0022-3565/02/3012-611–617$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 301, No. 2
4741/979885
JPET 301:611–617, 2002
*Printed in U.S.A.*




# Differential Actions of Peripheral Corticotropin-Releasing Factor (CRF), Urocortin II, and Urocortin III on Gastric Emptying and Colonic Transit in Mice: Role of CRF Receptor Subtypes 1 and 2

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Received November 9, 2001; accepted January 22, 2002 This article is available online at http://jpet.aspetjournals.org

## ABSTRACT

Peripheral CRF inhibits gastric emptying and stimulates colonic motor function in rats. We investigated the role of $CRF_1$ and $CRF_2$ receptors in i.p. CRF-induced alterations of gut transit in conscious mice using selective $CRF_1$ and $CRF_2$ ligands injected i.p. Gastric emptying 2 h after ingestion of a solid chow meal and colonic transit (time to expel a bead inserted into the distal colon) were determined simultaneously. Rat/human (r/h)CRF, which has $CRF_1 > CRF_2$ binding affinity, decreased distal colonic transit time at lower doses (6–12 $\mu$g/kg) than those inhibiting gastric emptying (20–60 $\mu$g/kg). Ovine CRF, a preferential $CRF_1$ receptor agonist (6–60 $\mu$g/kg), reduced significantly the colonic transit time without altering gastric emptying, whereas the selective $CRF_2$ receptor agonists mouse urocortin II (20–60 $\mu$g/kg) and urocortin III (120 $\mu$g/kg) inhibited significantly gastric emptying without modifying colonic transit. The $CRF_1$/$CRF_2$ receptor antagonist, astressin (30–120 $\mu$g/kg), dose dependently prevented r/hCRF (20 $\mu$g/kg)-induced inhibition of gastric emptying and reduction of colonic transit time. The selective $CRF_1$ receptor antagonists, NBI-27914 ($C_{18}H_{20}Cl_4N_4C_7H_8O_3S$) and CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]ethylamine) (5–30 mg/kg), dose dependently blocked r/hCRF action on the colon without influencing the gastric response, whereas the $CRF_2$ receptor antagonist, antisauvagine-30 (30–100 $\mu$g/kg), dose dependently abolished r/hCRF-induced delayed gastric emptying and had no effect on colonic response. These data show that i.p. r/hCRF-induced opposite actions on upper and lower gut transit in conscious mice are mediated by different CRF receptor subtypes: the activation of $CRF_1$ receptors stimulates colonic propulsive activity, whereas activation of $CRF_2$ receptors inhibits gastric emptying.

The 41-amino acid peptide corticotropin-releasing factor (CRF) was isolated from ovine hypothalamus and structurally characterized in 1981, as a novel hypothalamic releasing factor stimulating the release of pituitary proopiomelanocortin peptides (Vale et al., 1981). Recently, in addition to CRF, three other mammalian CRF-related peptides, namely, urocortin (Ucn), urocortin II (Ucn II) and urocortin III (Ucn III), have been identified in humans and rodents (Donaldson et al., 1996; Vaughan et al., 1996; Lewis et al., 2001; Reyes et al., 2001). Rat urocortin (rUcn) is a 40-amino acid peptide which shares 45% homology with rat/human (r/h)CRF (Vaughan et al., 1996). Human Ucn II and Ucn III have been discovered by sequence homology to r/hCRF from the Human Genome Database and the mouse orthologs have been cloned (Lewis et al., 2001; Reyes et al., 2001). Mouse (m)Ucn II and mUcn III display 34% and 26% sequence homology with r/hCRF and 42% and 18% identity with rUcn, respectively (Lewis et al., 2001; Reyes et al., 2001).

Two distinct seven-transmembrane domain $G_s$ protein-coupled receptors, designated $CRF_1$ and $CRF_2$, each encoded by distinct genes, have been cloned in mammals (Perrin and Vale, 1999). Alternative splicing of the $CRF_2$ receptor leads to $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$ variants in humans, which differ in their amino terminus, whereas in mice only the $CRF_{2\beta}$ form has been identified (Perrin and Vale, 1999). Although $CRF_1$ and $CRF_2$ receptors have considerable sequence similarity, they display distinct affinities for the four mammalian

This work was supported by the Department of Veterans Affairs Merit Review (Y.T.) and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-57238 (Y.T.) and DK-41301 (Animal Core, Y.T.), and DK-26741 (J.R.). V. Martínez was partially supported by the Conselleria de Cultura Educació i Ciència de la Generalitat Valenciana (Spain).

**ABBREVIATIONS:** CRF, corticotropin-releasing factor; DMSO, dimethyl sulfoxide; oCRF, ovine CRF; r/hCRF, rat/human CRF; Ucn, urocortin; mUcn II, mouse urocortin II; mUcn III, mouse urocortin III; rUcn, rat urocortin.

members of the CRF family characterized so far (Perrin and Vale, 1999; Lewis et al., 2001; Reyes et al., 2001). In vitro studies established that Ucn binds with high affinity to both CRF receptor subtypes, whereas r/hCRF exhibits higher affinity to $CRF_1$ than to $CRF_2$ receptors (Perrin and Vale, 1999). In contrast, human and mUcn II and mUcn III bind selectively to $CRF_2$ receptors, representing potential endogenous ligands for the receptor subtype 2 (Lewis et al., 2001; Reyes et al., 2001). In addition to their distinct pharmacological profiles, $CRF_1$ and $CRF_2$ receptors have different patterns of distribution in both the brain (Van Pett et al., 2000) and periphery (Baigent and Lowry, 2000; Muramatsu et al., 2000), indicating that they may subserve different biological functions.

Previous functional studies established that peripherally administered r/hCRF, rUcn, or the nonmammalian CRF-related peptides sauvagine and urotensin-I inhibit gastric emptying in rats, mice, and dogs (Pappas et al., 1985; Asakawa et al., 1999; Nozu et al., 1999; Wang et al., 2001). In contrast, r/hCRF and rUcn injected peripherally exert a stimulatory effect on colonic motor function as shown by the induction of spike burst activity in the proximal colon, acceleration of transit, and induction of defecation in rats (Williams et al., 1987; Maillot et al., 2000). The use of the nonselective $CRF_1/CRF_2$ receptor antagonists, $\alpha$-helical $CRF_{9-41}$ and astressin (Gulyas et al., 1995), showed that peripheral r/hCRF-induced delayed gastric emptying in rats and mice and accelerated colonic motor function in rats are CRF receptor-mediated (Williams et al., 1987; Shelton et al., 1990; Martínez et al., 1999; Nozu et al., 1999; Maillot et al., 2000). However, the CRF receptor subtype(s) mediating alterations of gastrocolonic motor activity induced by peripheral CRF remains to be ascertained. We recently reported that the selective $CRF_1$ receptor antagonist, CP-154,526 (Schulz et al., 1996), injected subcutaneously, prevented i.p. r/hCRF-induced clustered spike burst activity in the proximal colon and defecation in rats, indicative of a $CRF_1$ receptor-mediated action (Maillot et al., 2000). In contrast, the $CRF_1$ receptor antagonists antalarmin and NBI-27914 (Schulz et al., 1997; Maciejewski-Lenoir et al., 2000) administered intravenously in rats did not influence intravenous r/hCRF- and rUcn-induced delay of gastric emptying of a liquid meal, suggesting a possible mediation through $CRF_2$ receptors of the latter response (Nozu et al., 1999).

Up to now, the lack of selective $CRF_2$ receptor agonists and antagonists has hampered the direct pharmacological assessment of $CRF_2$ receptors in the actions of CRF. Recently, the highly specific $CRF_{2\beta}$ antagonist antisauvagine-30 has been developed (Eckart et al., 2001; Higelin et al., 2001), and the novel endogenous $CRF_2$-related peptides, Ucn II and Ucn III, have selective in vitro binding affinity to $CRF_2$ receptors (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001).

The aim of the present study was to establish the CRF receptor subtypes mediating peripheral r/hCRF-induced opposite actions on the upper and lower gut transit in mice. We first assessed whether r/hCRF and ovine CRF (oCRF), which have preferential binding affinity to the $CRF_1$ receptor (Behan et al., 1996), and mUcn II and mUcn III, which exhibit selective affinity to the $CRF_2$ receptor (Lewis et al., 2001; Reyes et al., 2001), exert differential effects on postprandial gastric and colonic transit. In addition, we investigated the blockade of r/hCRF actions by the nonselective $CRF_1/CRF_2$ receptor antagonist, astressin (Gulyas et al., 1995), the $CRF_1$ receptor antagonists NBI-27914 and CP-154,526 (Schulz et al., 1997; Maciejewski-Lenoir et al., 2000), and the $CRF_2$ receptor antagonist antisauvagine-30 (Eckart et al., 2001; Higelin et al., 2001). Gastric and colonic transits were monitored using a method that we developed to measure simultaneously the gastric emptying of a solid meal and the distal colonic propulsion in conscious mice.

## Materials and Methods

### Animals

Adult male C57BL/6 mice (6–8 weeks of age; Harlan, Indianapolis, IN) were maintained on a 12-h light/dark cycle with controlled temperature (21–23°C) and humidity (30–35%). Animals were group-housed in direct bedding cages with free access to food (Purina Lab Chow) and tap water. Mice were deprived of food for 18 to 20 h, with free access to water before the experiments, which were conducted under the Veteran Affairs Animal Component of the Research Protocol number 99-092-05.

### Experimental Compounds and Treatments

The following peptides were used: r/hCRF, oCRF, mUcn II, mUcn III, astressin (cyclo(30–33)-[D-$Phe^{12}$,$Nle^{21,38}$,$Glu^{30}$,$Lys^{33}$]r/h$CRF_{12-41}$) and antisauvagine-30 ([D-$Phe^{11}$,$His^{12}$]$sauvagine_{11-40}$) (Salk Institute, Clayton Foundation Laboratories for Peptide Biology, La Jolla, CA). Peptides were synthesized as previously described (Gulyas et al., 1995; Reyes et al., 2001) and kept in powder form at −80°C. Immediately before use, peptides were weighted and dissolved in sterile saline except astressin and antisauvagine-30, which were dissolved in double-distilled water (pH ~7.6). NBI-27914 (tosylate salt; Neurocrine Biosciences, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml, and further dilutions were performed in saline solution. A dilution of DMSO in saline [80% DMSO/20% saline (v/v), pH ~6.5) served as the control vehicle. CP-154,526 (hydrochloride salt; Pfizer Inc., Groton, CT) was dissolved to a concentration of 10 mg/ml in a vehicle consisting of saline/cremophor EL/DMSO at a 90:5:5 ratio, as in our previous study (Maillot et al., 2000). A solution of saline/cremophor EL/DMSO at pH adjusted to ~5.1 (similar to pH of CP-154,526 at 30 mg/kg) was used as vehicle control. Intraperitoneal injections were performed in 0.1 ml. In vitro receptor selectivity of the different CRF receptor agonists and antagonists used in this study is indicated in Table 1.

### Gastric and Distal Colonic Transit Measurements

Gastric emptying of a solid nutrient meal and distal colonic transit were simultaneously monitored in conscious mice by combining, with minor modifications, two techniques previously described (Yamada and Onoda, 1992; Barrachina et al., 1997). Fasted mice had free access to water and preweighed Purina chow for a 1-h period, then were briefly anesthetized with enflurane (1–2 min; Ethrane-Anaquest, Madison, WI) to insert a single 2-mm glass bead into the distal colon at 2 cm from the anus. Bead insertion was accomplished with a glass rod with a fire-polished end to avoid tissue damage. After bead insertion, mice were placed individually in their home cages without food and water. Mice regained consciousness within a 1- to 2-min period and thereafter showed normal behavior.

Distal colonic transit was determined to the nearest 0.1 min by monitoring the time required for the expulsion of the glass bead (bead latency). The percentage of gastric emptying of the ingested meal was assessed 2 h after the end of food exposure. Mice were euthanized by cervical dislocation followed by thoracotomy. The abdominal cavity was opened, the pylorus and cardia were clamped, and the stomach was removed. The stomach was weighed and opened, and the gastric content was washed out with tap water. The gastric wall was dried and weighed. The amount of food (grams)

TABLE 1
Inhibitory binding constant for CRF, CRF-related peptides, and CRF receptor antagonists used in this study

| | $K_i$[a] | | | References |
|---|---|---|---|---|
| | $CRF_1$ | $CRF_{2\alpha}$ | $CRF_{2\beta}$ | |
| | | *nM* | | |
| CRF (rat/human) | 2 | 44 | 30.7 | Behan et al., 1996; Perrin et al., 1999 |
| CRF (ovine) | 1 | 184 | 162.4 | Behan et al., 1996 |
| Ucn II (mice) | >100 | 2.1 | 0.66 | Lewis et al., 2001 |
| Ucn III (mice) | >100 | 5.0 | 1.8 | Lewis et al., 2001 |
| Astressin | 2.0 | 1.5 | 1.0 | Perrin et al., 1999 |
| Antisauvagine-30 | 370 | | 0.3 | Eckart et al., 2001 |
| NBI-27914 | 1.9 | | >10 $\mu$M | Maciejewski-Lenoir et al., 2000 |
| CP-154,526 | 2.7 | >10 $\mu$M[b] | | Schulz et al., 1996 |

[a] See original references for experimental conditions.
[b] No differentiation between $\alpha$ and $\beta$ variants.

contained in the stomach was calculated as the difference between the total weight of the stomach with content and the weight of the stomach wall after the content was removed. The solid food ingested by each animal was determined by the difference between the weight of the Purina chow before feeding and the weight of the pellet and spill at the end of the 1-h feeding period. All weight measurements were recorded with an accuracy of two decimal points. The gastric emptying for the 2-h period was calculated according to the equation: % of gastric emptying = (1 − gastric content/food intake) × 100.

### Experimental Protocols

**Effects of r/hCRF and CRF-Related Peptides on Gastric and Distal Colonic Transit.** At the end of the 1-h feeding period, mice were briefly anesthetized with enflurane, and the bead inserted into the distal colon followed by the i.p. injection of either saline, r/hCRF (2, 6, 12, 20 or 60 $\mu$g/kg; approximately 0.05–1.5 $\mu$g/mouse), oCRF (6, 12, 20 or 60 $\mu$g/kg), mUcn II (6, 12, 20 or 60 $\mu$g/kg) or mUcn III (12, 60 or 120 $\mu$g/kg). Animals were returned to their home cages without food or water and the bead expulsion time was monitored. Gastric emptying of the nutrient solid meal was determined 2 h after peptide or saline administration.

**Effects of CRF Receptor Antagonists.** Astressin (30, 60 or 120 $\mu$g/kg), antisauvagine-30 (30, 50 or 100 $\mu$g/kg), NBI-27914 (5, 10 or 20 mg/kg), or their respective vehicles were injected i.p. at 50 min and CP-154,526 (10 or 30 mg/kg) and its vehicle at 30 min after the start of the feeding period. At the end of the 1-h feeding period, mice were briefly anesthetized with enflurane for colonic bead insertion followed by the i.p. injection of r/hCRF (20 $\mu$g/kg) or vehicle (0.1 ml). Thereafter, the time for colonic bead expulsion and gastric emptying of the solid meal were determined as described above. In each daily experiment, vehicle control and several peptide doses, with or without antagonists, were included and repeated in multiple days. The doses of the r/hCRF and CRF antagonists were selected based on our previous studies in rats and mice (Nozu et al., 1999; Maillot et al., 2000; Wang et al., 2001) and adjusted according to the results of preliminary data. To avoid circadian variations, all experiments were performed during the morning, finishing no later than 2:00 PM.

### Statistical Analysis

Results are expressed as mean ± S.E. Comparisons within multiple groups were performed using one-way analysis of variance followed by a Student-Newman-Keuls multiple comparison test. $P$ values $< 0.05$ were considered statistically significant. $ED_{50}$ values for gastric emptying were determined by nonlinear regression to a sigmoidal equation with variable slope (Prism, version 2.0; GraphPad Software, San Diego, CA).

## Results

**Differential Actions of i.p. r/hCRF on Gastric Emptying and Distal Colonic Transit.** In nontreated mice, fasted for 18 to 20 h and re-fed for 1 h, the percentage of ingested food that had emptied from the stomach after 2 h was 43 ± 7% and the time for colonic bead expulsion was 14.6 ± 2.2 min ($n = 7$). Saline injected i.p. did not influence gastric emptying (44 ± 4%) or bead latency time (13.7 ± 0.6 min; $n = 7$). The i.p. injection of r/hCRF (2, 6, 12, 20, or 60 $\mu$g/kg, $n = 6$–12 for each dose) induced a dose-related reduction of gastric emptying to 36 ± 5%, 32 ± 7%, 29 ± 8%, 9 ± 3% ($P < 0.05$ versus vehicle), and 2 ± 2% ($P < 0.05$ versus vehicle), respectively (Fig. 1); the $ED_{50}$ value was 10.7 $\mu$g/kg (95% confidence interval, 5.6–20.4 $\mu$g/kg; $r^2 = 0.989$). Simultaneously, r/hCRF at 2, 6, 12, 20, or 60 $\mu$g/kg reduced the time latency for bead expulsion from the colon to 10.3 ± 1.4 min, 8.8 ± 1.0 min ($P < 0.05$), 7.0 ± 0.9 min ($P < 0.05$), 8.7 ± 0.7 min ($P < 0.05$), and 9.6 ± 1.4 min ($P < 0.05$), respectively (Fig. 1). The r/hCRF dose of 20 $\mu$g/kg, which significantly accelerated distal colonic transit while simultaneously inhibiting gastric emptying of a solid meal, was selected in subsequent studies.

**Effects of i.p. CRF-Related Peptides on Gastric Emptying and Distal Colonic Transit.** The preferential $CRF_1$ receptor agonist, oCRF, injected i.p. at 6, 12, 20, or 60 $\mu$g/kg ($n = 4$–5 for each dose) induced a linear, dose-related, significant 35 to 54% shortening of bead latency expulsion time at doses ranging from 6 to 12 $\mu$g/kg with a plateau of 51 to 55% reduction at doses from 12 to 60 $\mu$g/kg (Fig. 1). In contrast, gastric emptying of the solid meal was not significantly modified at any dose, although there was a trend toward a reduction at the two highest doses (Fig. 1). The selective $CRF_2$ receptor agonist mUcn II (6, 12, 20, or 60 $\mu$g/kg, i.p.; $n = 5$ for each dose) significantly reduced gastric emptying values to 13 ± 6%, 7 ± 7%, 0 ± 0%, and 0 ± 0%, respectively ($P < 0.05$ versus vehicle), with an $ED_{50}$ value of 4.1 $\mu$g/kg (95% confidence interval: 2.3–7.1 $\mu$g/kg; $r^2 = 0.998$). However, mUcn II, at all the doses tested, did not modify the time latency for bead expulsion from the distal colon (Fig. 1). The i.p. injection of mUcn III (12, 60, or 120 $\mu$g/kg; $n = 3$–4 for each dose) did not alter distal colonic transit while inhibiting gastric emptying of the solid meal only at the highest dose (11 ± 2%; $P < 0.05$ versus i.p. saline).

**Effects of i.p. CRF Receptor Antagonists on i.p. r/hCRF Actions.** In i.p. water-pretreated mice, r/hCRF (20 $\mu$g/kg, i.p., $n = 7$) inhibited gastric emptying of the solid meal (7.0 ± 4.0%; $P < 0.05$ versus 42 ± 6% in water + saline) and shortened the bead latency to 6.7 ± 0.9 min compared with 12.0 ± 0.8 min in the i.p. water + saline group ($n = 7$; $P < 0.05$). Pretreatment with the $CRF_1$/$CRF_2$ receptor antago-

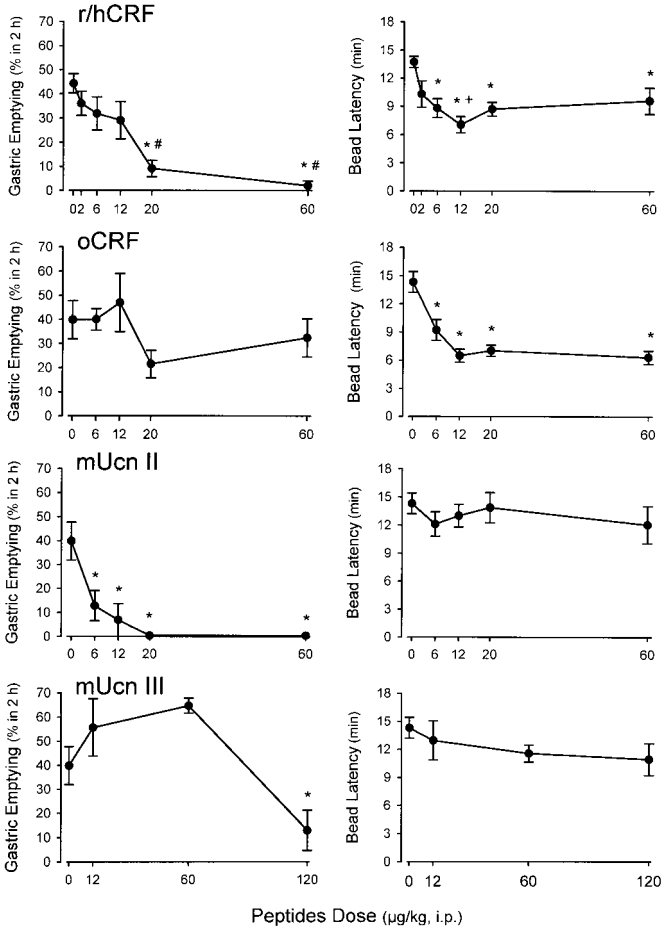


**Fig. 1.** Dose-related effects of r/hCRF, oCRF, mUcn II, and mUcn III injected i.p. on gastric emptying of a solid nutrient meal (left) and stimulation of distal colonic transit time (right) in conscious mice. Groups of fasted mice were given chow ad libitum for 1 h; then, under short anesthesia they were injected i.p. with either saline or peptide, and a glass bead was inserted into the distal colon 2 cm proximal from the anus. Gastric emptying of the ingested meal 2 h after peptide administration and the time for bead expulsion were monitored in the same animal. Data represent the mean ± S.E. of 6–12, 4–5, 5, and 3–4 mice per group for r/hCRF, oCRF, mUcn II, and mUcn III, respectively. r/hCRF: $F(5,39) = 10.1915$, $P = 0.0001$ for gastric emptying and $F(5,39) = 5.074$, $P = 0.001$ for colonic transit. Ovine CRF: $F(4,22) = 14.967$, $P < 0.01$ for colonic transit. Mouse Ucn II: $F(4,23) = 8.360$, $P < 0.001$ for gastric emptying. Mouse Ucn III: $F(3,14) = 4.96$, $P = 0.015$ for gastric emptying. ⋆, $P < 0.05$ compared with vehicle-treated animals; #, $P < 0.05$ compared with r/hCRF at 2, 6, or 12 μg/kg; +, $P < 0.05$ compared with r/hCRF at 2 μg/kg.

nist, astressin (30, 60, and 120 μg/kg, i.p.) dose dependently prevented i.p. r/hCRF-induced inhibition of gastric emptying and the acceleration of distal colonic transit (Fig. 2). Complete significant blockade of i.p. r/hCRF actions on gastric and distal colonic transit was achieved by astressin at doses of 60 and 120 μg/kg, respectively (Fig. 2).

The selective $CRF_2$ receptor antagonist antisauvagine-30 (30, 50, and 100 μg/kg) dose dependently prevented the i.p. r/hCRF (20 μg/kg, $n = 6$)-induced inhibition of gastric emptying, whereas the concomitant reduction in the distal colonic transit time was not influenced (Fig. 2). Complete reversal of i.p. r/hCRF-induced inhibition of gastric emptying was observed at 50 μg/kg of antisauvagine-30 (Fig. 2).

Pretreatment with the selective $CRF_1$ receptor antagonist NBI-27914 (5, 10, or 20 mg/kg, i.p.) dose dependently blocked the r/hCRF-induced simulation of distal colonic transit without influencing the gastric inhibitory response (Fig. 3). In vehicle (DMSO/saline) + saline-treated mice, bead latency was 13.5 ± 1.6 min ($n = 6$) and was reduced to 7.9 ± 0.8 min in the vehicle + r/hCRF group ($n = 8$; $P < 0.05$ versus vehicle + saline; Fig. 3). NBI-27914, at 10 and 20 mg/kg, dose dependently prevented the r/hCRF effect on the colon by 61% and 100% (bead latency; 11.3 ± 1.5 and 15.1 ± 1.8 min, respectively; $n = 7–8$ for each group; $P < 0.05$ versus vehicle + r/hCRF), whereas a lower dose (5 mg/kg) had no effect (bead latency: 7.6 ± 1.2 min, $n = 4$; $P > 0.05$ versus vehicle + r/hCRF; Fig. 3). In the same animals, r/hCRF-induced delay in gastric emptying (11 ± 4%; $P < 0.05$ versus vehicle

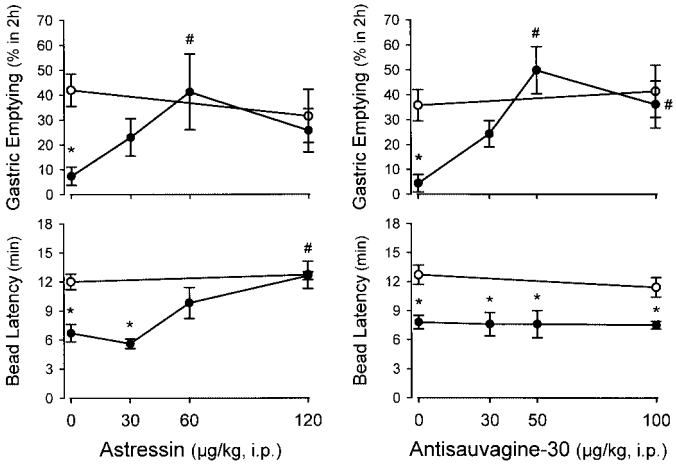


**Fig. 2.** Dose-related effects of the nonselective $CRF_1/CRF_2$ receptor antagonist, astressin, and the selective $CRF_2$ receptor antagonist, antisauvagine-30, on the inhibition of gastric emptying of a solid nutrient meal (top) and stimulation of distal colonic transit time (bottom) induced by peripheral r/hCRF in conscious mice. Protocols are the same as detailed in Fig. 1 except that astressin, antisauvagine-30, and vehicle (water) were injected i.p. 10 min before i.p. injection of r/hCRF or saline. Data represent the mean ± S.E. of five to seven animals per group. ⋆, $P < 0.05$ versus vehicle + saline, astressin + saline, or antisauvagine-30 + saline; #, $P < 0.05$ versus CRF + vehicle. ○, vehicle (0.1 ml, i.p.); ●, r/hCRF (20 μg/kg, i.p.).

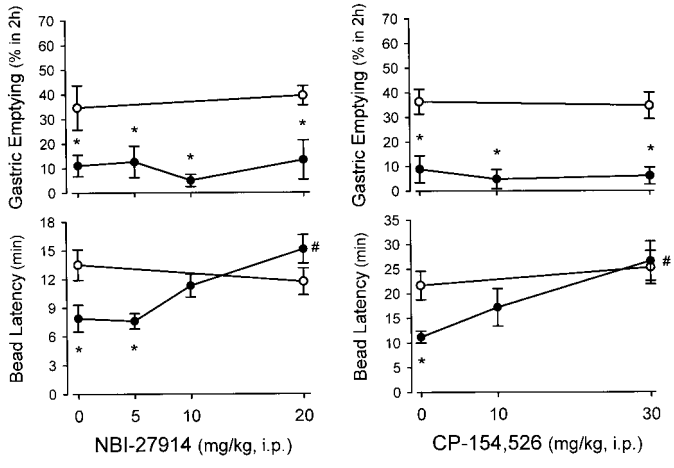


**Fig. 3.** Effects of the selective $CRF_1$ receptor antagonists NBI-27914 and CP-154,526 on the inhibition of gastric emptying of a solid nutrient meal (top) and stimulation of distal colonic transit time (bottom) induced by peripheral r/hCRF in conscious mice. Protocols are the same as in Fig. 2 except that CP-154,526 or its vehicle was injected i.p. 30 min before that of i.p. r/hCRF. Data represent the mean ± S.E. of four to eight animals per group. ⋆, $P < 0.05$ versus vehicle + saline, NBI-27914 (20 mg/kg) + vehicle, or CP-154,526 (30 mg/kg) + saline; #, $P < 0.05$ versus vehicle + CRF. ○, vehicle (0.1 ml, i.p.); ●, r/hCRF (20 μg/kg, i.p.).

+ saline, 35 ± 9%) was not influenced by NBI-27914 (13 ± 6%, 5 ± 2%, and 13 ± 8%, at the doses of 5, 10, and 20 mg/kg, respectively, $n = 4-8$ per group; Fig. 3).

Similar results were obtained with the selective $CRF_1$ receptor antagonist CP-154,526 (Fig. 3). In vehicle (saline/cremophor EL/DMSO, 90:5:5, pH ~5.1) + saline- treated mice, the bead latency time was 21.7 ± 2.9 min ($n = 7$). In vehicle-pretreated mice, r/hCRF (20 μg/kg) reduced bead latency to 11.2 ± 1.2 min ($n = 9$; $P < 0.05$ versus vehicle + saline). CP-154,526 at 10 and 30 mg/kg reversed by 43% and 100%, respectively, the effects of r/hCRF on the colonic bead latency time (17.2 ± 3.8 min and 26.6 ± 4.0 min, $n = 6$ and 9; $P < 0.05$ versus vehicle + r/hCRF; Fig. 3). The gastric emptying of a solid meal in vehicle + saline-treated mice was 36 ± 5%. R/hCRF reduced gastric emptying to 9 ± 5% ($n = 9$; $P < 0.05$ versus vehicle + saline). CP-154,526, either at the dose of 10 or 30 mg/kg, did not modify the r/hCRF-induced delay in gastric emptying (5 ± 4% and 6 ± 3%, respectively, $n = 6$ and 9; $P > 0.05$ versus vehicle + saline; Fig. 3). It is interesting to note that the control vehicle (saline/cremophor EL/DMSO, 90:5:5, pH ~5.1) used in this experiment resulted in higher basal values of colonic transit time compared with other vehicle groups (Fig. 3). However, r/hCRF had effects similar to those observed in other experimental conditions.

None of the CRF receptor antagonists, tested by themselves, influenced either the latency time of bead expulsion or the gastric emptying of the solid meal compared with their respective control vehicle-treated groups (Figs. 2 and 3).

## Discussion

The present study shows that r/hCRF administered peripherally exerts opposite propulsive effects on the proximal (stomach) and distal (colon) segments of the gastrointestinal tract, which were simultaneously monitored in conscious mice. The i.p. injection of r/hCRF at 2, 6, 12, 20, or 60 μg/kg resulted in a long-lasting (up to 2 h) dose-dependent 19 to 95% inhibition of gastric emptying of ingested chow and shortened the expulsion time of the bead inserted into the distal colon by 24, 36, 49, 36, and 30%, respectively. Consistent with our observations, a previous report showed that r/hCRF suppressed gastric emptying of a solid meal in mice up to 8 h after i.p. injection, although only a single high dose (400 μg/kg) was tested in this study (Asakawa et al., 1999). The long-lasting effect of r/hCRF may be related to its half-life, which was estimated to be 151 min from elimination kinetics studies in mice (Martins et al., 1997). Likewise, CRF induces a long-lasting and dose-dependent inhibition of gastric emptying of a non-nutrient liquid solution when administered i.p. in mice (Sheldon et al., 1990) and i.v. in dogs and rats (Pappas et al., 1985; Taché et al., 1987; Williams et al., 1987; Nozu et al., 1999). The present data provide the first evidence in mice that peripheral injection of r/hCRF stimulates colonic transit. In rats, i.p. or i.v. injection of r/hCRF dose dependently accelerates ceco-colonic motility and transit, and induces defecation (Williams et al., 1987; Maillot et al., 2000). Taken together, these findings show convergent evidence across species that peripherally administered r/hCRF exerts an inhibitory effect on gastric transit of either a solid meal or noncaloric solution, while simultaneously stimulating colonic propulsive activity.

The alterations of gastric and colonic transit induced by i.p. r/hCRF in mice are CRF receptor-mediated. The $CRF_1/CRF_2$ receptor antagonist astressin completely prevented both the 95% inhibition of gastric emptying of the solid meal and the 50% shortening of the distal colonic transit time induced by r/hCRF (20 μg/kg) at an antagonist/agonist ratio of 3:1 and 6:1, respectively. In rats, astressin also completely antagonized r/hCRF-induced delayed gastric emptying of a non-nutrient solution and defecation (Martínez et al., 1999; Maillot et al., 2000).

Convergent evidence using selective $CRF_2$ receptor agonists and antagonists demonstrates that i.p. r/hCRF-induced delayed gastric emptying of a solid meal in mice is $CRF_2$ receptor-mediated. Mouse Ucn II and mUcn III injected i.p. inhibited gastric emptying of a solid meal, providing the first evidence that these selective $CRF_2$ receptor agonists are biologically active upon peripheral administration in mice. We found that mUcn II is more potent than mUcn III as shown by the 83% inhibition of gastric emptying at 12 μg/kg, whereas a 10-fold higher dose is required for mUcn III to have a similar effect. The potency of mUcn II may have a bearing on the higher binding affinities and functional activity on $CRF_2$ receptors as shown in vitro on stably transfected cells (Lewis et al., 2001; Table 1). However, additional factors related to intrinsic pharmacodynamic and chemical properties of these peptides, as recently reported for r/hCRF, oCRF, and rUcn (Brauns et al., 2001), may also contribute to the observed differences in potencies. Consistent with the primary role of $CRF_2$ receptors, we also found that oCRF, which has preferential affinity to the $CRF_1$ receptor (Behan et al., 1998; Eckart et al., 2001; Table 1), did not delay gastric emptying.

The primary role of $CRF_2$ receptors on gastric emptying was further characterized by the use of selective CRF receptor subtype antagonists. The administration of the selective $CRF_1$ receptor antagonists NBI-27914 and CP-154,526 (Chen et al., 1996; Schulz et al., 1996) did not alter the i.p. r/hCRF-induced delayed gastric emptying of a solid meal in mice. In contrast, the newly developed, selective $CRF_2$ receptor antagonist, antisauvagine-30, injected i.p., completely prevented the i.p. r/hCRF-induced 97% inhibition of gastric emptying of a solid meal. Antisauvagine-30 has been previously used to ascertain the role of $CRF_2$ receptors in several behavioral tests and on peripheral rat Ucn-induced inhibition of gastric emptying and food intake in mice (Radulovic et al., 1999; Lu et al., 2000; Pelleymounter et al., 2000; Wang et al., 2001).

The present study also provides pharmacological evidence for a role of $CRF_1$ receptors in the stimulatory action of r/hCRF on distal colonic transit in mice. First, the preferential $CRF_1$ receptor agonist oCRF (Behan et al., 1996; Eckart et al., 2001) shortened significantly the distal colonic transit time by 50 to 55% at i.p. doses that did not influence gastric emptying. Second, r/hCRF significantly stimulated distal colonic transit at a lower dose (6 μg/kg) than that required to significantly inhibit gastric emptying (20 μg/kg). These results are consistent with r/hCRF displaying higher binding affinity for $CRF_1$ than for $CRF_2$ receptors (Behan et al., 1996; Vaughan et al., 1996; Perrin and Vale, 1999; Eckart et al., 2001; Table 1). Third, the selective $CRF_2$ receptor agonists mUcn II and mUcn III did not affect colonic transit when injected i.p. at doses that inhibited gastric emptying. Lastly, we showed that NBI-27914 and CP-154,526, injected periph-

erally, dose dependently prevented i.p. r/hCRF-induced shortening of the bead expulsion time, resulting in the complete normalization of the distal colonic transit time. In rats, we reported that CP-154,526, injected subcutaneously at a similar dose range, prevented peripheral r/hCRF-induced stimulation of colonic motor function (Maillot et al., 2000). Collectively these data show that $CRF_1$ receptors primarily mediate peripheral r/hCRF-induced stimulation of proximal and distal colonic motor activity in rodents.

These observations may have relevance to human pathophysiology. Intravenous injection of r/hCRF increased segmental contractions of sigmoid colon in healthy subjects and more so in patients with irritable bowel syndrome (Fukudo et al., 1998). In contrast, we reported that oCRF did not influence postprandial antral motility in healthy subjects (Mayer et al., 1992) when injected i.v. at a dose which significantly elevated serum cortisol levels, a response mediated by the activation of $CRF_1$ receptors (Turnbull and Rivier, 1997). These findings in humans are consistent with a $CRF_1$ receptor-mediated action of r/hCRF to stimulate colonic motility, whereas this receptor subtype does not influence gastric motor activity. The possible implications of $CRF_1$ receptors in the pathophysiology of irritable bowel syndrome have recently been reviewed (Heinrichs and Taché, 2001).

The exact sites at which peripherally injected r/hCRF induces a differential CRF receptor-mediated action on gastric and colonic transit need to be further elucidated. In vitro studies in rats established a direct action of r/hCRF in the stomach and the colon as shown by the reduction of spontaneous contractile activity through a tetrodotoxin-sensitive mechanism in longitudinal antral muscle strips (Raybould et al., 1990) and the increased mechanical and myoelectrical activity in isolated distal colonic preparations (Mancinelli et al., 1998; Maillot et al., 2000). Specific CRF binding has been reported in guinea pig cecal smooth muscles cells (Iwakiri et al., 1996), and $CRF_1$ and $CRF_2$ receptors have been found to be expressed in the lamina propria of the human colon (Muramatsu et al., 2000). However, the cellular distribution of CRF receptor subtypes in the stomach or colon is largely unknown, particularly at the level of the colonic myenteric nervous system where electrophysiological and functional studies indicate a possible direct excitatory action of r/hCRF (Hanani and Wood, 1992; Miampamba et al., 2002).

None of the nonselective and selective CRF receptor antagonists, at doses preventing exogenous CRF actions, influenced gastric emptying or distal colonic transit time on their own, indicating that CRF pathways do not modulate postprandial transit in mice. However, there are indications that peripheral administration of nonselective CRF receptor antagonists alleviates postoperative gastric ileus in rats and that activation of CRF receptors comes into play to activate colonic motility under stress (Martínez et al., 1999; Maillot et al., 2001).

In summary, we showed that r/hCRF injected i.p. dose dependently inhibited gastric emptying of a solid nutrient meal while stimulating distal colonic propulsion when monitored simultaneously in conscious mice. The newly identified selective ligands for the $CRF_2$ receptors mUcn II and, less potently, mUcn III, injected i.p., dose dependently inhibited gastric emptying while not influencing colonic transit. Conversely, the preferential $CRF_1$ receptor agonist oCRF increased distal colonic propulsion without altering gastric emptying. The actions of r/hCRF are CRF receptor-mediated as shown by the blockade of gastric and colonic responses by the $CRF_1/CRF_2$ receptor antagonist astressin. In addition, the $CRF_2$ receptor antagonist antisauvagine-30 selectively prevented i.p. r/hCRF-induced delayed gastric emptying, whereas the $CRF_1$ receptor antagonists, NBI-27914 and CP-154,526, selectively inhibited the stimulating action of r/hCRF on colonic transit. Collectively these data support a differential role of CRF receptor subtypes 2 and 1 in mediating peripheral r/hCRF-induced gastric stasis and stimulation of colonic propulsive activity, respectively. These findings may provide new venues for selective drug targeting of these receptor subtypes under stress-related activation of CRF pathways, leading to disturbances of gut motor function.

**Acknowledgments**

We thank Drs. E. B. De Souza and D. Grigoriadis (Neurocrine Biosciences, La Jolla, CA) for the generous donation of NBI-27914 and Dr. E. D. Pagani (Central Research Division, Pfizer Inc., Groton, CT) for the supply of CP-154,526. P. Kirsch is acknowledged for helping in the preparation of the manuscript.

**References**

Asakawa A, Inui A, Ueno N, Makino S, Fujino MA, and Kasuga M (1999) Urocortin reduces food intake and gastric emptying in lean and ob/ob obese mice. *Gastroenterology* **116:**1287–1292.

Baigent SM and Lowry PJ (2000) mRNA expression profiles for corticotrophin-releasing factor (CRF), urocortin, CRF receptors and CRF-binding protein in peripheral rat tissues. *J Mol Endocrinol* **25:**43–52.

Barrachina MD, Martínez V, Wei JY, and Taché Y (1997) Leptin-induced decrease in food intake is not associated with changes in gastric emptying in lean mice. *Am J Physiol* **272:**R1007–R1011.

Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, Liaw C, and De Souza EB (1996) Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. *Mol Psychiatry* **1:**265–277.

Brauns O, Liepold T, Radulovic J, and Spiess J (2001) Pharmacological and chemical properties of astressin, antisauvagine-30 and $\alpha$-helCRF: significance for behavioral experiment. *Neuropharmacology* **41:**507–516.

Chen C, Dagnino R Jr, De Souza EB, Grigoriadis DE, Huang CQ, Kim KKI, Liu Z, Moran T, Webb TR, Whitten JP, et al. (1996) Design and synthesis of a series of non-peptide high affinity human corticotropin-releasing factor receptor antagonists. *J Med Chem* **39:**4358–4360.

Donaldson CJ, Sutton SW, Perrin MH, Corrigan AZ, Lewis KA, Rivier JE, Vaughan JM, and Vale WW (1996) Cloning and characterization of human urocortin. *Endocrinology* **137:**2167–2170.

Eckart K, Jahn O, Radulovic J, Tezval H, Van Werven L, and Spiess J (2001) A single amino acid serves as an affinity switch between the receptor and the binding protein of corticotropin-releasing factor: implications for the design of agonists and antagonists. *Proc Natl Acad Sci USA* **98:**11142–11147.

Fukudo S, Nomura T, and Hongo M (1998) Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. *Gut* **42:**845–849.

Gulyas J, Rivier C, Perrin M, Koerber SC, Sutton S, Corrigan A, Lahrichi SL, Craig AG, Vale W, and Rivier J (1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc Natl Acad Sci USA* **92:**10575–10579.

Hanani M and Wood JD (1992) Corticotropin-releasing hormone excites myenteric neurons in the guinea-pig small intestine. *Eur J Pharmacol* **211:**23–27.

Heinrichs SC and Taché Y (2001) Therapeutic potential of CRF receptor antagonists: a gut-brain perspective. *Expert Opin Investig Drugs* **10:**647–659.

Higelin J, Py-Lang G, Paternoster C, Ellis GJ, Patel A, and Dautzenberg FM (2001) $^{125}$I-Antisauvagine-30 a novel and specific high-affinity radioligand for the characterization of corticotropin-releasing factor type 2 receptors. *Neuropharmacology* **40:**114–122.

Hsu SY and Hsueh AJ (2001) Human stresscopin and stresscopin-related peptide are selective ligands for the type-2 corticotropin-releasing hormone receptor. *Nat Med* **7:**1–7.

Iwakiri Y, Chijiiwa Y, Motomura Y, Akiho H, Osame M, and Nawata H (1996) Direct inhibitory effect of corticotropin releasing hormone on isolated caecal circular smooth muscle cells of guinea pig via adenylate cyclase system. *Life Sci* **58:**2243–2249.

Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, et al. (2001) 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 USA* **98:**7570–7575.

Lu L, Liu D, Ceng X, and Ma L (2000) Differential roles of corticotropin-releasing factor receptor subtypes 1 and 2 in opiate withdrawal and in relapse to opiate dependence. *Eur J Neurosci* **12:**4398–4404.

Maciejewski-Lenoir D, Heinrichs SC, Liu X-J, Ling N, Tucker A, Xie Q, Lappi DA, and Grigoriadis DE (2000) Selective impairment of corticotropin-releasing factor$_1$

($CRF_1$) receptor-mediated function using CRF coupled to saporin. *Endocrinology* **141:**498–505.
Maillot C, Million M, Wei JY, Gauthier A, and Taché Y (2000) Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. *Gastroenterology* **119:**1569–1579.
Mancinelli R, Azzena GB, Diana M, Forgione A, and Fratta W (1998) In vitro excitatory actions of corticotropin-releasing factor on rat colonic motility. *J Auton Pharmacol* **18:**319–324.
Martins JM, Banks WA, and Kastin AJ (1997) Transport of CRH from mouse brain directly affects peripheral production of β-endorphin by the spleen. *Am J Physiol* **273:**E1083–E1089.
Martínez V, Rivier J, and Taché Y (1999) Peripheral injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks peripheral CRF- and abdominal surgery-induced delayed gastric emptying in rats. *J Pharmacol Exp Ther* **290:**629–634.
Mayer EA, Sytnik B, Reddy NS, Van Deventer G, and Taché Y (1992) Corticotropin releasing factor (CRF) increases post-prandial duodenal motor activity in humans. *J Gastrointest Motil* **4:**53–60.
Miampamba M, Maillot C, Million M, and Taché Y (2002) Peripheral corticotropin-releasing factor (CRF) activates myenteric neurons in the proximal colon through $CRF_1$ receptor in conscious rats. *Am J Physiol*, in press.
Muramatsu Y, Fukushima K, Iino K, Totsune K, Takahashi K, Suzuki T, Hirasawa G, Takeyama J, Ito M, Nose M, et al. (2000) Urocortin and corticotropin-releasing factor receptor expression in the human colonic mucosa. *Peptides* **21:**1799–1809.
Nozu T, Martínez V, Rivier J, and Taché Y (1999) Peripheral urocortin delays gastric emptying: role of CRF receptor 2. *Am J Physiol* **276:**867–875.
Pappas T, Debas H, and Taché Y (1985) Corticotropin-releasing factor inhibits gastric emptying in dogs. *Regul Pept* **11:**193–199.
Pelleymounter MA, Joppa M, Carmouche M, Cullen MJ, Brown B, Murphy B, Grigoriadis DE, Ling N, and Foster AC (2000) Role of corticotropin-releasing factor (CRF) receptors in the anorexic syndrome induced by CRF. *J Pharmacol Exp Ther* **293:**799–806.
Perrin MH, Sutton SW, Cervini LA, Rivier JE, and Vale WW (1999) Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of CRF receptors. *J Pharmacol Exp Ther* **288:**729–734.
Perrin MH and Vale WW (1999) Corticotropin releasing factor receptors and their ligand family. *Ann N Y Acad Sci* **885:**312–328.
Radulovic J, Ruhmann A, Liepold T, and Spiess J (1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *J Neurosci* **19:**5016–5025.
Raybould HE, Koelbel CB, Mayer EA, and Taché Y (1990) Inhibition of gastric motor function by circulating corticotropin-releasing factor in anesthetized rats. *J Gastrointest Motil* **2:**265–272.
Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, and Sawchenko PE (2001) 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 USA* **98:**2843–2848.
Schulz DW, Mansbach RS, Sprouse J, Braselton JP, Collins J, Corman M, Dunaiskis A, Faraci S, Schmidt AW, Seeger T, et al. (1996) CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. *Proc Natl Acad Sci USA* **93:**10477–10482.
Sheldon RJ, Qi JA, Porreca F, and Fisher LA (1990) Gastrointestinal motor effects of corticotropin-releasing factor in mice. *Regul Pept* **28:**137–151.
Taché Y, Maeda-Hagiwara M, and Turkelson CM (1987) Central nervous system action of corticotropin-releasing factor to inhibit gastric emptying in rats. *Am J Physiol* **253:**G241–G245.
Turnbull AV and Rivier C (1997) Corticotropin-releasing factor (CRF) and endocrine response to stress: CRF receptors, binding protein, and related peptides. *Proc Soc Exp Biol Med* **215:**1–10.
Vale W, Spiess J, Rivier C, and Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and b-endorphin. *Science (Wash DC)* **213:**1394–1397.
Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, and Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* **428:**191–212.
Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, et al. (1996) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature (Lond)* **378:**287–292.
Wang L, Martínez V, Rivier JE, and Taché Y (2001) Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. *Am J Physiol* **281:**R1401–R1410.
Williams CL, Peterson JM, Villar RG, and Burks TF (1987) Corticotropin-releasing factor directly mediates colonic responses to stress. *Am J Physiol* **253:**G582–G586.
Yamada K and Onoda Y (1992) Effects of trimebutine on colonic propulsion in mice. *J Smooth Muscle Res* **28:**87–93.

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