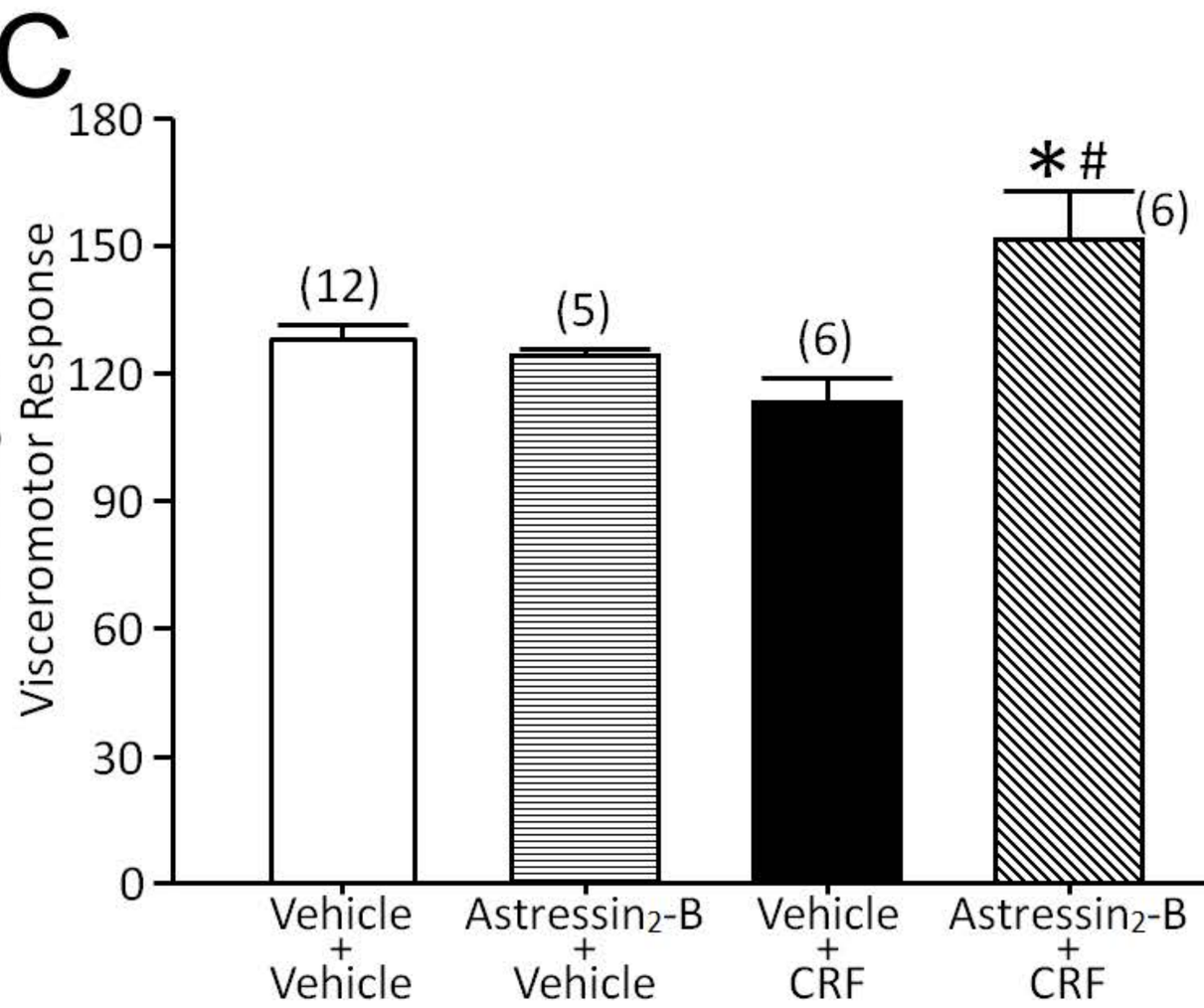
A


EMG amplitude (μV)
20
0
-20
Distended pressure (mmHg)
60
0
0
10
40
50
Time (min)

B


Visceromotor Response (μV×min)
120
100
80
60
40
20
0
*
1st CRD
2nd CRD

C


% change in Visceromotor Response
180
150
120
90
60
30
0
(12)
(5)
(6)
*#
(6)
Vehicle + Vehicle
Astressin2-B + Vehicle
Vehicle + CRF
Astressin2-B + CRF

Colonic contractions
Visceral sensation

CRF1

CRF2

CRF1

CRF2

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	N	Motor index change (%)
Vehicle + Vehicle	7	98.5 ± 11.7
Urocortin 2 (60 μg/kg) + Vehicle	9	98.9 ± 7.3
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Urocortin 2 (60 μg/kg) + CRF (60 μg/kg)	6	144.2 ± 16.3 *
Vehicle	5	93.2 ± 7.9
Cortagine (60 μg/kg)	5	130.9 ± 9.7 #

The motor index change was the % differences of area under the manometric trace of the colon for 1 h before and after drug(s) administration. N; The number of animals. *$p < 0.05$ vs. vehicle + vehicle-treated group. #$p < 0.05$ vs. vehicle-treated group.

Table 2. Effect of CRF receptor agonists/antagonists on enhanced visceromotor response (VMR) induced by colorectal distention (CRD) in rats.

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Astressin (200 μg/kg)	9	89.2 ± 8.1*
Vehicle	8	122.3 ± 6.9
Astressin$_2$-B (200 μg/kg)	7	119.6 ± 3.5
Vehicle	8	124.7 ± 9.2
CRF (60 μg/kg)	9	111.3 ± 3.2
Vehicle	8	122.6 ± 3.4
Urocortin 2 (60 μg/kg)	7	91.4 ± 5.7*
Vehicle	8	121.4 ± 7.8
Cortagine (60 μg/kg)	6	153.4 ± 12.8*

VMR was determined by measuring abdominal muscle contractions electrophysiologically. VMR change was the % differences of VMR during the first and the second CRD. N; The number of animals. *$p < 0.05$ vs. vehicle-treated group.