Antagonism of corticotrophin-releasing factor receptors in the fourth ventricle modifies responses to mild but not restraint stress

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Miragaya JR, Harris RB. Antagonism of corticotrophin-releasing factor receptors in the fourth ventricle modifies responses to mild but not restraint stress. Am J Physiol Regul Integr Comp Physiol 295: R404–R416, 2008. First published June 11, 2008; doi:10.1152/ajpregu.00565.2007.—Repetitive restraint stress (RRS; 3 h of restraint on 3 consecutive days) in rodents produces temporary hypophagia, but a long-term downregulation of body weight. The mild stress (MS) of an intraperitoneal injection of saline and housing in a novel room for 2 h also inhibits food intake and weight gain, but the effects are smaller than for RRS. Previous exposure to RRS exaggerates hypophagia, glucocorticoid release, and anxiety-type behavior caused by MS. Here we tested the involvement of brain stem corticotrophin-releasing factor receptors (CRFR) in mediating energetic and glucocorticoid responses to RRS or MS and in promoting stress hyperresponsiveness in RRS rats. Administration of 1.3 nmol chCRF1(9-41), a nonspecific CRFR antagonist, exaggerated hypophagia and weight loss in both RRS and MS rats, whereas 0.26 nmol had no effect in RRS or MS rats. In contrast, 2 nmol of the nonspecific antagonist astressin had no effect on weight loss or hypersensitivity to subsequent MS in RRS rats, but blocked weight loss and inhibition of food intake caused by MS alone. MS rats infused with 3 nmol antisauvagine-30, a CRFR2 antagonist, did not lose weight in the 48 h after MS, but 0.3 nmol did not prevent weight loss in MS rats. These data suggest that inhibition of food intake and weight loss induced by RRS or by MS involve different pathways, with hindbrain CRFR mediating the effect of MS on body weight and food intake. Hindbrain CRFR do not appear to influence stress-induced corticosterone release in RRS rats.

THE NEUROPEPTIDE CORTICOTROPHIN releasing factor (CRF) and its homologues urocortin (Ucn), Ucn II and Ucn III, have been identified as initiators of behavioral and physiological responses to stress (4, 50). CRF has two G protein-coupled receptor subtypes: CRFR1 and CRFR2 (8, 43), and CRFR2 presents two variants (CRFR2β and CRFR2α). CRFR2α prevails in neural tissue (31), whereas CRFR2β is expressed in areas such as heart, gastrointestinal tract, arteries, and muscle (26, 31). Studies with knockout and transgenic mice suggest that CRFR2 mediate behavioral responses (2–4, 38) to stress, including inhibition of food intake and body weight loss (14, 37, 38, 45), whereas CRFR1 appears to be more important in controlling activity of the hypothalamic-pituitary-adrenal (HPA) axis and anxiety behaviors (40, 47). Many sites in the brain are involved in the initial response to a stressor and multiple sites express CRFR. Nuclei that have been demonstrated to control food intake or energy expenditure (18, 55, 56) and also express CRFR1 and/or CRFR2 include the dorsomedial, arcuate, paraventricular, lateral, and ventromedial nuclei of the hypothalamus, the area postrema, the nucleus of the solitary tract, and the dorsal raphe nuclei (53).

Humans exposed to severe stress may either gain or lose body weight (13) because food intake can either increase or decrease in adults according to the type and severity of stress that is experienced (54). In contrast to humans, rats and mice consistently lose weight in response to stress. Several investigators have demonstrated a decrease in body weight of rats exposed to acute stress (13, 46) or following infusion of CRF into the third ventricle (28). We have previously demonstrated that rats exposed to repeated restraint stress (RRS) for 3 h a day for three consecutive days, decrease their food intake and lose body weight on the days of stress (25). Three days of restraint is used because increasing the number of days of restraint does not exaggerate the weight loss experienced by the rats (23). The rats are not hyperphagic in the poststress period and do not compensate for the weight loss (25). Therefore, the weight of RRS rats remains lower than that of controls for extended periods of time (24). Others have reported that chronically stressed animals show an exaggerated release of ACTH and corticosterone in response to a novel stress (15, 17). Similarly, animals submitted to a mild physical stress (MS) 12 days after RRS show exaggerated hypophagia, corticosterone release, and anxiety-type behavior compared with those that have not previously been subjected to RRS (11, 22).

In a previous study, we found that injections of a nonspecific receptor antagonist [chCRF1(9-41)] into the third ventricle of rats before restraint on each day of RRS blocked hypophagia and weight loss, but could not stop stress-induced activation of the HPA axis (46), suggesting that the area responsible for hypophagia is in or near the hypothalamus and functions independently of pathways that activate the HPA axis. Because injections into the fourth ventricle could potentially diffuse into the fourth ventricle and act on nuclei in the hindbrain, we cannot exclude the brain stem as a mediator of changes in food intake and body weight of stressed rats. Grill and Kaplan (19) demonstrated that infusion of Ucn in the fourth ventricle inhibited food intake in rats (20), although the inhibition was less than that caused by Ucn infusion into the lateral ventricle. Therefore, the objective of this study was to test whether antagonism of CRFRs located adjacent to the fourth ventricle would modify the changes in food intake, body weight, and corticosterone release that are induced by RRS or by a less severe MS and whether antagonism of CRFRs during restraint would prevent the subsequent hyperresponsivity of RRS rats towards MS. The MS used in the studies described here consisted of an intraperitoneal injection of saline and housing in a novel room for 2 h. We have previously shown that this stress causes a significant increase in serum corticoste-
rone, inhibits food intake, and causes transient weight loss in rats and mice (22, 29), and others have reported that a saline injection causes a significant increase in serum corticosterone and ACTH in mice (41).

METHODS

All experiments described here used male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) that weighed ~275 g on arrival at University of Georgia, Athens. The rats were housed in wire hanging cages in temperature and humidity-controlled rooms (23°C, 55% humidity) with lights on from 7:00 AM to 7:00 PM. The rats had free access to chow (Purina Rodent Chow 5012: Purina Mills, MO) and water, unless stated otherwise. All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guiding Principles of the American Physiological Society (1).

Experiment 1: fourth ventricle infusions of 1.3 nmol ohCRF(9-41) in RRS rats. This experiment tested whether infusions of a nonselective CRFR antagonist, α-helical CRF(9-41) (ohCRF), into the fourth ventricle would block the weight loss, inhibition of food intake, and subsequent increased adrenal responsiveness to MS caused by RRS. The dose of antagonist used in this study was selected because it was one-half of the amount that had been shown to prevent sustained weight loss in RRS rats when it was infused into the third ventricle (46). It was also twice the molar amount of Ucn that was used to show an effect of fourth ventricle CRFR activation on food intake and body weight of rats (20).

Experimental design is illustrated as a flow chart in Fig. 1. Ninety-five male rats were included in this experiment in three cohorts with treatment groups equally represented within each cohort. All rats were fitted with 26-gauge guide cannulas (Plastics One, Roanoke, VA) aimed at the fourth ventricle. Rats were anesthetized with ketamine/xylazine mix (90 mg/kg ketamine, 10 mg/kg xylazine) given by intraperitoneal injection. Coordinates for cannula placement in relation to the midline at the occipital suture were 2.5 mm anterior, 0 mm lateral, and 5.2 mm ventrodorsal (46), according to the Paxinos and Watson brain atlas (36). Immediately after surgery and again the next day the rats were injected with analgesic [2 mg/kg ketoprofen (Ketofen); Fort Dodge Animal Health, Fort Dodge, Iowa]. One week after surgery the position of the cannula was confirmed by measuring glucoprivation-induced hyperglycemia. Baseline blood glucose concentration was measured from tail blood using glucose strips (Accu- met glucometer; Boehringer-Mannheim). Each rat received an intracerebroventricular infusion of 210 μg 5-thio-D-glucose (Sigma-Aldrich, St Louis, MO) in 2 μl sterile isotonic saline over 1 min. All infusions were from Hamilton syringes controlled by an infusion pump (model PHD 2000; Harvard Apparatus, Holliston, MA). Those rats that showed a doubling of blood glucose 60 min after the infusion were included in the experiment. Approximately 10% of rats were excluded from the experiment based on this test. Rats were allowed 1 wk of recovery before the beginning of the experiment.

Daily food intake, water intake, and body weight were recorded throughout the experiment. Baseline measurements were made for 6 days, and then the rats were divided into four weight-matched groups of 20–22 rats: RRS/ohCRF, RRS/saline, control/ohCRF, and control/saline. On each day of the 3 days of restraint, 10 min before the beginning of each restraint, all rats received a 2-μl infusion of saline or 1.3 nmol (5 μg) of ohCRF(9-41) (Bachem Bioscience, King of Prussia, PA) into the fourth ventricle over 1 min. RRS rats were placed in Perspex restraining (21.6 × 6.4 cm) tubes (Plas Labs, Lansing, MI) for 3 h on each of three consecutive days. Infusions started at 8:30 AM each day so that the rats were restrained during the early part of the light phase. The control rats were placed in shoe box cages in the same room as the restrained rats. All rats were food and water deprived during the 3 h of restraint. Corticosterone levels were
measured at 0 and 60 min on day 2 of restraint in blood samples collected by tail bleeding. Twelve days after the end of RRS, one-half of the rats from each group were submitted to a MS, whereas the other one-half served as controls (n = 10 or 11). Starting at 9:00 AM, rats exposed to MS received a 2-ml ip injection of saline and were placed in new cages in a novel room for 2 h. Control rats were picked up but replaced in their home cages. Both groups were food and water deprived during the 2 h of MS. Corticosterone levels were measured at 0, 15, 30, 60, 90, and 120 min after the start of MS in blood samples collected by tail-bleeding.

Experiment 2: fourth ventricle infusions of a lower dose of \( \text{hCRF}_{(9-41)} \) in RRS rats. The results of the previous experiment suggested that fourth ventricle \( \text{hCRF}_{(9-41)} \) had agonist-like properties, exaggerating the effects of stress on body weight and food intake in RRS rats. Menzaghi et al. (35) reported development of agonist-like activity when increasing doses of \( \text{hCRF} \) were infused into the lateral ventricle; therefore, this study tested the effects of one-fifth the amount of \( \text{hCRF}_{(9-41)} \) that was used in experiment 1 on body weight and food intake in RRS rats. The rats were not exposed to MS at the end of the study because we did not find any effect of the high dose of \( \text{hCRF}_{(9-41)} \), in the previous experiment. In addition, we did not collect blood to measure corticosterone on the day 2 of RRS to minimize exposure to nonspecific stressors.

Forty rats were fitted with fourth ventricle cannulae and cannula placement tested as described above. Baseline measures of food intake and body weight were recorded for 7 days starting 1 wk after confirming cannula placement. The rats were divided into four weight-matched groups of 10 rats each: control/saline, RRS/saline, control/\( \text{hCRF} \), and RRS/\( \text{hCRF} \). The saline rats received fourth ventricle infusions of 2 \( \mu \)l saline, and the \( \text{hCRF} \) groups received infusions of 0.26 nmol (1 \( \mu \)g) \( \text{hCRF}_{(9-41)} \) in 2 \( \mu \)l saline. Infusions started at 8:00 AM. Ten minutes after infusion, the RRS rats were placed in restraint tubes and the controls were placed in shoe box cages in the same room as RRS rats, as described above. After 3 h, the rats returned to their home cages. The same procedure was followed for two more days. Daily body weights and food intakes of the rats were recorded for 10 days after the end of RRS (day 13 of the experimental period).

Experiment 3: fourth ventricle infusion of \( \text{hCRF}_{(9-41)} \) in MS rats. This experiment tested whether \( \text{hCRF}_{(9-41)} \) infusions into the fourth ventricle could block body weight loss and inhibition of food intake in rats exposed to MS.

Thirty-six rats were fitted with fourth ventricle cannulae, and, 7 days after testing cannula placement, the rats were divided into four weight-matched groups of nine rats each: control/saline, control/\( \text{hCRF} \), MS/saline, and MS/\( \text{hCRF} \). Starting at 9:00 AM, rats received a 2-\( \mu \)l infusion of either 1.3 nmol \( \text{hCRF} \) or saline in the fourth ventricle 10 min before the beginning of MS. Food intake was recorded for 2 days before and at 2, 4, 6, 12, and 12 h after exposure to MS. Body weight was also measured on the day of MS and 24 h after MS.

A second set of 66 rats were fitted with fourth ventricle cannulae. Seven days after testing, cannula placement rats were divided into six weight-matched groups of 11 rats each: control/saline, control/low \( \text{hCRF} \), control/high, \( \text{hCRF} \)/saline, MS/low \( \text{hCRF} \), and MS/high \( \text{hCRF} \). Starting at 9:00 AM rats received a 2-\( \mu \)l infusion of either saline or \( \text{hCRF} \) in the fourth ventricle 10 min before the beginning of MS. The low dose of \( \text{hCRF} \) was 0.26 nmol and the high dose was 1.3 nmol, the same doses as had been tested in experiments 1 and 2. A small blood sample was collected by tail bleeding immediately before infusion, and a second was collected 45 min after the start of MS. The rats were returned to their home cages after 2 h. Food intakes were recorded 2, 4, 12, 24, and 48 h after the end of MS. Body weights were recorded before and 24 and 48 h after the start of MS.

Experiment 4: fourth ventricle infusions of astressin in RRS rats. Results from experiment 1 suggested that \( \text{ohCRF} \) was acting as a partial agonist and exaggerating some aspects of the response to stress. For that reason, we repeated the experiment using a different nonselective CRFR antagonist, astressin (AST; American Peptides, Sunnyvale, CA). AST has similar binding affinity for CRFR1 and CRFR2 as \( \text{ohCRF} \) (57) but is a more potent antagonist, both in vivo and in vitro, possibly because it is more metabolically stable (21). The dose of AST used in this study was the same as that given intracerebroventricularly to block CRF-induced increases in gastric motility (33), but was twice the amount used in the lateral ventricle to block CRF-induced anxiety-type behavior (51).

Thirty-six rats were fitted with fourth ventricle cannulae. Experimental design was similar to experiment 1, except that 2 nmol AST was used as the CRFR antagonist. The number of animals was lower than in experiment 1 because we did not have control animals during MS; instead, all rats (n = 9/group) were submitted to MS 12 days after the end of RRS. Corticosterone levels were measured at 0, 30, 60, and 120 min during MS.

Experiment 5: fourth ventricle infusion of AST in MS rats. In experiment 3, \( \text{hCRF}_{(9-41)} \) appeared to produce a partial agonist effect, exaggerating the effects of MS on food intake. Therefore, the study was repeated using AST.

Thirty-four rats were fitted with fourth ventricle cannulae. Experimental design was similar to experiment 3, except that 2 nmol AST was used and food intake was measured at 2, 4, 6, 12, 24, and 48 h after the end of MS. Body weight also was measured before and 24 and 48 h after MS.

Experiment 6: fourth ventricle infusion of antisauvagine-30 in MS rats. AST infusion into the fourth ventricle blocked hypoglycemia and weight loss caused by MS in experiment 5. Because CRFR2 may mediate the effects of stress on food intake, this study tested the effects of specific antagonism of CRFR2 receptors with antisauvagine-30 (ASV-30; Phoenix Pharmaceuticals, Belmont, CA) in rats exposed to MS. The dose of ASV-30 used initially was one-third the lateral ventricle dose required to block stress-induced inhibition of food intake (44). Subsequently, we tested the effects of lower concentrations of ASV-30.

Thirty-three rats were used in the first part of this experiment. Experimental design was the same as experiment 5, except that 2.2 nmol (10 \( \mu \)g) ASV-30 was used to selectively inhibit CRFR2 (n = 8 or 9 per group). A second set of 64 rats was subjected to the same procedure except that they were infused with saline, 0.3 or 0.15 nmol (1 \( \mu \)g or 0.5 \( \mu \)g) ASV-30 (n = 10 or 11/group). Food intakes were measured at 2, 4, 12, 24, and 48 h after the end of MS and body weight was measured at 24 and 48 h after MS.

Statistical analysis. Animals tested in RRS experiments had their food intake and body weight analyzed by repeated-measures ANOVA (Statistica; Stat Software, Tulsa, OK). Baseline food intake and body weight measured immediately before the first restraint were used as covariates. A repeated-measures ANOVA was also used to analyze the body weight data in MS animals. Two-way ANOVA was used to compare cumulative food intakes at different time points in MS animals and to compare corticosterone concentrations. Time 0 for corticosterone was used as a covariate. Duncan’s Multiple Range Test was used for post hoc comparisons among all groups. Differences were considered statistically significant at P < 0.05.

RESULTS

Experiment 1: fourth ventricle infusions of \( 1.3 \) nmol \( \text{hCRF}_{(9-41)} \) in RRS rats. All RRS rats lost weight on the days of restraint, but RRS/\( \text{hCRF} \) rats lost more than RRS/saline rats (Fig. 2A; stress, P < 0.0001; time, P < 0.0001; stress \times time, P < 0.03; stress \times infusion, P < 0.03). The two groups of control rats weighed more than RRS rats, but were not
different from each other. Food intake of all of the rats was inhibited on the days of restraint, presumably due in part to the stress of handling and infusion. RRS/ohCRF rats ate less than any other group on the first 2 days of restraint and less than control/ohCRF rats on the 3rd day of restraint (Fig. 2B; stress, $P < 0.01$; time, $P < 0.0001$; and stress $\times$ infusion, $P < 0.05$). There was no difference in the daily food intakes of the rats on any other day of the experiment. When total intake for 3 days before restraint, during restraint, and after restraint was calculated, intakes on the days before and after stress were not different, but on the days of restraint RRS/ohCRF rats ate less than any other group (Fig. 2C). On the 2nd day of restraint, both groups of RRS rats had higher levels of corticosterone at 60 min compared with controls, but there was no effect of ohCRF in either RRS or control rats (Fig. 3A; stress, $P < 0.0001$). There was no effect of either ohCRF or of RRS on food intake or body weight measured 24 h after MS 12 days after the end of RRS (data not shown). MS caused a significant increase in corticosterone levels 15 min after the start of MS (Fig. 3B; MS, $P < 0.0001$), and RRS/saline/control rats had lower corticosterone levels than control/saline/MS, RRS/ohCRF/MS, or RRS/saline/MS rats (Fig. 3B). When saline- and ohCRF-infused rats were combined into groups that had been exposed to RRS or that had been controls, there was a significant effect of MS on serum corticosterone concentrations measured 15 and 30 min after the start of stress, but no additional effect of RRS (Fig. 3C: MS: $P < 0.003$).

Experiment 2: fourth ventricle infusions of a lower dose of ohCRF(9-41) in RRS rats. On the 1st day of restraint only the RRS/saline rats weighed less than controls, but from day 2 to the end of the experiment, both groups of RRS rats weighed less than the control groups and there was no effect of ohCRF on the weights of control or RRS rats (Fig. 4A; stress, $P < 0.0001$; time, $P < 0.0001$; stress $\times$ time, $P < 0.0001$). Both groups of RRS rats ate less than controls on each of the 3 days of restraint, but there were no differences between groups on any of the remaining days of the experiment (Fig. 4B; stress, $P < 0.06$; day, $P < 0.0001$; stress $\times$ time, $P < 0.0001$). When food intakes were averaged for 3 days before, during, and after restraint, the RRS/saline rats ate less than control/saline rats both during stress and during the 3 days after stress, whereas the food intake of RRS/ohCRF rats was inhibited only on the days of stress (Fig. 4C; stress, $P < 0.002$; time, $P < 0.0001$; stress $\times$ time, $P < 0.001$).

Experiment 3: fourth ventricle infusion of ohCRF(9-41) in MS rats. There was no effect of either MS or of 1.3 nmol ohCRF on 24-h weight gain of rats (Fig. 5A). Food intake during the 2 h after the end of MS and 4–6 h after MS was lower in MS/ohCRF than MS/saline rats (Fig. 5B; $P < 0.04$). Cumulative food intake of MS/ohCRF rats was significantly lower than for any other group at both 4 and 6 h after MS (Fig. 5C; $P < 0.03$), but there were no differences in food intakes 6 to 24 h after the end of MS or in cumulative intake 24 h after MS. In the second part of the study, when rats were infused with either 0.26 or 1.3 nmol ohCRF, there was no effect of MS or of ohCRF on body weights or food intakes of the rats at any time point (data not shown). All rats lost weight, and this may have been because the tail bleeding procedure for measuring serum corticosterone was a stressor in itself. Serum corticosterone tended to be increased in MS rats with the difference

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**Fig. 2.** Daily body weight (A), daily food intake (B), and cumulative food intake for 3 consecutive days before, during, and after repeated restraint stress (RRS) for rats in experiment 1 infused with 1.3 nmol a-helical corticotrophin-releasing factor (ohCRF; C). Values are means ± SE for 15–16 rats. A: *significant difference in body weight of RRS/ohCRF rats and all other groups ($P < 0.0001$); and #significant difference between control and RRS groups at $P < 0.05$. B: *significant difference between RRS/ohCRF rats and all other groups; $\Delta$significant difference between RRS/ohCRF and control/ohCRF rats. C: values for 3-day cumulative food intake on the days of RRS that do not share a common superscript are significantly different ($P < 0.002$).
being significant only in rats infused with 1.3 nmol ohCRF (Fig. 6; MS, \( P \leq 0.004 \)).

**Experiment 4: fourth ventricle infusions of AST in RRS rats.**

RRS rats lost weight on the days of restraint and regained weight after RRS at the same rate as controls, but did not return to the same body weight as the control rats, resulting in a significant difference in weight between control and RRS rats from *day 1* to the end of the experiment (Fig. 7A; stress, \( P < 0.0002 \); time, \( P < 0.0001 \)). AST had no effect on body weight of control or RRS animals. There was a significant decrease in
food intake during the stress period for all rats compared with baseline. RRS rats ate less than controls for the first 2 days of restraint, but there was no effect of AST (Fig. 7; time, $P < 0.0001$; stress × time, $P < 0.003$). When 3-day food intakes were compared before, during, and after RRS, it could be seen that intake of all of the rats returned to baseline levels as soon as restraint ended (Fig. 7C). At the end of the experiment, MS inhibited food intake and caused weight loss in all groups of rats, but there were no significant differences between the groups 24 h after MS (data not shown). There was no effect of AST infusion during RRS on corticosterone concentrations measured during MS; therefore, AST and saline groups were combined for RRS and for control treatment groups. Rats previously exposed to RRS had higher circulating concentrations of corticosterone 60 min after the start of MS compared...
Fig. 6. Serum corticosterone concentrations measured in rats infused with 0.3 or 0.15 nmol αhCRF (9-41) before MS in experiment 3. Data are means ± SE. Values for the 45-min time point that do not share a common superscript are significantly different.

with animals that had not been previously restrained (Fig. 7D; stress, P < 0.02).

Experiment 5: fourth ventricle infusion of AST in MS rats.

All control and MS rats lost weight after MS, but AST blocked the effect of MS on both food intake and body weight. MS/saline rats lost more weight than any other group at both 24 (Fig. 8A; infusion, P < 0.01; stress × infusion, P < 0.01) and 48 h after MS (Fig. 8B; infusion, P < 0.04; stress × infusion, P < 0.01). AST also blocked an MS-induced inhibition of food intake at 2–4 h after the end of MS. AST increased food intake of both control and MS rats between 12 and 24 h after MS (Fig. 8C; P < 0.02). In the period 24–48 h after MS the intake of MS/AST rats was higher than that of MS/saline rats (Fig. 8B; P < 0.03), and this was also represented as an increase in cumulative food intake of MS/AST rats compared with control/AST rats 48 h after MS (Fig. 8D; P < 0.03). Statistical analysis also showed an interaction between stress and AST for 24 h cumulative intake (P < 0.03), but post hoc analysis showed no difference between specific groups.

Experiment 6: fourth ventricle infusion of ASV-30 in MS rats.

There was no effect of MS or of ASV-30 on body weight change of rats during the 24 h after MS in experiment 6 when rats were infused with 2.2 nmol ASV-30 (Fig. 9A), but ASV-30 did prevent weight loss in MS rats 48 h after stress (Fig. 9B; stress × infusion, P < 0.04). MS/saline rats ate less than MS/ASV-30 rats 6–12 h after MS (Fig. 9C; stress × infusion, P < 0.04), but there were no other differences in food intake at any other time interval. When cumulative food intake was considered, food intake of MS/saline rats was inhibited compared with control/saline rats at 12, 24, and 48 h after MS (Fig. 9D; stress × infusion, P < 0.001). There was no difference in cumulative food intake of MS/ASV-30 and control/saline rats at any time, but 48-h cumulative intake was greater in control/saline than control/ASV-30 rats (Fig. 9D; P < 0.009).

In the second group of rats, there was no effect of MS or of low doses of ASV-30 on weight gain during the 24 h after MS (Fig. 10A). In contrast, 48 h after the end of MS both MS/saline and MS/High ASV-30 rats lost weight, whereas all other groups gained a small amount of weight (Fig. 10B; infusion, P < 0.008; stress × infusion, P < 0.05). Two hours after the end of MS, the food intake of MS/saline rats was lower than that of control/saline rats, but there were no other significant differences between groups (Fig. 10C; infusion, P < 0.04). There was no effect of MS or of ASV-30 on food intake at any other time interval or on cumulative intake over 48 h (Fig. 10D).

DISCUSSION

Rats that are exposed to RRS are hypophagic and lose weight on the days of restraint and do not overeat or return to the weight of their nonstressed controls in the poststress period (24). These observations suggest that RRS rats adjust the level at which they regulate body weight and also are consistent with reports of a decrease in body weight of rats exposed to acute stress (13, 42, 46) or ventricular infusion of CRF (28). RRS animals not only have a reduced body weight, but also are hyperresponsive to novel mild stressors (22) and show increased anxiety-type behavior in the postrestraint period (11). Rats that are subjected to MS, such as intraperitoneal saline injections, also have a reduced food intake and lose weight, but, in contrast to the sustained change in body weight that is caused by exposure to RRS, the effects of MS are relatively small and short lived (29). The objective of the studies described here was to determine whether CRF receptors located adjacent to the fourth ventricle mediated any of the energetic responses to RRS or to MS in rats. The results suggest that antagonism of hindbrain CRFR does not change the effects of the more severe RRS on food intake or body weight, but that antagonism of CRFR2 does prevent acute changes in food intake and body weight in rats exposed to MS. Therefore, it seems likely that there is an important role for hindbrain CRFR2 in mediating the energetic responses to MS, but the data does not exclude an additional role for CRFR1.

Previously, we reported that injection of αhCRF, a nonselective antagonist for the CRFRs (35), in the third ventricle blocked the sustained reduction in body weight of rats exposed to RRS (46). Because it is possible that αhCRF diffused from the third into the fourth ventricle, the experiments described here tested whether inhibition of RRS-induced weight loss was due to antagonism of CRFRs adjacent to the fourth ventricle. A potential role for the brain stem in the energetic response to stress is supported by the observation that infusion of Ucn in the fourth ventricle caused weight loss and decreased food intake of rats (20).

The results from studies described here suggest that antagonism of CRFR in areas adjacent to the fourth ventricle do not modify the acute or long-term effects of RRS on food intake, body weight, or corticosterone release. In other studies we found that 2.2 nmol of ASV-30 infused into the third ventricle before the start of RRS prevented stress-induced hypophagia (10). The experimental protocol was the same as that used for experiment 5, except for the site of infusion, and included blood collection by tail bleeding; therefore, it is unlikely that the doses of antagonists used in experiments described here...
were insufficient to block the activity of CRFR ligands. Similarly, fourth ventricle infusion of CRFR antagonists during RRS did not change the corticosterone response to MS in the post restraint period. In contrast, MS rats infused with a nonspecific antagonist, AST, or a CRFR2-specific antagonist, ASV-30, had the same weight gain as control rats, suggesting an important role for CRFR2 in mediating energetic or metabolic responses to MS. Because both food intake and weight gain in ASV-30-infused rats were the same as in control rats, then either weight loss in MS rats is a result of an inhibition in food intake or CRFR2 is also responsible for additional changes in energy expenditure or metabolism that contribute to the weight loss. Although it has been shown that fourth ventricle infusions of CRF inhibit gastric emptying (49), it is unlikely that the weight loss represented a significant change in gut fill of the rats because stimulation of CRFR2 causes gastric stasis (34). Therefore, antagonism of CRFR2 would be expected to support normal gut motility, and this would not be consistent with a body weight gain that was determined solely by gut content.

In the experiments described here, significant effects on food intake within specific time periods after the end of MS varied between experiments, but the effect of MS on cumulative intake over 48 h tended to be more consistent. This extended effect of a 2-h MS on body weight and food intake was similar to that caused by fourth ventricle injection of Ucn (20), and the ASV study described here suggests that this is mediated by CRFR2. The time course of this response contrasts with that in which hypophagia was induced in CRFR knockout mice by lateral ventricle infusion of Ucn. Ucn inhibited food intake of wild-type mice for 10 h but inhibited intake of CRFR2 knockout mice for only 2 h, suggesting that the early response to Ucn was mediated by CRFR1 (12). In another study, CRFR1 knockout mice did not respond to the hypophagic effects of Ucn for the first 1.5 h after injection, again suggesting that the immediate effect of stress on food intake is mediated by CRFR1 (6). The earliest that we measured food intake was 2 h after the end of MS, which would be on the borderline of when CRFR1 effects would be expected to be attenuating and the inhibition of food intake mediated by CRFR2 would be initiated. Because single injections of Ucn into the lateral ventricle (12) and of CRF into the third ventricle (30) inhibit food intake for periods of hours rather than days, further studies are needed to determine the mechanism by which stress-related peptides in the brain stem mediate long-term inhibition of food intake.

None of the antagonists tested here modified corticosterone release during RRS, consistent with our previous observation that third ventricle aCRF prevented sustained weight loss in RRS rats, but did not change restraint-induced corticosterone release (46). We only measured corticosterone release on day 2 of restraint because tail bleeding is perceived as a stressor by the rats. Previous measurements have shown that corticosterone in restrained rats peaks at 30 min and may be back to baseline levels by the end of the 3-h restraint (25). The single
measure of corticosterone concentration made during restraint (60 min) would not have identified any change in the pattern of adrenal response to stress unless there was an attenuation or extension of the peak response. Blood samples were collected on day 2 of restraint because corticosterone release during restraint starts to attenuate by day 3 of RRS, but is the same on day 2 as day 1 (23). The effect of a CRFR antagonist on corticosterone release during MS was measured only in experiment 2 because the procedures required to collect blood appeared to be a stressor, and all rats, including controls, lost weight during the 48 h after MS when blood was collected. We previously found a similar confounding effect of blood sampling when tail bleeding inhibited food intake of the controls for rats that received third ventricle infusions of CRF (30).

Unexpectedly, in experiment 1, αhCRF exaggerated hypophagia and weight loss in RRS rats. These results are surprising, since αhCRF is a CRF receptor antagonist and would be expected to inhibit behavioral and endocrine changes that are induced by stress. Experiment 1 was conducted in three equally divided cohorts, and each cohort had the same outcome, making it unlikely that the exaggerated energetic response to RRS was a one-time phenomenon. There are several possible explanations for these results. The first is that antagonism of CRFR in the brain stem lifts negative feedback in some areas of the midbrain, such as the hypothalamus. CRF connections between the hypothalamus and the brain stem have already been described (5, 9), and these CRF-containing neurons could potentially be responsible for the exaggerated decrease in food intake and increase in corticosterone release during MS. Measurements made in this study did not identify the mechanisms responsible for the exaggerated energetic and endocrine responses in αhCRF-treated rats.

![Graphs A and B](image1.png)

**Fig. 8.** Body weight change 24 h (A) and 48 h (B) after MS in rats infused with 2 nmol AST before MS in experiment 5. C: food intake during specific time intervals. D: cumulative food intake. Data are means ± SE. Values in A or B or within the 6- to 12-h time interval in C that do not share a common superscript are significantly different. D: ★significant difference between MS/saline and control/saline rats; ■significant difference between the 2 groups of MS rats; and *significant difference between the 2 control groups.
but experiment 2 used a lower dose of αhCRF, and experiment 3 used a different, more potent, nonselective CRFR antagonist, AST (7, 48), and neither of these had any effect on the change in food intake or body weight of RRS rats. Therefore it seems more likely that the higher dose of αhCRF acted as an agonist in stressed rats, rather than allowing the upregulation of other stress-activated pathways. This would be consistent with a previous report that a 25-μg injection of αhCRF into the lateral ventricle increased anxiety-type behavior of rats in an Elevated Plus Maze (35). If αhCRF had acted as a simple agonist, then we also would expect the control/αhCRF rats to have experienced a decrease in body weight and food intake during the period of the infusions. Control/αhCRF rats, however, showed a nonsignificant increase in food intake and a significant increase in body weight during the stress period, compared with control/saline rats. Others have suggested that handling animals is a mild form of stress (16), and it is possible that αhCRF infusions in control rats blocked this MS, resulting in an apparent weight gain. A third explanation of the exaggerated weight loss in RRS/αhCRF rats is that the partial agonist activity of αhCRF is specific to conditions in which stress-related pathways are already activated and that it represents an interaction between stress-induced peptides. An example of this type of interaction is that low concentrations of CRF or Ucn II inhibit activity of serotonergic neurons in the dorsal raphe nucleus, whereas high concentrations of these peptides increase activity of serotonergic neurons in this area (27, 52). The differences in neuronal activation have been associated with selective activation of CRFR1 with low doses of ligand and activation of both CRFR1 and CRFR2 with
higher doses (27, 32). Since the infusions in the study described here were into the fourth ventricle, it is theoretically possible that the partial agonist effect of dhCRF on CRFR1 inhibited serotonergic neurons, leading to an exaggerated inhibition of food intake in both RRS and MS rats. Another explanation is that dhCRF has a higher affinity for CRF binding protein (CRF-BP) (21) than for CRFR1 and CRFR2 (57); therefore it would displace CRF and Ucn from the binding protein, increasing the amount of ligand available for binding to CRFR. CRF-BP is widely distributed in the brain, including some areas of the brain stem (39). In nonstress conditions, the amount of free ligand present and displaced from CRF-BP may not be enough to initiate stress-like behaviors, but in stressed animals the concentrations of CRF and Ucn would be expected to increase, and it is possible that various aspects of the stress response would be exaggerated if CRF-BP was not able to sequester CRF or Ucn. The nuclei responsible for the changes in food intake or body weight were not identified, but we assume that there was an initiation of the response in a brain stem nucleus because the antagonist was infused into the fourth ventricle.

In conclusion, the results from this study suggest that changes in food intake and body weight in rats that have been exposed to RRS or to MS are mediated by different pathways. It appears that the brain stem may play an important role in regulating the effects of MS but not RRS. Maintenance of a

Fig. 10. Weight change 24 h (A) and 48 h (B) after MS of rats infused with 0.3 or 0.15 nmol ASV before exposure to MS in experiment 6. C: intervalled food intake. D: cumulative intake. Data are means ± SE. Values for 48 h weight change or food intake 0–2 h after MS that do not share a common superscript are significantly different.
normal food intake and weight gain in MS rats infused with the antagonist ASV-30 suggests an important role of CRFR2 in the initiation of energetic and/or metabolic response to MS. In experiments 1 and 3 we found that a relatively high dose of ohCRF infused into the fourth ventricle exaggerated weight loss and hypophagia in RRS and hypophagia in MS rats, but not in control rats, implying that ohCRF may act as a partial agonist only in conditions of stress.

Perspectives and Significance

The results from the studies described here suggest that changes in food intake and body weight induced by different types of stressors in rats are mediated by different pathways. It appears that the brain stem may play an important role in regulating the effects of mild, but not a more severe, stressor and that CRFR2 is a critical part of this pathway. Further studies are required to identify specific nuclei and other neurotransmitters that are involved in the pathway. In addition to providing new information on brain areas that influence energy expenditure, these observations add to a growing body of evidence that the hindbrain is a primary determinant of food intake and energy expenditure in nonstressful conditions (18). One surprising observation described here is that a relatively high dose of ohCRF infused into the fourth ventricle exaggerated weight loss and hypophagia in RRS rats and hypophagia in MS rats, but not in control rats, implying that ohCRF may act as a partial agonist, but only in conditions of stress. The mechanistic basis of this agonism and whether it is caused by activation of a specific CRFR subtype remains to be determined.

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GRANTS

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