Antagonism of Corticotrophin-Releasing Factor Receptors in the Fourth Ventricle Modifies Responses to Mild but not Restraint Stress

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ABSTRACT

Repeated restraint stress (3 hours of restraint on 3 consecutive days: RRS) in rodents produces temporary hypophagia, but a long-term down-regulation of body weight. The mild stress (MS) of an intraperitoneal injection of saline and housing in a novel room for 2 hours 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 brainstem corticotrophin releasing factor receptors (CRFR) in mediating energetic and glucocorticoid responses to RRS or MS and in promoting stress hyper-responsiveness in RRS rats. Administration of 1.3 nmol αhCRF(9-41), a non-specific 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 non-specific 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 hours 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 influence stress-induced corticosterone release in RRS rats.

Key words: brainstem, αhCRF(9-41), astressin, antisauvagine-30, rats
INTRODUCTION

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 are expressed in areas such as heart, gastrointestinal tract, arterioles 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 appear 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 in following infusion of CRF into the third ventricle (28). We have previously demonstrated that rats exposed to restraint stress for 3 hours a day for 3 consecutive days (RRS), 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 post-stress 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.
Others have reported that chronically stressed animals show an exaggerated release of adrenocorticotropic hormone (ACTH) and corticosterone in response to a novel stress (15, 17). Similarly, animals submitted to a mild physical stress (MS) twelve 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 non-specific receptor antagonist ($\alpha$hCRF$_{(9-41)}$) 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 third ventricle could potentially diffuse into the fourth ventricle and act on nuclei in the hindbrain, we cannot exclude the brainstem as a mediator of changes in food intake and body weight of stressed rats. Grill et al (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 hypersensitivity 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 two hours. We have previously shown that this stress causes a significant increase in serum corticosterone, inhibits food intake and causes transient weight loss in rats and mice (22, 29) and others have reported that a saline injection in mice 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 approximately 275 g on arrival at UGA. The rats were housed in wire hanging cages in temperature and humidity controlled rooms (23°C, 55% humidity) with lights on from 7.00 a.m. to 7.00 p.m. 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 αhCRF(9-41) in RRS rats

This experiment tested whether infusions of a non-selective CRFR antagonist, alpha helical CRF(9-41) (αhCRF), into the 4th 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 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 urocortin 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 Figure 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 26G guide cannulas (Plastics One, Roanoke, VA) aimed at the 4th ventricle. Rats were anaesthetized 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 ventro-dorsal (46), according to Paxinos & Watson Brain Atlas (36). Immediately after surgery and again the next day the rats were injected with analgesic (2mg/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 (Accumet glucometer; Boehringer Mannheim, Gmg). Each rat
received an i.c.v. infusion of 210 \( \mu g \) 5-thio-D-glucose (Sigma-Aldrich, St Louis, MO) in 2 \( ul \) sterile
isotonic saline over one minute. All infusions were from Hamilton syringes controlled by an infusion
pump (PHD 2000 Infusion pump, Harvard Apparatus, Holliston, MA). Those rats that showed a
doubling of blood glucose 60 minutes after the infusion were included in the experiment.
Approximately 10\% of rats were excluded from the experiment based on this test. Rats were allowed
one week 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 4 weight-matched
groups of 20 – 22 rats: RRS/\( \alpha \)hCRF, RRS/Saline, Control/\( \alpha \)hCRF, Control/Saline. On each day of
the three days of restraint, 10 minutes before the beginning of each restraint, all rats received a 2 \( ul \)
infusion of saline or 1.3 nmol (5 \( \mu g \)) of \( \alpha \)hCRF\(_{(9-41)} \) (Bachem Bioscience, King of Prussia, PA) into
the fourth ventricle over one minute. RRS rats were placed in Perspex restraining (21.6 x 6.4 cm)
tubes (Plas Labs, Lansing, MI) for three hours on each of three consecutive days. Infusions started at
8.30 a.m. 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 hours of restraint. Corticosterone levels were measured at 0 and 60
minutes on Day 2 of restraint in blood samples collected by tail-bleeding. Twelve days after the end
of RRS, half of the rats from each group were submitted to a mild stress, whereas the other half
served as controls (n= 10 or 11). Starting at 9.00 a.m., rats exposed to mild stress (MS) received a
2ml i.p. injection of saline and were placed in new cages in a novel room for 2 hours. Control rats
were picked up but replaced in their home cages. Both groups were food and water deprived during
the 2 hours of MS. Corticosterone levels were measured at 0, 15, 30, 60, 90, and 120 minutes after the start of MS in blood samples collected by tail-bleeding.

**Experiment 2: Fourth ventricle infusions of a lower dose of αhCRF(9-41) in RRS rats**

The results of the previous experiment suggested that fourth ventricle αhCRF(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 αhCRF were infused into the lateral ventricle, therefore, this study tested the effects one fifth the amount of αhCRF(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 αhCRF(9-41) in the previous experiment. In addition we did not collect blood to measure corticosterone on the second day of RRS in order to minimize exposure to non-specific stressors.

Forty rats were fitted with 4th ventricle cannulae and cannula placement tested as described above. Baseline measures of food intake and body weight were recorded for seven days starting one week after confirming cannula placement. The rats were divided into four weight-matched groups of 10 rats: Control/Saline, RRS/Saline, Control/αhCRF, RRS/αhCRF. The saline rats received 4th ventricle infusions of 2ul saline and the αhCRF groups received infusions of 0.26 nmol (1 μg) αhCRF(9-41) in 2 ul saline. Infusions started at 8.00 a.m. 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 hours the rats were 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 αhCRF(9-41) in MS rats**
This experiment tested whether αhCRF(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, seven days after testing cannula placement, the rats were divided into 4 weight matched groups of nine rats; Control/Saline, Control/αhCRF, MS/Saline, MS/αhCRF. Starting at 9.00 a.m., rats received a 2 ul infusion of either 1.3 nmol αhCRF or saline in the fourth ventricle 10 minutes before the beginning of MS. Food intake was recorded for 2 days before and at 2h, 4h, 6h, and 12h after exposure to MS. Body weight was also measured on the day of MS and 24 hours after MS.

A second set of sixty-six rats were fitted with 4th ventricle cannulae. Seven days after testing cannula placement the rats were divided into six weight-matched groups of 11 rats: Control/Saline, Control/Low αhCRF, Control/High αhCRF MS/Saline, MS/Low αhCRF, MS/High αhCRF. Starting at 9.00 a.m. rats received a 2 ul infusion of either saline or αhCRF in the fourth ventricle 10 minutes before the beginning of MS. The low dose of αhCRF 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 minutes after the start of MS. The rats were returned to their home cages after 2 hours. Food intakes were recorded 2, 4, 12, 24, and 48 hours after the end of MS. Body weights were recorded before and 24 and 48 hours after the start of MS.

Experiment 4: Fourth ventricle infusions of astressin in RRS rats.

Results from Experiment 1 suggested that αhCRF 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 non-selective CRFR antagonist, astressin (AST). AST has similar binding affinity for CRFR1 and CRFR2 as αhCRF (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 intracisternally to block icv 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 4th ventricle cannulae. Experimental design was similar to Experiment 1, except that 2 nmol AST (American Peptides Company, Sunnyvale, CA) 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 minutes during MS.

Experiment 5: Fourth ventricle infusion of astressin in MS rats.

In Experiment 3, αhCRF(9-41) appeared to produce a partial agonist effect, exaggerating the effects of MS on food intake. Therefore, the study was repeated using astressin.

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 hours after the end of MS. Body weight also was measured before and 24 and 48 hours after MS.

Experiment 6: Fourth ventricle infusion of antisauvagine-30 in MS rats

AST infusion into the fourth ventricle blocked hypophagia 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 in rats exposed to MS. The dose of antisauvagine-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 antisauvagine-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 ug) antisauvagine-30 (ASV30; Phoenix
Pharmaceuticals, Belmont, CA) was used to selectively inhibit CRFR2 (n= 8 or 9 per group). A second set of sixty-four rats was subjected to the same procedure except that they were infused with saline, 0.3 or 0.15 nmol (1 ug or 0.5 ug) ASV-30 (n= 10 or 11/group). Food intakes were measured at 2, 4, 12, 24 and 48 hours after the end of MS and body weight was measured at 24 and 48 hours 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 αhCRF(9-41) in RRS rats

All RRS rats lost weight on the days of restraint, but RRS/αhCRF rats lost more than RRS/Saline rats (Fig 2A: stress: P< 0.0001, time: P<0 .0001, stress X time: P< 0.03, stress X 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/αhCRF rats ate less than any other group on the first two days of restraint and less than Control/αhCRF rats on the third day of restraint (Fig 2B: stress: P< 0.01, time P< 0.0001, and stress X 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 three 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/αhCRF rats ate less than any other group (Fig 2C). On the second day of restraint, both groups of RRS rats had higher levels of corticosterone at 60 minutes compared to controls, but there was no effect of αhCRF in either RRS or Control rats (Fig 3A: stress: P< 0.0001). There was no effect of either αhCRF or of RRS on food intake or body weight measured 24 hours after MS twelve days after the end of RRS (data not shown). MS caused a significant increase in corticosterone levels 15 minutes 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/αhCRF/MS, or RRS/Saline/MS rats (Fig 3B). When saline- and αhCRF-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 minutes 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 αhCRF(9-41) in RRS rats

On the first 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 αhCRF on the weights of Control or RRS rats (Figure 4A: stress: P<0.0001, time: P<0.0001, stress X time: P<0.0001). Both groups of RRS rats ate less than Controls on each of the three 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 X time: P<0.0001). When food intakes were averaged for three days before, during and after restraint, the RRS/Saline rats ate less than Control/Saline rats both during stress and during the three days after stress whereas as the food intakes of RRS/αhCRF rats was inhibited only on the days of stress (Figure 4C: stress: P<0.002, time: P<0.0001, stress X time: P<0.001).
Experiment 3: Fourth ventricle infusion of αhCRF(9-41) in MS rats

There was no effect of either MS or of 1.3 nmol αhCRF on 24 hour weight gain of rats (Fig 5A). Food intake during the 2 hours after the end of MS and 4-6 hours after MS was lower in MS/αhCRF than MS/Saline rats (Fig 5B: P< 0.04). Cumulative food intake of MS/αhCRF rats was significantly lower than for any other group at both 4 and 6h after MS (Fig 5C: P< 0.03), but there were no differences in food intakes 6-24h 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 αhCRF, there was no effect of MS or of αhCRF 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 being significant only in rats infused with 1.3 nmol αhCRF (Figure 6: MS: P<0.004).

Experiment 4: Fourth ventricle infusions of astressin 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 two days of restraint, but there was no effect of AST (Fig 7B: time: P< 0.0001, stress X time: P< 0.003). When three 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 hours 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 minutes after the start of MS compared with animals that had not been previously restrained (Fig 7D: stress: P<0.02).

Experiment 5: Fourth ventricle infusion of astressin 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 X infusion: P< 0.01) and 48 hours after MS (Fig 8B: infusion: P< 0.04, stress X infusion: P< 0.01). AST also blocked an MS-induced inhibition of food intake at 2-4 hours after the end of MS. AST increased food intake of both Control and MS rats between 12 and 24 hours 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 48h after MS (Fig 8D: P< 0.03). Statistical analysis also showed an interaction between stress and AST for 24 hour cumulative intake (P< 0.03), but post-hoc analysis showed no difference between specific groups.

Experiment 6: Fourth ventricle infusion of antisauvagine-30 in MS rats

There was no effect of MS or of ASV-30 on body weight change of rats during the 24 hours 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 hours after stress (Fig 9B: stress X infusion: P< 0.04). MS/Saline rats ate less than MS/ASV-30 rats 6-12 hours after MS (Fig 9C: stress X 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 X 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 hour 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 hours after MS (Fig 10A). In contrast, 48 hours 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 X 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 (Figure 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 hours (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 non-stressed controls in the post-stress 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 hyper-responsive to novel mild stressors (22) and show increased anxiety-type behavior in the post-restraint period (11). Rats that are subjected to mild stress, such as i.p. 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 mild stress 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 non selective 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 the 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 brainstem 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 non-specific antagonist, AST, or a CRFR2-specific antagonist, ASV30, 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 are 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 hours tended to be more consistent. This extended effect of a 2 hour mild stress 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 hours but inhibited intake of CRFR2 knockout mice for only two hours, 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 hours after injection, again suggesting that the immediate effect of stress on food intake is mediated by CRFR1 (6). The earliest we measured food intake was 2 hours after the end of MS, which would be on the border-line 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 brainstem 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 αhCRF 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 measurement have shown that corticosterone in restrained rats peaks at 30 minutes and may be back to
baseline levels by the end of the 3 hour 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 hours 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 stress was a one-time phenomenon. There are several possible explanations for these results. The first is that antagonism of CRFR in the brainstem lifts negative feedback in some areas of the midbrain, such as the hypothalamus. CRF connections between the hypothalamus and the brainstem have already been described (5, 9), and these CRF containing neurons could potentially be responsible for the exaggerated decrease in food intake during both RRS and MS stress and the 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, but Experiment 2 used a lower dose of αhCRF and Experiment 3 used a different, more potent, non-selective 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 up-regulation of other stress-activated pathways. This would be consistent with a previous report that a 25 ug 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 non-significant increase in food intake and a significant increase in body weight during the stress period, when compared to 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 mild stress, 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 αhCRF on CRFR1 inhibited serotonergic neurons, leading to an exaggerated inhibition of food intake in both RRS and MS rats. Another explanation is that αhCRF 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 brainstem (39). In non-stress 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 brainstem 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 brainstem may play an important role in regulating the effects of MS, but not RRS. Maintenance of a normal food intake and weight gain in MS rats infused with the antagonist antisauvagine-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 \( \alpha \text{hCRF} \) infused into the fourth ventricle exaggerated weight loss and hypophagia in RRS and hypophagia in MS rats, but not in Control rats, implying that \( \alpha \text{hCRF} \) 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 brainstem may play an important role in regulating the effects of mild, but not a more severe stressor, and that CRFR2 are 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 balance and metabolism in conditions of stress, these observations add to a growing body of evidence that the hindbrain is a primary determinant of food intake and energy expenditure in non-stressful conditions (18). One surprising observation described here is that a relatively high dose of \( \alpha \text{hCRF} \) 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 \( \alpha \text{hCRF} \)
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 sub-type remains to be determined.
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REFERENCES


FIGURE LEGENDS

**Figure 1**: Flow chart for Experiment 1

**Figure 2**. Daily body weight (Panel A), daily food intake (Panel B) and cumulative food intake for 3 consecutive days before, during and after RRS for rats in Experiment 1 infused with 1.3 nmol αhCRF (Panel C). Values are means ± SEM for 15-16 rats. In Panel A, asterisks indicate a significant difference in body weight of RRS/αhCRF and all other groups (P< 0.0001), and # indicates a significant difference between control and RRS groups at p< 0.05. In Panel B, an asterisk indicates a significant difference between RRS/αhCRF rats and all other groups. $ indicates a significant difference between RRS/αhCRF and Control/αhCRF rats. Values for three day cumulative food intake on the days of RRS that do not share a common superscript are significantly different (P< 0.002).

**Figure 3**. Serum corticosterone concentration measured before and 60 minutes after the start of RRS (Panel A) in Experiment 1 when rats were infused with 1.3 nmol αhCRF before RRS. Values that do not share a common superscript are significantly different at P< 0.05. Panel B: Serum corticosterone concentration measured in eight groups of rats before and during MS at the end of Experiment 1. Data are means ± SEM and significant differences are not marked. Panel C represents the same data as Panel B except that animals were divided only by previous exposure to RRS and subsequent exposure to MS or control conditions. # indicates a significant difference between control and RRS groups.

**Figure 4**: Daily body weight (Panel A), daily food intake (Panel B) and cumulative food intake for 3 consecutive days before, during and after RRS (Panel C) for rats in Experiment 2 infused with 0.26 nmol αhCRF. Values are means ± SEM for 10 rats. In Panel A, # indicates a significant difference between control and RRS groups at p< 0.05. In Panel B, % indicates that the RRS/Saline rats weighed
less than both groups of Controls but RRS/αhCRF rats weighed less only than RRS/Saline rats. ★ indicates that RRS/Saline rats ate less than Control/Saline rats. Values for three day cumulative food intake on the days of RRS or on the post-stress days that do not share a common superscript are significantly different (p< 0.002).

**Figure 5:** Weight change (Panel A), intervalled food intake (Panel B) and cumulative food intake (Panel C) of rats infused with 1.3 nmol αhCRF(9-41) in Experiment 3. Data are means ± SEM. Values for food intake within a specific time interval in Panel B that do not share a common superscript are significantly different. An asterisk in Panel C indicates that the intake of MS/αhCRF rats was lower than for any other group.

**Figure 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 ± SEM. Values for the 45 minutes time point that do not share a common superscript are significantly different.

**Figure 7:** Daily body weight (Panel A), daily food intake (Panel B), food intake averaged over three day intervals before, during and after RRS (Panel C) and corticosterone release during MS 12 days after the end of RRS (Panel D) in rats infused with 2 nmol AST before exposure to restraint stress in Experiment 4. Data are means ± SEM. # indicates a significant difference between Control and RRS rats. Values for food intake within a specific time interval in Panel C that do not share a common superscript are significantly different.

**Figure 8:** Body weight change 24 hours (Panel A) and 48 hours (Panel B) after MS in rats infused with 2 nmol AST before MS in Experiment 5. Panel C shows food intake during specific time
intervals and Panel D shows cumulative food intake. Data are means ± SEM. Values in Panel A or B or within the 6-12 hour time interval in Panel C that do not share a common superscript are significantly different. In Panel D ★ indicates a significant difference between MS/Saline and Control/Saline rats, $ indicates a significant difference between the two groups of MS rats and † indicates a significant difference between the two Control groups.

**Figure 9:** Weight change 24 hours (Panel A) and 48 hours (Panel B) after MS of rats infused with 2.2 nmol ASV-30 before exposure to MS in Experiment 6. Panel C shows intervalled food intake and Panel D shows cumulative intake. Data are means ± SEM. Values for 48 hour weight change or food intake 6-12 hours after MS that do not share a common superscript are significantly different. In Panel D ★ indicates a significant difference between MS/Saline and Control/Saline rats and † indicates a significant difference between the two Control groups.

**Figure 10:** Weight change 24 hours (Panel A) and 48 hours (Panel B) after MS of rats infused with 0.3 or 0.15 nmol ASV before exposure to MS in Experiment 6. Panel C shows intervalled food intake and Panel D shows cumulative intake. Data are means ± SEM. Values for 48 hour weight change or food intake 0-2 hours after MS that do not share a common superscript are significantly different.
96 rats fitted with 4th ventricle cannulas

One week recovery

Cannula placement tested

85 rats enter experiment

6 days baseline

21 RRS rats infused with saline

22 RRS rats infused with αhCRF

22 Controls infused with saline

20 Controls infused with αhCRF

12 days post-RRS

11 MS Rats

10 Control Rats

11 MS Rats

11 Control Rats

11 MS Rats

11 Control Rats

10 MS Rats

10 Control Rats
A: Daily Body Weight Experiment 1

B: Daily Food Intakes Experiment 1

C: Three Day Food Intakes Experiment 1
Figure 3

A: Corticosterone during RRS in Experiment 1

B: Corticosterone during MS in Experiment 1

C: Corticosterone during MS in Experiment 1
Figure 4

A: Daily Body Weight Experiment 2

B: Daily Food Intakes Experiment 2

C: Three Day Food Intakes Experiment 2
A: 24 Hour weight Change Experiment 3

![Graph showing weight change](image)

B: Intervalled Food Intake Experiment 3

![Graph showing food intake](image)

C: Cumulative Food Intake Experiment 3

![Graph showing cumulative food intake](image)
Figure 6

Corticosterone (ng/ml)

- Control/Saline
- MS/Saline
- Control/Low αhCRF
- MS/Low αhCRF
- Control/High αhCRF
- MS/High αhCRF

Time after start of MS (min)
A: Daily Body Weight Experiment 4

B: Daily Food Intake Experiment 4

C: Three Day Food Intakes Experiment 4

D: Corticosterone during MS in Experiment 4
**A: 24 Hour Weight Change in Experiment 5**

- Control/Saline
- Control/AST
- MS/AST
- MS/Saline

**B: 48 Hour Weight Change in Experiment 5**

**C: Intervalled Food Intake in Experiment 5**

**D: Cumulative Intake in Experiment 5**
Figure 9

A: 24Hour Weight Change 3 nmol ASV-30

B: 48Hour Weight Change 3 nmol ASV-30

C: Intervalled Food Intake 3 nmol ASV-30

D: Cumulative intake 3 nmol ASV-30
Figure 10

A: 24 hr Weight Change 0.3 and 0.15nm ASV30

B: 48 Hour Weight Change 0.3 and 0.15nm ASV30

C: Intervalled intake 0.3 and 0.15nm ASV30

D: Cumulative intake 0.3 and 0.15nm ASV30