Enhanced pelvic responses to stressors in female CRF-overexpressing mice

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Abstract

Acute stress affects gut functions through the activation of corticotropin-releasing factor (CRF) receptors. The impact of acute stress on pelvic viscera in the context of chronic stress is not well characterized. We investigated the colonic, urinary, and locomotor responses monitored as fecal pellet output (FPO), urine voiding, and ambulatory activity, respectively, in female and male CRF-overexpressing (CRF-OE) mice, a chronic stress model, and their wild-type littermates (WTL). Female CRF-OE mice, compared with WTL, had enhanced FPO to 2-min handling (150%) and 60-min novel environment (155%) but displayed a similar response to a 60-min partial restraint stress. Female CRF-OE mice, compared with WTL, also had a significantly increased number of urine spots (7.3 ± 1.4 vs. 1.3 ± 0.8 spots/h) and lower locomotor activity (246.8 ± 47.8 vs. 388.2 ± 31.9 entries/h) to a novel environment. Male CRF-OE mice and WTL both responded to a novel environment but failed to show differences between them in colonic and locomotor responses. Male WTL, compared with female WTL, had higher FPO (113%). In female CRF-OE mice, the CRF_1/CRF_2 receptor antagonist astressin B and the selective CRF_2 receptor agonist mouse urocortin 2 (injected peripherally) prevented the enhanced defecation without affecting urine or locomotor responses to novel environment. RT-PCR showed that CRF_1 and CRF_2 receptors are expressed in the mouse colonic tissues. The data show that chronic stress, due to continuous central CRF overdrive, renders female CRF-OE mice to have enhanced pelvic and altered behavioral responses to superimposed mild stressors and that CRF_1-initiated colonic response is counteracted by selective activation of CRF_2 receptor.

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Keywords

chronic stress; colon; urine; locomotor activity; astressin B; mouse urocortin 2

EARLY ON, CLINICAL OBSERVATIONS and experimental studies established a link between psychological stress and motor function alterations of gut (1) and bladder (62). Recent studies in humans showed the role of psychosocial factors in the onset or development of functional bowel and bladder diseases, particularly irritable bowel syndrome (IBS) (40) and interstitial cystitis (IC) (55). This led to the notion that the level of chronic life stress predicts the clinical outcome in these patients (6, 19). Numerous studies in rodents have characterized the alterations of motor and mucosal barrier functions in the gastrointestinal and urinary tracts, mainly under acute stress conditions (13, 58, 65).

Persistent elevation of central drive of the hypothalamicpituitary-adrenal (HPA) axis is one of the distinguishing characteristics of chronic stress in animals and humans (9). Corticotropin-releasing factor (CRF) is a 41-amino acid hypothalamic peptide that initiates HPA axis activation by stimulating the secretion and biosynthesis of pituitary ACTH, which, in turn, causes adrenal glucocorticoid secretion (4). In addition, activation of CRF signaling pathways induces a battery of acute behavioral, autonomic and visceral stress responses (4, 17, 65). Hypersecretion of CRF in the central nervous system has been implicated in stress-related human pathologies, including major depressive disorders (2). The actions of CRF and other related endogenous peptides, urocortin 1 (Ucn 1), Ucn 2, and Ucn 3, are mediated through the activation of two G-protein-coupled receptors, CRF_1 and/or CRF_2 (4, 21). Convergent reports indicate that acute stress and central injection of CRF and Ucn 1 stimulate propulsive colonic motility, primarily through the activation of brain CRF_1 receptors as established by the use of selective CRF_1 and CRF_2 receptor antagonists in rodents (67) and monkeys (17).

Recent studies also support a role for peripheral CRF receptors in the colonic response to stress in rats (68). Peripheral injection of CRF_1/CRF_2 agonists such as CRF and Ucn 1 and exposure to stressors such as restraint or water avoidance stimulate motility and increase permeability of the colon. These responses are blocked in rats by peptide CRF antagonists acting peripherally (68). In contrast to CRF and Ucn 1, peripheral injection of selective CRF_2 agonists, Ucn 2 or Ucn 3, had no measurable effects on basal colonic function in rats and mice (39, 43). However, emerging evidence points to a role for CRF_2 receptors in counterbalancing CRF_1-initiated anxiety, stimulation of the pituitary adrenal axis, and visceral hyperalgesia responses to stress, thereby contributing to the restoration of homeostasis (3, 11, 12, 50), although recent studies indicate that brain septal CRF_2 plays different roles (anxiogenic or no effect) depending on the level of background stress (25). With regard to the gut, there are recent reports showing that peripheral injection of Ucn 2 inhibits the CRF_1-mediated hyperalgesia induced by intestinal distension in rats (42, 44, 47).

CRF injected daily or intermittent exposure to stressors has been used to model a state of chronic stress (26, 33). An alternative strategy is to use transgenic mice that overexpress CRF (CRF-OE) (59). CRF-OE mice were generated by a chimeric CRF transgene comprising the methallothionein promoter driving the rat CRF gene, including introns (59)

or the Thy-1 promoter (15, 16). These transgenic mice overexpress CRF primarily in the brain and show several features that parallel those characteristic of chronic stress (12), including the chronic stimulation of pituitary ACTH release and elevated corticosterone levels (16, 59). CRF-OE mice are also a validated model of chronic stress that bear an anxiety-like behavioral profile featuring hypoactivity when placed in a novel environment (60, 72), hyperemotionality and impaired learning (23, 70), and diminished sexual receptivity (22). CRF-OE mice display immunologic alterations (12, 46) and increased body temperature and heart rate during the inactive phase of the diurnal cycle (15). Although CRF-OE mice have been largely characterized to assess the consequences of chronic constitutive CRF overdrive on the endocrine, behavioral, cardiac, and immune systems under basal or acute stress, to date, this model has not been used to explore alterations of pelvic function well known to be susceptible to stress (28, 40, 65).

In the present study, we examined pelvic visceral functions, primarily propulsive colonic motility, and, to a lesser extent, bladder function and locomotor responses in CRF-OE mice after exposure to acute stressors that were superimposed on their life-long CRF overexpression. Studies were conducted in male and female CRF-OE mice and wild-type littermates (WTL) because studies suggest the presence of sex differences in intestinal motor responses to chronic wrap restraint in rats (76), as well as in the prevalence of IBS (24) and IC (10). A link between IBS and overactive bladder has also been suggested (45). The role of peripheral CRF signaling pathways in mediating the pelvic changes induced by exposing CRF-OE mice and WTL to a novel environment stressor was assessed by peripheral pretreatment with the long-acting CRF_1/CRF_2 peptide antagonist astressin B (54). We also investigated whether acute stress-induced stimulation of pelvic propulsive motor activity in CRF-OE mice is modulated by activation of CRF_2 receptors using peripheral injection of Ucn 2.

MATERIALS AND METHODS

Animals.

Adult male and female CRF-OE mice and WTL (Oregon Health and Science University, Portland, OR) and male C57BL/6 mice (Harlan, Indianapolis, ID), aged 3–6 mo, were used. CRF-OE mice were generated as previously detailed (59, 60) with the use of a chimeric CRF transgene comprising the methallothionein protomer driving the rat CRF gene and backcrossed eight generations onto the C57BL/6 mouse strain. Mice were housed in standard plastic cages (outer dimension: $27 \times 12 \times 16$ cm) in groups of three or four of the same sex, in a temperature-, humidity-, and light cycle (lights on 0600)-controlled environment. Mice were provided ad libitum access to water and a commercially available laboratory rodent diet (Prolab RMH 2500–5P14; Purina LabDiet, St. Louis, MO). All of the procedures were approved by the Animal Research Committees at UCLA and Veteran Affairs Greater Los Angeles Healthcare System (Protocols 05058–02 and 11084–03).

Peptides.

Mouse Ucn 2 and astressin B (Peptide Biology Laboratory, The Salk Institute, La Jolla, CA) were synthesized by the solid-phase approach and the Boc strategy as previously described

(54) and stored in powder form at −80°C. Immediately before the experiments, astressin B was dissolved in sterile water and Ucn 2 in saline (pH 7.0). Subcutaneous and intraperitoneal injections were performed (in 0.1 ml).

Acute stressors.

Three stressors were used: brief handling, novel environment, and partial restraint. Brief handling consisted of handling for 2 min. The novel environment consisted of transferring mice from their home cage (in <20 s) to a new clean plastic cage (outer dimension: $27 \times 12 \times 16$ cm). The cage floor was lined with filter paper instead of bedding, and each mouse stayed alone in this novel environment for 60 min without food or water. Partial restraint stress consisted of placing the mouse head first into a ventilated 50-ml falcon tube for 60 min, as previously described (38).

Monitoring colon, bladder, and behavioral parameters.

Fecal pellet output (FPO) was recorded as the number of fecal pellets expelled at 2 min for the handling stressor; at 5, 15, 30, 45, and 60 min for the novel environment; and at 15, 30, 45, and 60 min for the partial restraint stressor. Urine voiding was monitored essentially as previously described (14) with some modifications. Mice were placed individually in a new cage with the floor lined with filter paper that was divided with a marker in 12 equal squares of 5.2×5.2-cm size. Urine spots left on the filter paper were viewed at the end of the 60-min novel environment with a UV transilluminator and photographed. The number and area of spots were determined with NIH Image software (Scion, Frederick, MD). Locomotor activity was monitored in mice placed individually in a new cage with the floor lined with filter paper that was divided with a marker in 12 equal squares of 5.2×5.2-cm size. Locomotor activity was assessed as the number of squares crossed during the first 5, 15, 30, 45, and 60 min, as previously described (5).

CRF receptor gene expression in the colon.

Proximal and distal colon tissue samples were harvested from naive female CRF-OE mice and female WTL to assess CRF receptor gene expression by RT-PCR. For RNA isolation and cDNA synthesis, total RNA was extracted from the proximal and distal colons of CRF-OE female mice ($n = 5$) and their WTL ($n = 3$) using RNA-Bee (TEL-TEST, Friendswood, TX), according to the manufacturer's protocol, which included a DNase step. Briefly, the colonic tissue was homogenized in RNA-Bee with the FastPrep system (FP120A homogenizer and lysing matrix D; QBiogene, Carlsbad, CA), 200 μl of chloroform were added, and the solution was centrifuged for 15 min at 4°C. The aqueous layer was precipitated with isopropanol. The RNA pellet was suspended in diethyl pyrocarbonate water and digested for 60 min at 37°C using DNase I (Promega, Madison, WI). The quality and amount of final total RNA yield were estimated based on the ratio of absorbance at 260 to 280 nm by UV spectrophotometer (ND-1000; NanoDrop, Wilmington, DE). Total RNA (5 μg) was denatured at 65°C for 5 min and used to synthesize first-strand cDNA in a total volume of 20-μl reaction by the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). The reverse transcription was terminated by incubation at 85°C for 5 min. RNase H was then added to the reaction mixture to remove the total RNA template.

The following specific mouse CRF_1, CRF_2, and ribosomal protein S16 oligonucleotide primers were used in the PCR: for mouse CRF_1, 5′-GGTGTGCCTTTCCCCATCATT-3′ and 5′-CAACATGTAGGTGATGCCCAG-3′ corresponding to nucleotides 740–760 (sense) and 998–1018 (antisense) (74) and the predicted size of the band of 279 bp; for mouse CRF_2, 5′-GGCAAGGAAGCTGGTGATTTG-3′ and 5′-GGCGTGGTGGTCCTGCCAGCG-3′ corresponding to nucleotides 957–977 (sense) and 1314–1334 (antisense) (61) and the predicted size of the band of 378 bp; and for ribosomal protein S16, 5′-TGCGGTGTGGAGCTCGTGCTTGT-3′ and 5′-GCTACCAGGCCTTTGAGATGGA-3′ corresponding to nucleotides 369–391 (sense) (75) and the predicted size of the band is 309 bp. PCR reactions were performed in a final volume of 30 ml using the RedTaq system (Sigma-Aldrich, St. Louis, MO). The reaction was predenatured at 95°C for 2 min and then amplified 34 times (94°C, 40 s; 59°C, 40 s; 72°C, 2 min), followed by a 5-min extension at 72°C in Thermal Cycler (PTC-200; MJ Research, San Francisco, CA). S16 was used as an internal control to ensure cDNA quality and equal loading. Negative control contained all reagents, except that 1 μl of H_2O was substituted for reverse transcriptase in the RT reaction to exclude the possibility of genomic or other DNA contamination. The agarose gel image of PCR products corresponding to CRF receptors was acquired by the Kodak EDAS 290 system and semiquantified by densitometric measurement using NIH Image software (Scion). All results were normalized by the internal control.

Experimental protocols.

All experiments were performed during the light phase between 0830 and 1200 in nonfasted mice.

Effects of various acute stressors on FPO and urine voiding.

Female CRF-OE mice (22.1 ± 0.9 g) and WTL (21.2 ± 0.3 g) were handheld for 2 min (handling stress), and the FPO during the 2-min handling period was monitored. Female CRF-OE mice (22.6 ± 1.1 g) and WTL (21.7 ± 0.3 g) were restrained for 60 min (partial restraint stressor), and FPO was monitored every 15 min for 60 min. Female (24.7 ± 1.3 g) and male (32.4 ± 2.4 g) CRF-OE mice and female (21.4 ± 0.4 g) and male (31.8 ± 1.0 g) WTL were placed in the novel environment for 60 min (novel environment stressor). FPO and locomotor activity were monitored at 5, 15, 30, 45, and 60 min. Urine output during the 60-min period was determined after the end of experiment. To avoid the confounding effect of pheromone, experiments were conducted in isolated individual cages for each mouse. Male and female mice were not housed together under any circumstances, either for regular housing or during the experiment. The cages used for each study were cleaned and autoclaved at the Animal Facility where experiments were conducted. No single cage was used more than once.

Effects of CRF receptor antagonist on colonic response to acute stressors in female CRF-OE mice and WTL and male C57BL/6 mice.

Astressin B (100 μg/kg) or water was injected subcutaneously 30 min before the 60-min novel environment exposure of female CRF-OE mice (24.2 ± 2.2 g) and WTL (22.1 ± 0.8 g), and FPO, locomotor activity, and urine voiding were monitored for 60 min. A similar

injection protocol was used with male C57BL/6 mice (34.3 ± 0.6 g) before the 60-min exposure to partial restraint stress, and FPO was monitored. The dose and route of CRF antagonist administration were selected based on previous experiments in mice and rats that characterized the regimen of injection for astressin B to block peripheral CRF-induced stimulation of distal colonic transit or endocrine response (39, 54).

Effects of Ucn 2 on colonic response to a novel environment in female CRF-OE mice and WTL.

Female CRF-OE mice (28.9 ± 1.7 g) and WTL (23.9 ± 0.5 g) were injected intraperitoneally with vehicle or Ucn 2 (1, 3 and 10 μg/kg) 10 min before exposure to a novel environment for 60 min. The FPO, urine voiding, and behavioral locomotor responses were monitored for 60 min. Doses and route of Ucn 2 administration were chosen on the basis of previous studies on gut functions in rodents (38, 43).

Statistical analysis.

All values are reported as means $\pm$ SE. One-way repeated-measures ANOVA followed by the Newman-Keuls multiple comparison test were used to analyze the time courses of FPO and locomotor activity. The number of urine spots and volume were analyzed by one-way ANOVA followed by Student's t-test to compare differences at one time point. Two-way repeated-measures ANOVA was used when two-factor effects, genotype and treatment, were analyzed. A P value of <0.05 was considered statistically significant.

RESULTS

Differential FPO responses to acute stressors in female and male CRF-OE and WTL mice.

Female CRF-OE mice responded to 2-min handling with a higher FPO than female WTL (1.0 ± 0.2 vs. 0.4 ± 0.2 pellets/2 min, respectively, $P < 0.05$; Fig. 1A). Similarly, female CRF-OE mice exposed to a novel environment for 60 min displayed significantly higher cumulative FPO/h than did the WTL (6.9 ± 0.9 pellets/h, $n = 12$, vs. 2.7 ± 0.9 pellets/h, $n = 11$, respectively, $P < 0.05$; Fig. 1B). Time-course studies showed that the peak FPO in female CRF-OE mice occurred within the first 15 min of the novel environment exposure and was sevenfold higher than that of the corresponding WTL (5.4 ± 0.8 vs. 0.7 ± 1.0 pellets/15 min, respectively, $P < 0.05$; Fig. 2A). Thereafter, FPO/15 min in female CRF-OE mice remained low and was not significantly different from that of female WTL, with values between 0.6 and 1 pellet/15 min (Fig. 2A). In response to partial restraint, however, both female CRF-OE mice and WTL had similarly high cumulative FPO (12.3 ± 0.4 pellets/h, $n = 7$, vs. 11.8 ± 1.2 pellets/h, $n = 6$, respectively, $P > 0.05$) with a similar peak response at 15 min (5.7 ± 0.6 vs. 5.3 ± 1.0 pellets/min) and significantly decreased plateau response during the remaining 45 min (Fig. 1C). Because the novel environment stressor showed a significant difference between the female CRF-OE mice and WTL, we selected this stressor for all subsequent studies involving CRF-OE mice and their WTL. In male mice exposed to a novel environment, both CRF-OE mice and WTL had a significantly higher FPO score during the first 15 min (7.0 ± 0.5 vs. 4.9 ± 1.1 pellets/15 min, respectively) than that shown in the remaining 45 min, monitored every 15 min (Fig. 3A). However, neither the peak (7.0 ± 0.5 vs. 4.9 ± 1.1 pellets/15 min, $P > 0.05$) nor the cumulative defecation response ($10.9 \pm$

1.2 vs. 8.6 ± 1.1 pellets/h, $P > 0.05$) reached significant difference between male CRF-OE mice and WTL (Fig. 3A).

Comparison of the total 60-min FPO to novel environment by sex showed that male WTL mice had significantly higher FPO response (8.6 ± 1.1, $n = 7$) than did the female WTL (2.7 ± 0.9 pellets/h, $n = 11$, $P < 0.05$) (Figs. 1B and 3B). Likewise, male CRF-OE mice tended to have a higher increased FPO to a novel environment than did female CRF-OE mice (10.9 ± 1.2 pellets/h, $n = 7$, vs. 6.9 ± 0.9 pellets/h, $n = 12$, $P > 0.05$) (Figs. 1B and 3B), although the difference did not reach significance. Because the body weight was higher in male than in female mice, FPO per 10 g body wt was computed. Analysis of FPO normalized to body weight yielded similar results. In response to a novel environment, there was no significant difference between male CRF-OE mice and WTL (3.5 ± 0.5 vs. 2.8 ± 0.4 pellets·10 $g^{-1}h^{-1}$, $P > 0.05$), whereas female CRF-OE mice had a significantly higher response than their WTL (2.9 ± 0.5 vs. 1.2 ± 0.4 pellets·10 $g^{-1}h^{-1}$, $P > 0.05$). Male WTL also had a significantly higher response to a novel environment than did female WTL (2.8 ± 0.4 vs. 1.2 ± 0.4 pellets·10 $g^{-1}h^{-1}$, $P < 0.05$). These data argue against body weight as a significant contribution to the observed sex difference in FPO in response to a novel environment in both WTL and CRF-OE mice.

Differential urine output and locomotor activity responses to novel environment between female CRF-OE and WTL mice.

Female CRF-OE mice exposed to the novel environment had a higher number of urine spots than did the WTL (7.3 ± 1.4 vs. 1.3 ± 0.8 spots/h, $P < 0.05$; Fig. 2B). No significant difference was identified in total area under the curve of the urine spots between female CRF-OE mice (32.2 ± 9.6 cm^2, $n = 7$) and WTL (21.4 ± 9.9 cm^2, $n = 6$, $P > 0.05$). The urine output response in male mice was not studied.

The ambulatory activity in response to a novel environment, assessed as the total number of squares crossed during the 60-min period, was significantly lower in the female CRF-OE mice than in the WTL (246.8 ± 47.8 vs. 388.2 ± 31.9/h, $P < 0.05$; Fig. 2C). The time course study showed that the activity of CRF-OE female mice was reduced starting in the first 15 min and continued to be lower than that of the WTL throughout the 60-min period with a significant effect at 30–45 min (Fig. 2C). In male CRF-OE mice, the total number of squares crossed during the 60 min of novel exposure was not different from that of WTL (404.7 ± 99.1 vs. 468.0 ± 27.3/h, respectively, $P > 0.05$; Fig. 3B). Inspection of the time course of the response showed similar values in both male CRF-OE and WTL mice during the first 45-min period, with a significant reduction in activity during the last 15 min in the CRF-OE mice (Fig. 3B). The ambulatory activity in male CRF-OE mice and WTL tended to be higher than that of female CRF-OE mice and WTL (Figs. 2C and 3B). Because female CRF-OE mice were more responsive to the novel environment than male CRF-OE mice compared with their WTL, further studies were mainly performed in female mice.

Effects of astressin B on acute stress-induced stimulation of pelvic function in CRF-OE and WTL mice.

Female CRF-OE mice injected subcutaneously with vehicle and exposed to a novel environment had a significantly higher FPO than vehicle injected subcutaneously in female WTL (0–30 min: 7.3 ± 0.9 vs. 1.7 ± 1.7 pellets/30 min, $P < 0.05$; 30–60 min: 2.3 ± 0.6 vs. 0.3 ± 0.2 pellets/30 min, $P < 0.05$; Fig. 4, A and B) consistent with previous observations in nonpretreated animals. Astressin B (100 µg/kg sc) injected 30 min before exposure to a novel environment did not significantly modify the low defecation score in female WTL but prevented the significant increase in FPO in CRF-OE mice, and values were similar to those observed in astressin B-pretreated female WTL (Fig. 4A). Likewise, astressin B (100 µg/kg sc) injection in male C57BL/6 mice as a 30-min pretreatment significantly inhibited the FPO rise induced by partial restraint stress compared with subcutaneous injection of vehicle (vehicle + restraint: 8.7 ± 0.8 pellets/h, $n = 13$; astressin B + restraint: 3.8 ± 0.2 pellets/h, $n = 5$/group, $P < 0.05$). Astressin B did not significantly influence FPO compared with vehicle-injected control male C57BL/6 mice (2.4 ± 1.3 pellets/h, $n = 7$, vs. 1.7 ± 0.9 pellets/h, $n = 7$; $P > 0.05$). By contrast, astressin B pretreatment under the same conditions affected neither the number of urine spots (vehicle: 6.3 ± 2.4 spots/h vs. astressin B: 9.0 ± 0.8 spots/h; Fig. 4C) nor the locomotor response (vehicle: 232.0 ± 89.8 entries/h vs. astressin B: 178.0 ± 34.8 entries/h; Fig. 4D) induced by a novel environment in female CRF-OE mice.

Peripheral mouse Ucn 2 blocked FPO response to novel environment in female CRF-OE mice.

CRF-OE mice injected intraperitoneally with saline had a significant 3.1- to 3.3-fold higher FPO score than did the saline-injected WTL during the first 15 or 30 min of novel environment (Fig. 5, A–C). Ucn 2 (1, 3, and 10 µg/kg ip) blocked the novel environment-induced 60-min FPO response in CRF-OE female mice (pellets/h: 4.0 ± 1.5, 3.8 ± 0.8, and 2.6 ± 0.4, respectively, vs. 9.0 ± 2.1 in vehicle, $P < 0.05$; Fig. 5B). By contrast, Ucn 2 (10 µg/kg ip) did not significantly affect the urine response as shown by the similar spot number in WTL and CRF-OE mice (Fig. 5D). Ucn 2 (10 µg/kg ip) treatment in female CRF-OE mice significantly reduced the novel environment-induced overall locomotor response (106.3 ± 23.5 vs. 218.4 ± 62 entries/h; Fig. 5E) with no significant effect during the first 15 min when the FPO response was maximal (Fig. 5C).

CRF$_1$ and CRF$_2$ gene expression in proximal and distal colons of female CRF-OE and WTL mice.

Using the primers specific for mouse CRF$_1$ and CRF$_2$, we found RT-PCR products for CRF$_1$ and CRF$_2$ with the predicted sizes in all samples of the proximal and distal mice colons that were examined (Fig. 6A). Quantitative analysis of the proximal vs. distal colon for CRF$_1$ signal showed that CRF$_1$ mRNA levels were significantly higher in proximal colon than in distal colon in both WTL (94.2%) and CRF-OE mice (96.1%). CRF$_1$ mRNA levels in CRF-OE mice tended to decrease by 22.6% in the proximal colon and by 48.5% in the distal colon compared with WT mice. In contrast, similar levels of CRF$_2$ expression was found in the proximal and distal colons with increased tendency of CRF$_2$ mRNA level in the proximal colon of CRF-OE mice (21.4%) (Fig. 6B).

DISCUSSION

Stimulation of colon and bladder motor functions (transit, defecation, urination) is a hallmark of the acute visceral response in rodents exposed to various stressors (38, 63, 65). The present study shows that female CRF-OE mice exposed to a novel environment for 60 min displayed a 6.7-fold peak increase in FPO compared with WTL. The response had a rapid onset and was short lasting. Likewise, female CRF-OE mice responded to handling for 2 min with a significant increase in FPO, compared with their WTL. By contrast, both female CRF-OE and their female WTL mice had a robust FPO response with a similar time course and magnitude during partial restraint stress. Female CRF-OE mice displayed enhanced urine voiding and decreased ambulation during exposure to the novel environment compared with their female WTL. These data are significant because they demonstrate that exposure to a novel environment triggers pelvic and behavioral manifestations of stress in female CRF-OE mice, which are under conditions of chronic central drive of CRF (12). Therefore, female CRF-OE mice provide an experimental model of enhanced pelvic responsiveness to a novel environment in the context of chronically activated stress pathways that may have relevance to stress modulation of functional disorders (19, 40, 55).

The present data also provide evidence that peripheral activation of CRF signaling pathways is part of the underlying mechanisms of acute stress-related activation of motor function in the colon. The CRF receptor antagonist, astressin B (54), prevented the significant increase in FPO of female CRF-OE mice compared with their WTL when exposed to the novel environment stressor. Similarly, in male C57BL/6 mice, the partial restraint-induced 4.1-fold increase in FPO compared with control was prevented by astressin B injected subcutaneously. Consistent with these observations, studies in female and male rats have also shown that the CRF_1/CRF_2 peptide antagonists, α-helical CRF_{9-41}, or astressin (51, 53) injected intraperitoneally or intravenously prevented wrap restraint or water avoidance stress-induced acceleration of colonic transit and defecation (7, 36, 43, 77).

The blunting of the FPO response to acute stress by subcutaneous astressin B is likely to reflect a peripheral site of action (38, 68). In our study, the subcutaneous injection of astressin B did not influence the decreased locomotor activity in CRF-OE mice exposed to a novel environment. Such a decrease in exploratory activity is part of the anxiety-like behavior in CRF-OE mice, which has been well characterized in different novel environment paradigms, including open field, light-dark exploration, 16-hole board task, and elevated plus maze (35, 60, 70, 72), and shown to be mediated by brain CRF_1 receptors (12, 60). CRF injected into the cerebral ventricles in as low as 0.15 nmol was previously reported to decrease locomotion in an open-field test, whereas subcutaneous injection of similar doses of CRF failed to affect locomotion (64). Thus, although there is no immunohistochemical evidence for the overexpression of CRF peptide in the peripheral tissue or elevated CRF levels in the circulation of CRF-OE mice (12, 59), an acute mild stressor superimposed on their chronic central CRF overproduction could engage the peripheral urocortin signaling (20, 31) to contribute to the colonic response.

Although astressin B binds to both CRF_1 and CRF_2 receptors (52), the astressin B-induced dampening of colonic motor response to a novel environment is likely to occur primarily

through peripheral blockade of CRF_1 receptors. This has been shown by the blockade of stress or CRF-induced colonic motor stimulation by intraperitoneal injection of astressin and CRF_1 receptor antagonists (39) but not by peripheral injection of the selective CRF_2 antagonist astressin B (38, 42). In addition, the CRF_2 agonists Ucn 2 (6–60 μg/kg ip) and Ucn 3 (6–120 μg/kg ip) did not influence basal distal colonic transit, although they dose-dependently inhibited gastric transit monitored simultaneously in male mice (39). CRF_1 receptors are localized at peripheral sites that subserve the regulation of propulsive motor activity in the colon. These include the myenteric neurons in the rat and guinea pig colon, where CRF induced a CRF_1-dependent activation (8, 34, 41). We found by RT-PCR that CRF_1 receptors are also expressed in CRF-OE mouse and WTL proximal colon and to a lesser extent in the distal colon, consistent with a possible site of action as established in rats. Lastly, female CRF-OE mice injected intraperitoneally with Ucn 2 in doses ranging from 1 to 10 μg/kg no longer displayed a significant increase in FPO during the 60 min of novel environment, whereas vehicle-injected CRF-OE mice did. Collectively, these findings support a role for peripheral CRF_1 and not CRF_2 receptors in mediating the FPO response to an acute stressor in mice, in line with previous reports in rats (36, 43).

Some of the phenotypic attributes of the CRF-OE mice, particularly those related to immunological abnormalities, derive exclusively from the CRF_1-mediated chronic increase in circulating glucocorticoids (12). Other attributes, such as decreased exploratory activity and decreased sexual receptivity in females, are unrelated to the hypercorticosteronemia (12, 22, 23). Existing data do not support a glucocorticoid modulation of the CRF_1-mediated colonic motor response in female CRF-OE mice. Hypophysectomy does not alter the stimulation of colonic transit by an acute stressor, and peripheral injection of ACTH or glucocorticoids does not result in colonic motor activation (66). The FPO of female CRF-OE mice was enhanced during exposure to the novel environment compared with WTL but was similar to WTL during the partial restraint stressor, indicative of differential responsiveness between the endocrine (12, 16, 60) and colonic motor changes to acute stressors (present study).

The selective CRF_2 receptor agonist, Ucn 2 injected intraperitoneally, prevented the CRF_1-initiated colonic motor response to a novel environment in female CRF-OE. Ucn 2 injected intravenously into mice has been reported to reach the brain parenchyma in an intact form at a moderate rate through passive diffusion (27), and a direct or indirect central action could occur. However, intraperitoneal Ucn 2 is likely to act peripherally. First, CRF_2 receptors are expressed in the proximal and distal colons of both CRF-OE mice and WTL, as has been observed in rats (8). In addition, intraperitoneal Ucn 2 did not block the decrease in locomotor activity in CRF-OE mice exposed to the novel environment, a response well established to be mediated by CRF receptors in the brain (12, 60). These data support the emerging concept that activation of CRF_2 receptors may counterbalance CRF_1-initiated endocrine and visceral responses and thereby maintain allostasis (3, 44, 50).

Female CRF-OE mice exposed to a novel environment responded by a 4.6-fold increase in the frequency of urine voiding compared with WTL. CRF-OE mice are reported to have increased water intake (72), which may lead to increased voiding frequency. However, the fact that voiding frequency, but not the total urine output, was increased in CRF-OE mice

suggests that the increased spotting in CRF-OE mice is related to an altered voiding and/or guarding reflex rather than factors affecting the overall urine volume (14). A recent review linked disorders of voiding with anxiety (29), and data in conscious rats established an important positive relationship between the state of arousal and micturition (28). The increased number of urine spots from CRF-OE mice, which also display anxiety-like behavior, is in line with the idea that their state of anxiety and/or arousal increased voiding frequency. The Barrington's and sacral parasympathetic nuclei are synaptically linked to modulate motor function in both colon and bladder (56, 57, 71), and CRF serves as a major neurotransmitter in this pathway (56, 73). However, the precise role of CRF in the micturition reflex remains to be clarified. An inhibitory effect of central and spinal CRF on the micturition pathway in conscious and anesthetized male Sprague-Dawley rats (28, 48) and increased micturition frequency and threshold of micturition by intrathecal or intraperitoneal injection of CRF in conscious Wistar female rats have been reported (30). In the present study, astressin B or Ucn 2 injected intraperitoneally at doses that counteracted the FPO response to novel environment did not alter the voiding response in CRF-OE mice. These data suggest that peripheral CRF receptors play little role in triggering or modulating the enhanced voiding frequency under these conditions. The lack of CRF_1 receptor expression in the rat bladder under noninflamed conditions (32) may explain the differential effects of peripheral astressin B on colonic vs. bladder responses, since CRF_1 receptors are located on myenteric neurons in the colon, where they exert an excitatory action in response to peripheral injection of CRF agonists (8, 34, 41). Clearly, further studies will be needed to address the underlying mechanisms regulating voiding function in CRF-OE mice exposed to novel environments. Whether a reflex response from the possible cross-talk between the colon and bladder contribute to the observed effects is also not known and requires further studies.

The present data suggest that chronically stressed female mice tend to have enhanced behavioral and autonomic responses to a superimposed acute mild stress compared with male. Female CRF-OE mice, compared with their female WTL, displayed higher FPO and anxiogenic-like responses than did male CRF-OE mice, compared with their male WTL, during exposure to the novel cage stressor. Alterations of brain CRF pathways induced by restraint stress during the last week of pregnancy result in a sex difference in the offspring, whereby female offspring display a prolonged HPA axis response and increased anxiety to stressors vs. that shown in the male offspring (49). Such in utero impact of stress may have a bearing on the observed sex difference in our study. The observation that chronic stress enhances the colonic and anxiogenic responses to a superimposed acute, novel stressor, mainly in the female CRF-OE mice has relevance to sex differences observed in chronic disease conditions in humans. It is well documented that women under stress conditions develop functional diseases such as IBS (24), IC (55), and comorbid disorders (6, 78) with anxiety more prevalently than men. Whether estrogen receptors, known to influence the activity of the CRF promoter and transcriptional activity of the urocortin gene (18, 69), play a role in this sex difference warrants investigation.

In summary, the present study established that female CRF-OE mice exhibit enhanced FPO and urine voiding and reduced locomotor activity when submitted to novel environmental stress compared with female WTL. This enhanced pelvic responsiveness of female CRF-OE

mice was mainly observed in the context of mild stressors and was sex specific. The present findings also point to differential peripheral mechanisms in the colon vs. bladder of female CRF-OE mice exposed to a novel environment. The FPO response to novel cage and restraint stressors involved primarily peripheral CRF_1 and could be blunted by peripheral Ucn 2, strengthening the notion of a possible modulation of CRF_1 stimulatory action by CRF_2 receptors. By contrast, the enhanced urine spotting was largely independent of peripheral CRF_1 receptors and not modulated by intraperitoneal Ucn 2, suggesting the presence of different peripheral or central mechanisms that will require further detailed investigations. The data also add to a possible link between IBS and overactive bladder, as suggested by Monga et al. (45). In view of the enhanced response to acute novel mild stressors in chronically CRF-OE female mice, this model has an important bearing on the understanding of several functional diseases in women, such as IBS and IC, which are known to be linked to chronic stress and for which growing evidence indicates that CRF signaling pathways may be of significant relevance (6, 37).

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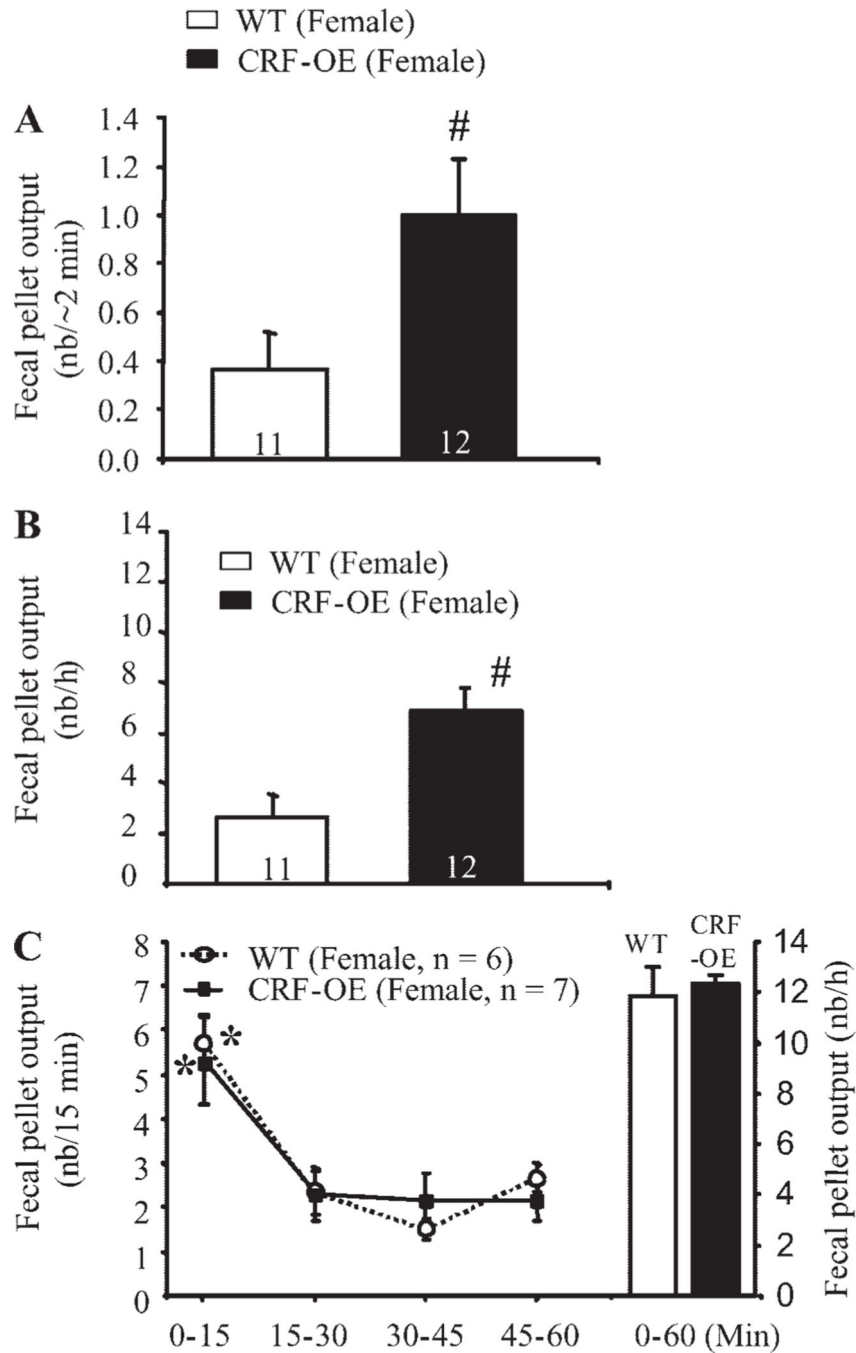
Fig. 1.
Mild stress (*A* and *B*) but not strong restraint stress (*C*) induces enhanced fecal pellet output (FPO) in female corticotropin-releasing factor overexpressing (CRF-OE) mice compared with their wild-type (WT) littermates. Mice were handled for 2 min (*A*), placed in individual new cages for 60 min (*B*), or partially restrained for 60 min (*C*). Each bar or point represents the mean ± SE of the number of mice indicated at the bottom of columns or in parentheses. #$P < 0.05$ vs. WT littermates (*t*-test); *$P < 0.05$ vs. all other time points (repeated one-way ANOVA). nb, number.
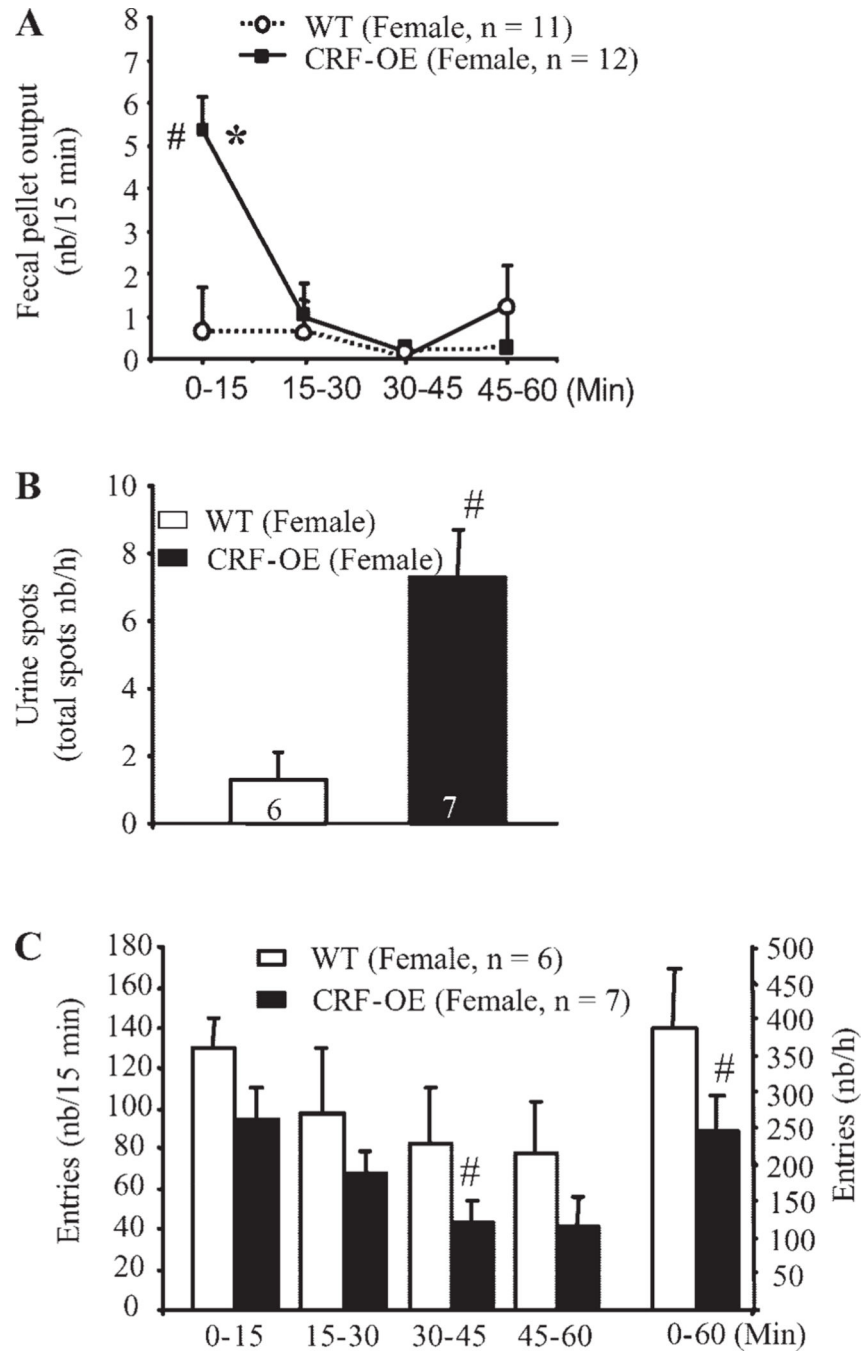
Fig. 2.
Novel environment stress for 60 min induces a rapid defecatory peak response in the first 15 min (*A*), increased urine spots (*B*), and decreased ambulatory response (*C*), as monitored concomitantly in female CRF-OE mice compared with their WT littermates. Each bar or point represents the mean ± SE of the number of mice indicated at the bottom of columns or in parentheses. *$P < 0.05$ vs. all other time points (repeated one-way ANOVA); #$P < 0.05$ vs. WT littermates (*t*-test).
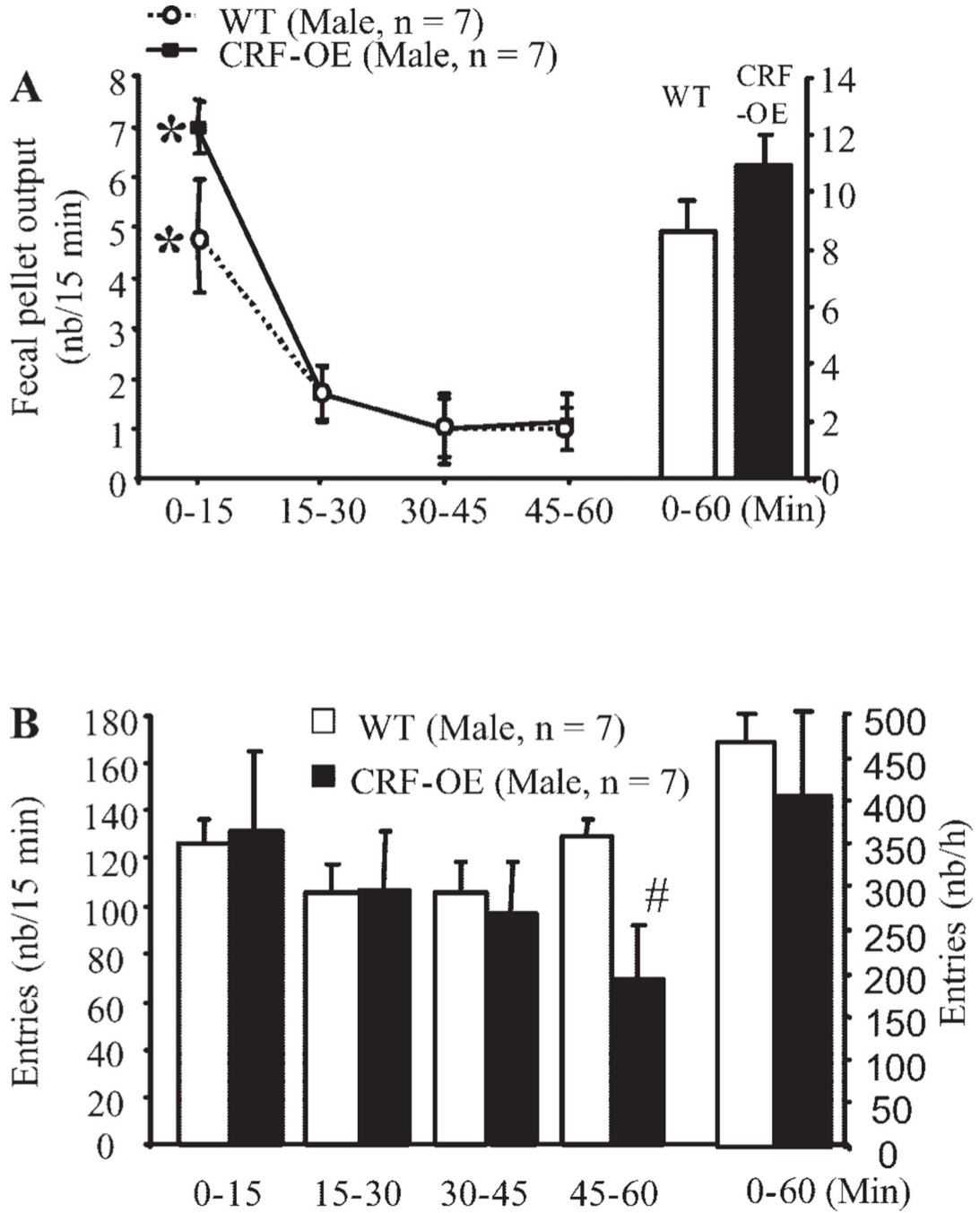
Fig. 3.
Effects of novel environment on colonic (*A*) or locomotor (*B*) responses in male CRF-OE mice and WT littermates. Time course analysis shows enhanced colonic response during the first 15 min in both CRF-OE mice and WT littermates (*A*) and decreased locomotor response during the 45–60 min (*B*) of the stress session monitored concomitantly. Each bar or point represents the mean ± SE of number of mice indicated in parentheses. *$P < 0.05$ vs. all other time points (repeated one-way ANOVA); #$P < 0.05$ vs. WT littermates at 45–60 min time point (*t*-test).
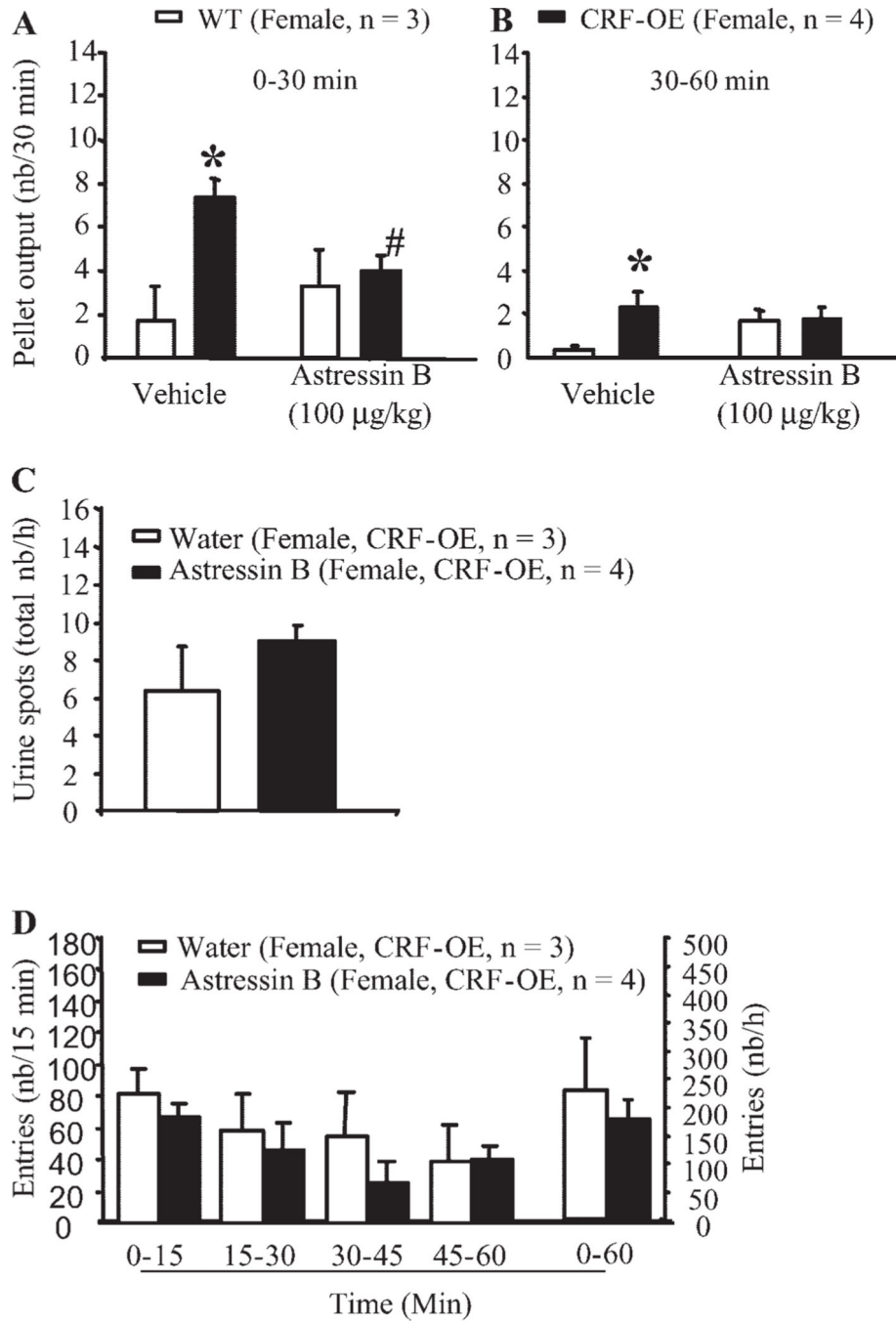
Fig. 4.

Subcutaneous injection of a CRF_1 and CRF_2 receptor antagonist, astressin B, prevents novel environment stress-induced colonic response in female CRF-OE mice. The subcutaneous injection of vehicle or astressin B in female CRF-OE mice was performed 30 min before the 60-min exposure to a novel environment (*A*). Urinary voiding (*B*) and locomotor (*C*) responses were monitored concomitantly. Each bar represents the mean ± SE of number of mice indicated in parentheses. *$P < 0.05$ vs. vehicle-treated WT littermates (repeated one-way ANOVA); #$P < 0.05$ vs. vehicle-treated CRF-OE mice (*t*-test).
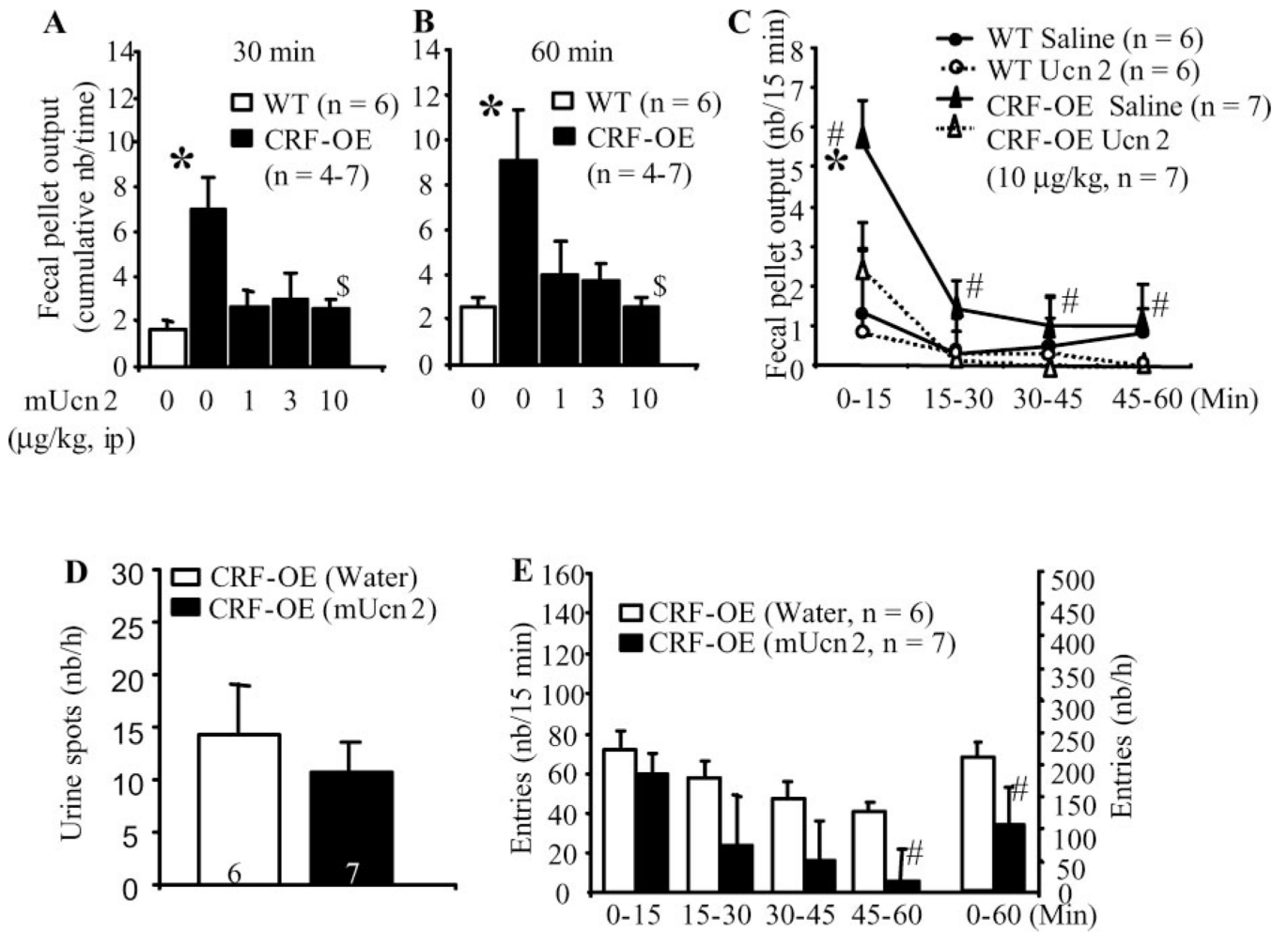
Fig. 5.
Intraperitoneal injection of selective CRF₂ receptor agonist mouse urocortin 2 (mUcn 2), blocked novel environment stress-induced colonic response. Shown are dose and time courses (*A–C*), lack of effect on urinary voiding (*D*), and time course of locomotor response (*D*) in female CRF-OE mice compared with WT littermates. mUcn was injected intraperitoneally 10 min before exposure for 60 min to novel environment. $*P < 0.05$ vs. all other time points (repeated two-way ANOVA); $\#P < 0.05$ vs. the corresponding WT littermates (one-way ANOVA); $\$P < 0.05$ vs. vehicle CRF-OE mice (one-way ANOVA).
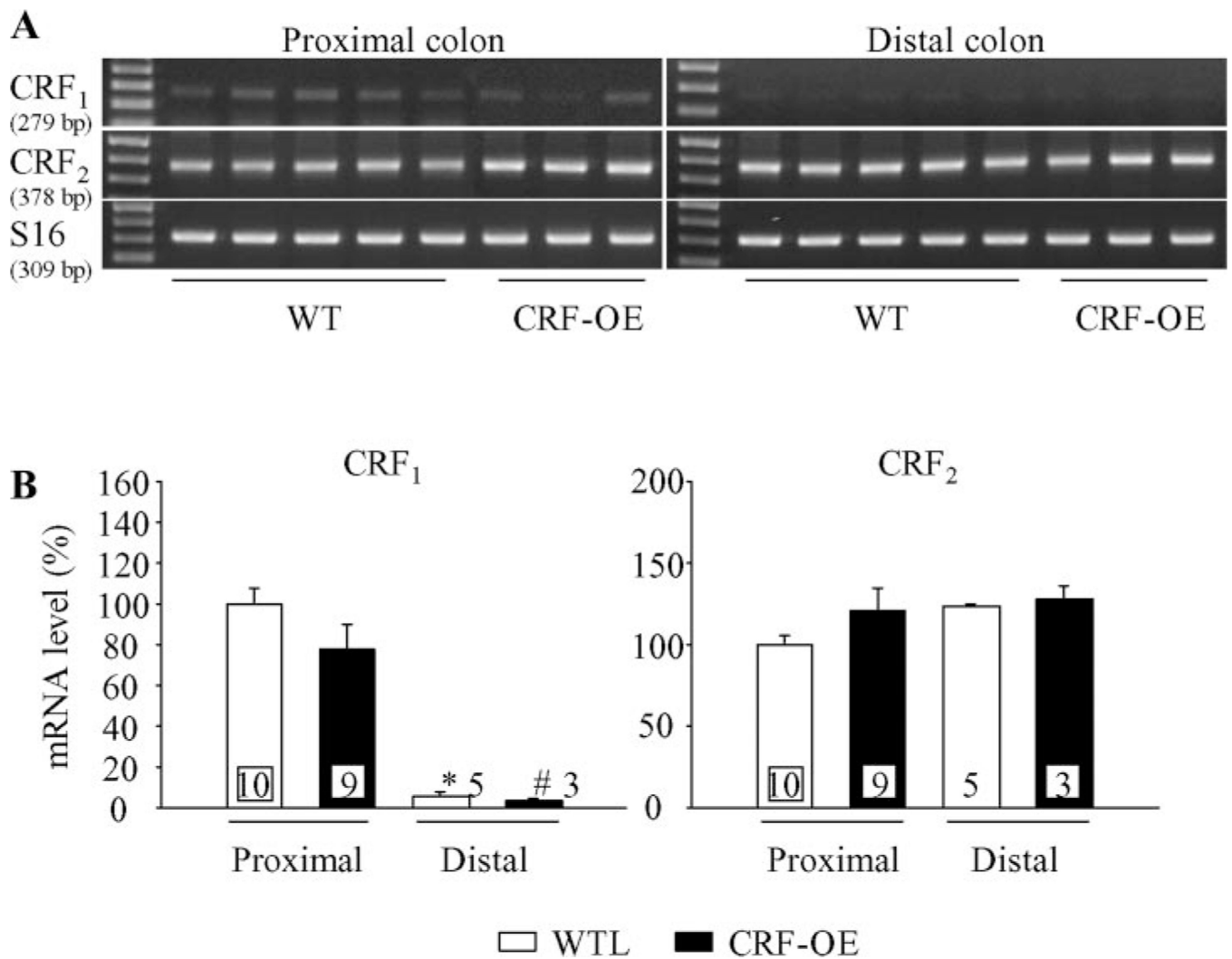
Fig. 6.

CRF_1 and CRF_2 gene expression in the proximal and distal colonic tissues of female CRF-OE mice and WT littermates (WTL). *A*: gel images of RT-PCR for mouse CRF_1 and CRF_2 acquired by the Kodak EDAS 290 system. The representative total RNA samples were extracted from whole thickness proximal ($n = 9$–10) and distal colons ($n = 3$–5) of WTL and CRF-OE mice. *B*: quantitative analysis of RT-PCR for CRF_1 and CRF_2 in proximal and distal colon using the NIH Image system (Scion, Frederick, MD) and standardized by taking the ratio to ribosomal protein S16 in each sample, respectively. Each column represents the mean ± SE of the number of mice indicated at the bottom of bars. $P < 0.05$ compared with proximal colon in WTL (*) or CRF-OE (#) mice.