Corticotropin-releasing factor type 1 receptors mediate the visceral hyperalgesia induced by repeated psychological stress in rats

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1Department of Medicine, 2Center for Neurovisceral Sciences and Women’s Health, 3Center for Ulcer Research and Education: Digestive Disease Research Center, 4Departments of Physiology, Psychiatry, and Behavioral Sciences, and 5Brain Research Institute, David Geffen School of Medicine at University of California, Los Angeles; 6Veterans Affairs, Greater Los Angeles Healthcare System, Los Angeles, California; and 7GlaxoSmithKline (Neurology and Gastrointestinal Centre for Excellence for Drug Discovery), Harlow, United Kingdom

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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 294: G1033–G1040, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00507.2007.—Visceral hypersensitivity has been implicated as an important pathophysiological mechanism in functional gastrointestinal disorders. In this study, we investigated whether the sustained visceral hyperalgesia induced by repeated psychological stress in rats involves the activation of CRF1 signaling system using two different antagonists. Male Wistar rats were exposed to 10 consecutive days of water avoidance stress (WAS) or sham stress for 1 h/day, and the visceral motor response to phasic colorectal distension (CRD) was assessed before and after the stress period. Animals were injected subcutaneously with the brain penetrant CRF$_1$ antagonist, CP-154,526, acutely (30 min before the final CRD) or chronically (via osmotic minipump implanted subcutaneously, during stress) or with the peripherally restricted, nonselective CRF$_1$ and CRF$_2$ antagonist, astressin, chronically (15 min before each stress session). Repeated WAS induced visceral hypertenderness to CRD at 40 and 60 mmHg. CP-154,526 injected acutely significantly reduced stress-induced visceral hyperalgesia at 40 mmHg but not at 60 mmHg. Chronic subcutaneous delivery of astressin reduced the stress-induced visceral hyperalgesia to baseline at all distension pressures. Interestingly, chronically administered CP-154,526 eliminated hyperalgesia and produced responses below baseline at 40 mmHg and 60 mmHg, indicating a hypoalgesic effect of the compound. These data support a major role for CRF$_1$ in both the development and maintenance of visceral hyperalgesia induced by repeated stress and indicate a possible role of peripheral CRF receptors in such mechanisms.

Visceral nociception; astressin; CP-154,526; water avoidance

IRRITABLE BOWEL SYNDROME (IBS) is a common functional bowel disorder in which recurrent abdominal pain or discomfort occurs in the absence of any detectable organic abnormalities (48). Symptom-related anxiety and comorbidity with anxiety disorders or depression are common in patients with IBS (29, 56). In addition, many studies have highlighted the importance of psychological stress in the onset (13), maintenance, and exacerbation of IBS symptoms (13, 33).

Corticotropin-releasing factor (CRF) is a key mediator in the body’s stress response (58). CRF and the endogenous related peptides, urocortin 1 (Ucn1 or Urocortin), urocortin 2 (Ucn2), and urocortin 3 (Ucn3), exert their biological actions by binding to two CRF receptors, subtype 1 (CRF$_1$) and subtype 2 (CRF$_2$), which have distinct affinity for CRF ligands (21). In rodents, CRF$_1$ is distributed in brain areas involved in affective, stress, and nociceptive circuitries, including the paraventricular nucleus of the hypothalamus (PVN), the locus coeruleus (LC), and the amygdala (9, 46, 47). At the spinal level, in rats, lumbar CRF$_1$ receptors are present in highest concentrations in laminae I and II (3), where visceral primary afferents terminate (1). In the rat and guinea pig colon, CRF$_1$ is essentially found in the colonic mucosa and in the myenteric and submucosal nervous plexi, whereas CRF$_2$ expression is mainly localized in the submucosal/myenteric layers (11, 28, 66).

It is now well established that the brain CRF/CRF$_1$ signaling system modulates pain responses although the exact sites mediating this modulation remain unidentified (25, 38). The first evidence that brain CRF system plays a role in the modulation of visceral pain in rats was shown by the elimination of visceral hyperalgesia to colorectal distention (CRD) induced by acute partial restraint stress by intracerebroventricular (ICV) injection of the nonselective CRF receptor antagonist, α-hCRF$_{9-41}$ (19). Conversely, CRF$_1$-injected ICV mimicked partial restraint stress-induced visceral hyperalgesia to CRD (19). Later on, the specific participation of CRF$_1$ was established using the non-peptide CRF$_1$-selective antagonist, NBI-35965, which, when given peripherally, prevented visceral hypersensitivity occurring immediately after an acute session of water avoidance stress (WAS) in maternally separated Long-Evans rats (34). Subsequently, with the use of peripheral injection of a different non-peptide CRF$_1$-selective antagonist, CP-154,526, it was shown that CRF$_1$ played a role in the development of delayed visceral hyperalgesia induced by WAS in Wistar rats and maternally separated Long-Evans rats (52, 53). Likewise, antalarmin, another selective nonpeptide CRF$_1$ antagonist, injected intraperitoneally, prevented the visceral hypersensitivity induced by repeated tonic CRD in Fisher rats (35) or the ICV injection of CRF, as well as the one occurring spontaneously in a high anxiety strain of rats (18). Since all of these non-peptide CRF$_1$-selective antagonists injected peripherally cross the blood-brain barrier (BBB), they could act at both peripheral and central sites.

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Studies in humans have corroborated a role for CRF1 in visceral hypersensitivity and suggested a potential role of peripheral CRF/CRF1 signaling in the process. Specifically, peripheral injection of ovine CRF, a preferential CRF1 agonist (42), was shown in healthy controls to lower pain thresholds to colonic distension, reproducing the effect of stress (17). Conversely, systemic administration of the nonselective and peripherally restricted CRF receptor antagonist, α-hCRF9-41, was shown to significantly reduce visceral hyperalgesia in IBS diarrhea-predominant patients subjected to colonic electrical stimulation (50).

To date, the evidence for a role of brain CRF/CRF1 signaling system in the modulation of visceral sensitivity in rodents has been based on the study of visceral hypersensitivity in acute models of stress (32). Given that chronic stress is more relevant in stress-related visceral hypersensitivity in humans (4), we aimed to determine the role of CRF1 in a chronic intermittent WAS-related visceral hyperalgesia model that we recently developed and characterized to have high face and construct validity to IBS (7). In addition, in view of the growing evidence of peripheral CRF signaling mechanisms in the colon (66) and the recent clinical evidence of a peripheral role for CRF1 in patients with IBS (50), we determined the central vs. peripheral site of action of CRF1 in chronic intermittent (1-h/day) WAS-related visceral hyperalgesia by using two CRF1 antagonists with different properties: 1) a selective nonpeptide antagonist CP-154,526, which crosses the BBB (22), and 2) since there are no peripherally restricted CRF1 selective antagonists available yet, we used a peripherally acting nonselective peptide antagonist, astressin (20, 49).

**Materials and Methods**

**Animals.** Adult male Wistar rats (200–250 g) were purchased from Harlan (Indianapolis, IN). Animals were kept on a 12-h:12-h light-dark cycle (lights on at 0600). They were housed in pairs and maintained on a standard rodent food diet (Purina rat chow) with a water ad libitum. The animals were allowed to acclimatize to the animal facility for 1 wk before surgery. All protocols were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA (03025-04) and the University of California at Los Angeles (2003-060-02).

**Drugs and chemicals.** The CRF1 antagonist CP-154,526 was a gift from Pfizer (Groton, CT). For acute treatment, the CP-154,526 was dissolved in vehicle (5% DMSO, 5% cremophor, and saline adjusted to pH 2.5 using 5N HCl) and administered subcutaneously at a dose of 32 mg/kg in 0.2 ml. For chronic treatment, the CP-154,526 (20 mg/kg per day) was dissolved in 5% DMSO-5% cremaphor-90% saline, 0.2 ml, n = 11) or CRF1 antagonist, CP-154,526 (32 mg/kg, n = 12) 30 min before the VMR recording (CRD1). From day 1 to day 10, rats were subjected to either WAS or sham WAS (controls). The VMR (CRD2) was recorded 24 h after the last stress or sham stress session. Different groups of animals were used for the three pharmacological studies.

**Effect of acute subcutaneous injection of CP-154,526 after daily WAS on visceral response to CRD.** Two groups of rats were subjected to the first baseline CRD (CRD1) and 24 h later exposed to WAS (1 h/day) for 10 days. On day 11, rats were subjected to a CRD (CRD2) to assess the effect of chronic intermittent WAS on the VMR response. After 1 h of rest, rats were injected subcutaneously with vehicle (5% DMSO-5% cremaphor-90% saline, 0.2 ml, n = 11) or CRF1 antagonist, CP-154,526 (32 mg/kg, n = 12) 30 min before CRD3. All experiments were repeated in a sham WAS group to exclude a possible effect of CP-154,526 per se.

**Effect of chronic CP-154,526 treatment during the daily WAS on visceral response to CRD.** After baseline VMR recording (CRD1), each rat was equipped in the afternoon with one osmotic minipump filled with vehicle (80% PEG 400) or CP-154,526 at a concentration sufficient to deliver a daily dose of 20 mg/kg sc. Two days later, groups were subjected to daily WAS or sham WAS for 10 days, and,
on the following day, the VMR (CRD2) was recorded. All experiments were repeated in a sham WAS group to exclude a possible effect of CP-154,526 per se.

Effect of repeated subcutaneous injections of astressin before daily WAS on visceral response to CRD. A baseline VMR recording (CRD1) was performed, and, 24 h later, rats were injected subcutaneously daily with astressin (20 μg/kg per day, n = 12) or vehicle (sterile deionized water, 0.2 ml, n = 11) 15 min before each daily session of WAS for 10 days. The VMR (CRD2) was recorded 24 h after the last WAS procedure on day 11. All experiments were repeated in a sham WAS group to exclude a possible effect of astressin per se.

Statistical analysis. Visceral pain data are presented as mean difference ± SED (standard error of the difference), and the statistical significance was analyzed by using two different methods as described below. In all cases, rats with an EMG signal-noise ratio of <0.5 were excluded from the data and statistical analysis. To examine the pressure-response relationship, EMG amplitudes were normalized as percents of the baseline response at the highest pressure (60 mmHg) for each rat and averaged for each group of rats. Such a normalization has generally been used to adjust for interindividual variations of the EMG signal (39). The effects of WAS and/or pharmacological treatments on EMG response to CRD within one group of animals were analyzed by comparing the poststress or posttreatment measurements to the baseline or pretreatment values at each distention pressure by using a repeated measure two-way ANOVA followed by Bonferroni posttest comparisons. We have presented the data showing the EMG response at day 11 for rats treated with CRF receptor antagonists or vehicle as the mean change from baseline for different pressures of distention as validated in our previous studies (7). These data were analyzed with an ANOVA followed by a post hoc t-test with Bonferroni correction for multiple comparisons. The second method of analysis determined the overall effect of stress by calculating the AUC of the raw EMG amplitude as a function of pressure for each animal at different times of testing. The change in overall response after stress with and without different treatments was determined by dividing the AUC values by the baseline value for each rat. The resulting ratios were then averaged for each group of rats. Significance was determined using one-way ANOVA followed by Tukey’s posttest comparisons or as unpaired t-tests as appropriate.

RESULTS

Seventy-two percent of the rats subjected to chronic WAS developed hyperalgesia, i.e., increased VMR over baseline response to graded CRD. The degree of hyperalgesia was evaluated by calculating the AUC of the EMG response to CRD as a function of pressure after WAS and dividing it by the baseline value for each rat. Thirty-three percent exhibited an increase in AUC between 1 and 50% over baseline, 22% showed increased AUC in the range of 51–100%, and 17% had an increase in AUC ≥101%. These changes are similar to those described previously (7).

Acute subcutaneous injection of CRF1 antagonist CP-154,526. As shown in Fig. 1, A and C, daily 1-h exposure to WAS induced a significant increase in VMR to CRD on day 11 (CRD2) compared with baseline (CRD1) at pressures of 40 and 60 mmHg (P < 0.05, n = 9). CP-154,526 (32 mg/kg) injected subcutaneously 24 h after the last stress session abolished the WAS-induced increase in the VMR response at 40 mmHg (ΔEMG response after CP-154,526 vs. baseline: 4.8 ± 13.9 vs. 54.2 ± 23.6 at 40 mmHg for vehicle; P < 0.05) while not significantly decreasing the VMR at 60 mmHg (ΔEMG response from baseline: 34.6 ± 22.6 vs. 59.7 ± 29.8 at 60 mmHg for CP-154,526 vs. vehicle-treated) (Fig. 2A). Injection of vehicle per se had no significant effect on the VMR compared with baseline (Fig. 2A).

To determine the effect of the CRF1 antagonist in control conditions, we tested the response to CP-154,526 or vehicle injection in animals previously subjected to repeated sham
WAS. Compared with baseline VMR responses, repeated exposure to sham WAS had no significant effect on the VMR to CRD (Fig. 1, B and C), and injection of vehicle or CP-154,526 did not change the response to CRD under these conditions (see Fig. 2B). In addition, there was no difference in the EMG response to CRD3 compared with CRD2 after vehicle injection in the WAS group (WAS day 11 vs. WAS + vehicle day 11, Figs. 1C and 2A) or in the sham WAS group (sham WAS day 11 vs. sham WAS + vehicle day 11, Figs. 1C and 2B), indicating that repeated phasic CRD at day 11 does not affect the EMG response after chronic sham WAS compared with vehicle. Data are presented as mean change ± SE of difference, n = 9 in each group.

Chronic subcutaneous administration of CRF1 antagonist CP-154,526. Chronic CP-154,526 (calibrated to give 20 mg/kg per day through an osmotic minipump) abolished the WAS-induced increase of the VMR to CRD (P < 0.05, n = 9–10; Fig. 3A). Chronic subcutaneous astressin reduced the EMG response to a level similar to baseline (ΔEMG response after astressin over baseline vs. vehicle: −9.42 ± 17.11 vs. 50.26 ± 15.20 at 40 mmHg and 15.72 ± 22.25 vs. 66.35 ± 14.90 at 60 mmHg for vehicle). Daily injections with astressin or vehicle had no effect on the VMR of rats subjected to sham WAS (Fig. 3B).

Chronic subcutaneous injection of astressin, a CRF1 and CRF2 peptide antagonist. Repeated subcutaneous injections of astressin (20 μg/kg per day) before each WAS session for 10 days abolished the stress-induced increase of the VMR compared with vehicle (P < 0.05, n = 7–10; Fig. 4A). Chronic subcutaneous astressin reduced the EMG response to a level lower than baseline at 40 and 60 mmHg distention, with a significant effect at 40 mmHg (ΔEMG response after astressin over baseline vs. vehicle: 9.42 ± 17.11 vs. 50.26 ± 15.20 at 40 mmHg and 15.72 ± 22.25 vs. 66.35 ± 14.90 at 60 mmHg for vehicle). Daily injections with astressin or vehicle had no effect on the VMR of rats subjected to sham WAS (Fig. 4B).
intermittent WAS. These data extend further the previous findings on the role of CRF1 receptor signaling in acute stress-related visceral hypersensitivity in rats (19, 34, 52) and provide further evidence for the role of CRF1 in the onset, development and maintenance of stress-induced visceral hypersensitivity following repeated psychological stress in rats. Interestingly, the chronic administration of CP-154,526, but not astressin, induced a small degree of analgesia at the two highest volumes of distention, which was significant at 40 mmHg. One explanation to these different results may be that both central and peripheral CRF1 participate to the visceral hypersensitivity induced by chronic WAS. As CRF2 in the periphery participate to the induction of analgesia (35, 37, 40), the blockade of visceral hypersensitivity by astressin is likely to be mediated by antagonism of peripheral CRF1. In addition, by being able to access the central nervous system, it may be speculated that CP-154,526 abolishes both the central and the peripheral components of the pronociceptive CRF1 pathway, whereas astressin, being restricted to the periphery due to its chemical properties (20, 49), only partially blocks the CRF1 pathway. Alternatively, our results may also indicate that chronic blockade of CRF1 with CP-154,526 unmasks the analgesic influence of CRF2 on visceral nociceptive pathways (35, 37, 40) leading to the development of analgesia, an effect that was not seen with astressin, which blocks both types of receptors.

The involvement of CRF1 in the maintenance of chronic stress-induced visceral hyperalgesia is supported by our observation that acute treatment with CP-154,526 normalized the VMR to CRD at a pressure of 40 mmHg (but not 60 mmHg), both these pressures being over the pain threshold level in healthy human subjects and rats (10, 55). In previous studies with the use of a single stress session, we found that the same dose of CP-154,526 normalized stress-induced visceral hyperalgesia at distention pressures of both 40 and 60 mmHg (2, 40, 52, 53). These data suggest that a chronic intermittent stress may cause upregulation of the CRF1 signaling system either on central and/or peripheral components of visceral afferent pathways mediating visceral pain at a distention pressure of 40 mmHg but less so at 60 mmHg. Hypothesis supported by the fact that chronic CP-154,526 and astressin did not have any significant effect per se in animals submitted to sham WAS, confirming that visceral pain pathways are not modulated by peripheral and/or central CRF1 and CRF2 under basal conditions, but only during stress sensitization as previously suggested (18, 40). However, stress, depending on its time course (acute vs. chronic) or the type of stressor used, affects the regulation of the CRF1 signaling system in a highly variable manner (6, 23, 31, 43, 44). How exactly chronic WAS and the chronic CRF1 antagonist treatment influence the expression of CRF1 at sites involved in the regulation of visceral pain in our model will need to be assessed in further experiments.

From our results, it is interesting to note that independently from a difference in the efficacy of the acute vs. chronic CRF1 antagonism regimens in reducing the VMR to CRD at 40 and/or 60 mmHg, the groups of rats receiving acute vs. chronic CP-154,526 exhibit a higher variability in their visceral response. Even though this variability could be linked to the acidity of the solvent used for acute injections, it could also be due to the greater efficacy of the chronic treatment in reducing visceral hypersensitivity. It is known that in rats CRD activates

**DISCUSSION**

The present study demonstrates that the activation of the CRF1 signaling system plays a major role in both the development and maintenance of chronic WAS-induced hyperalgesia to CRD as investigated in a previously validated rodent model of visceral pain (7). The enhanced VMR to CRD was abolished by acute and chronic peripheral administration of a selective CRF1 antagonist with CNS access, as well as by daily subcutaneous injection, before stress exposure, of a peripherally acting nonselective CRF1 and CRF2 antagonist. The data show that chronic intermittent psychological stress-induced visceral hyperalgesia involves CRF1 receptor activation in the brain but also in the peripheral tissue.

Chronic administration of either the nonselective CRF1 and CRF2 antagonist, astressin, or the selective CRF1 antagonist, CP-154,526 (22), during the entire stress exposure completely eliminated the enhanced VMR to CRD induced by chronic CRF1 antagonism regimens in reducing the VMR to CRD at 40

![Fig. 4. Chronic treatment with astressin. A: EMG amplitude expressed as mean change from baseline after chronic treatment with vehicle or astressin (20 μg/kg per day sc) in rats exposed to repeated WAS. Astressin administered daily 15 min before each stress session abolished the stress-induced enhancement of VMR to CRD at both 40 and 60 mmHg. *P < 0.05 significantly different compared with vehicle, 2-way ANOVA and Bonferroni’s post hoc t-test. B: EMG amplitude expressed as mean change from baseline after chronic treatment with vehicle or astressin (20 μg/kg per day sc) in rats submitted to chronic sham WAS. Daily injection of astressin before the sham WAS session did not affect the EMG response compared with vehicle, both of which were unchanged from the VMR recorded on day 0. Data are expressed as mean change ± SE of difference, n = 7–10 in each group.](http://ajpgi.physiology.org/)

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the LC (14, 59), which receives strong CRF innervation from the pontine Barrington’s nucleus (BN) (61). CRF receptors are present in the LC (45), and activation of LC neurons by low levels of colonic distension are mediated by CRF release from the BN within the LC, whereas activation elicited by higher magnitudes of distension involve other mediators, such as glutamate (26). A recent report revealed the involvement of CRF₁ in the increased spontaneous discharge rate of LC neurons induced by intracranial injection of CRF or phasic CRD of 45 mmHg in anesthetized rats (24). These data are in agreement with our results and support the involvement of the CRF₁ system only for low volumes of distensions (40 mmHg in our study). On the other hand, there is growing literature suggesting that the normal synaptic glutamatergic transmission in different brain regions such as the lateral septum, central amygdala, LC, BN, and dorsal raphe nucleus are tightly controlled by endogenous levels of CRF and related peptides, urocortin 1 and urocortin 2, which establish a “tone” or homeostasis for normal excitatory glutamatergic transmission (27, 41, 65). It is well known that glutamate and N-methyl-D-aspartate (NMDA) receptors are major players in central sensitization and visceral hyperalgesia (60), particularly at high colonic distension pressures (26). Under stressful conditions, release of large amounts of CRF or related peptides could perturb this synaptic homeostasis by exacerbating the glutamatergic transmission, thereby leading to enhanced visceral sensitivity. Interestingly, CRF mRNA expression was shown to be decreased in the PVN and BN after chronic but not acute administration of CP-154,526 (2). This would be expected to dampen glutamatergic transmission and restore synaptic homeostasis, leading to a decrease in visceral pain at high distension pressures (60 mmHg). This effect, combined with the more direct effect of CRF₁ receptor antagonism at lower pressure (40 mmHg) in chronically stressed animals, could explain the disparity in the efficacy of the acute and chronic treatment regimens.

Last, a role for CRF₁ at the spinal level is also excluded. Recent preliminary data from our group (8) show activation of spinal microglia in the chronic WAS model and its possible contribution to the sustained visceral hyperalgesia. Analogous findings have been reported recently in another chronic visceral pain model (63). There is evidence for CRF₁ and CRF₂ expression on glial cells (64) and the participation of both receptors, as well as CRF ligands in the immune activation of spinal microglia (57).

Taken together, our results show for the first time the involvement of CRF₁ in the chronic intermittent stress-induced visceral hyperalgesia in rats. Although the exact site(s) of CRF₁ modulation of visceral nociception could not be identified in our experimental setting, the data suggest that both central and peripheral CRF₁ are involved in the observed effect. This is supported by the fact that CP-154,526 is characterized as a highly selective antagonist of the rat CRF₁ (51, 54) with penetration into the central nervous system under basal conditions (22), whereas astressin is a peptide antagonist with similar affinity for both CRF₁ and CRF₂ with poor ability to penetrate into the central nervous system under basal conditions (20, 49). These data, coupled with the fact that the brain-penetrating antagonist exerted higher effect than the peripherally acting peptide CRF₁/₂ antagonist, point to the role of CRF₁ signaling both centrally and peripherally in mediating the chronic intermittent stress-related visceral hyperalgesia in rats. However, given that stress increases BBB permeability (15, 16), we cannot exclude the possibility that astroinjected peripherally may have also acted at spinal and/or supraspinal sites. In conclusion, our results support the use of CRF₁ antagonists in human conditions, such as IBS, where visceral hyperalgesia is associated with anxiety and symptoms are commonly exacerbated by chronic stress (5).

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