THE EFFECTS OF BLOCKING BRAINSTEM CRF RECEPTORS ON STRESS RESPONSIVENESS IN RATS

by

JOANNA RODRIGUES MIRAGAYA

(Under the Direction of Ruth Harris)

ABSTRACT

Stress is a reaction of the body to a critical situation and it produces many different responses, including endocrine, autonomic, metabolic, immune, and behavioral, which vary according to the type and period of exposure to a stressor. Centrally released corticotrophin-releasing factor (CRF) and its homologues Urocortin (Ucn), Ucn II, and Ucn III, appear to be the initiators of responses to stress. There are two receptor subtypes for these neuropeptides, CRFR1 and CRFR2, and each receptor plays a different role in stress-induced responses. It is well established that acute stress can decrease body weight and inhibit food intake of rats. Several brain nuclei known to control energy balance respond to stress, however, the major initiator and/or regulator of stress-induced changes in energy balance has not been identified. Therefore, we investigated whether areas adjacent to the fourth ventricle were involved in regulating body weight and food intake during acute stress. Experiments described here used two types of acute stress to produce weight loss and inhibit food intake; repeated restraint and mild stress. The first set of Experiments tested whether CRF infusions into the fourth ventricle would inhibit food intake in overnight food-deprived rats, and whether CRFRs antagonists would prevent this decrease in food intake. The second set of Experiments tested whether antagonism of CRFRs in

brain nuclei adjacent to the fourth ventricle would prevent stress-induced changes in body weight and food intake. In the third set of Experiments, we tested whether antagonism of CRFRs in areas adjacent to the fourth ventricle before stress would prevent activation of the hypothalamus and/or brain nuclei known to respond to stress. The outcome of these Experiments lead us to conclude that: (1) CRF in areas adjacent to the fourth ventricle plays a role in regulating feeding behavior in unstressed animals, (2) stress-induced decrease in body weight and inhibition of food intake are mediated by different pathways, depending upon the type of stress, (3) the brainstem may play an important role in regulating energetic responses to mild stress, (4) and that nuclei in the brainstem may be responsible for some of these stress-induced responses.

INDEX WORDS: stress, CRF, CRF receptors, brainstem, rats, food intake

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DEDICATION

I dedicate this dissertation to my parents, Joao and Maria Helena, my anchor and biggest source of support throughout these years. They have always inspired me and believed in me. Nothing I have done would ever been possible without their love, patience, and understanding. I love you both with all my heart.

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CHAPTER 1

INTRODUCTION

Stress was first characterized in 1936 by Seyle (30), as an important and complex model that regulates the homeostasis of the body during an event. By that time, Seyle had already predicted the important role that stress and its consequences can play in our lives (29). Since then, stress has been extensively studied because of its impact on health status. Stress has been implicated in producing endocrine, autonomic, immune, and behavioral responses; all of which appear to be regulated by different organs and brain nuclei (7). Multiple systems in the body are maintained in physiological equilibrium, known as homeostasis. McEwen (22, 23) then, describes in more detail the term allostasis, which was first introduced by Sterling (35), and it means the activation of some mechanisms to maintain the homeostasis of the body during a stressful situation. A failure to adapt to a stressful situation is known as allostatic load; which can lead to a collapse of the allostatic systems (23).

Dysfunctional regulation of stress-responses to maintain homeostasis can lead to different pathologies in the brain, the cardiovascular, the metabolic, and the immune systems (22). The brain is the central pathway regulating all systems in the body, and itself (19). Stress can down- or up-regulate some neuropeptides, neurotransmitters, and receptors in specific brain nuclei (6, 20); thus, inducing emotional and behavioral diseases. Both cardiovascular and metabolic systems can also be affected by stress, leading to an increased risk for atherogenesis, hypertension, myocardial infarction, obesity, and diabetes (13, 28). Lastly, the immune response tends to be decreased in stress, resulting in a delayed or deficient response against infections and

tumors. Nevertheless, the stress-induced responses in these systems vary according to the type and period of exposure. Acute stress can be defined as a short period of exposure to a stressor, and some examples include trauma and abuse; whereas, chronic stress is a continuous exposure to a stressor, such as in a job.

Different hormones, receptors, and neurotransmitters play a role in regulating the stress response, in which corticotrophin-releasing factor (CRF) appears to be the initiator. CRF was first discovered in 1981 (34), and has two receptor-subtypes: CRFR1 and CRFR2 (4, 25, 27). Both receptors can play different roles in stress- induced responses (3, 8). Some additional agonists for these receptors include Urocortin (Ucn), Ucn II, and Ucn III (21, 36). CRF is released from the paraventricular nucleus of hypothalamus in response to stress (28), and stimulates the anterior pituitary to release adrenocorticotropic-hormone (ACTH) (34), which will then, stimulate the release of glucocorticoids by the adrenals. This is known as the Hypothalamic-Pituitary-Adrenal (HPA) axis (2, 33). Additionally, CRF can activate the adrenergic system by regulating catecholamine release from the adrenal medulla. Stress can also down-regulate other hormones in the body (9), and stimulate different biochemical pathways to make energy available (1) for a threatening situation. Stress, and thus, CRF can also regulate the behavioral response to stress, such as anxiogenic and anxiolytic effects (31). Those effects, though, are dependent on the type of CRFR involved and its location in the brain (3, 8).

As previously reported, acute stress can induce a sustained decrease in body weight, and a decrease in food intake of rats during the stress period (24), and the rats do not tend to compensate for their body weight loss in the post-stress period (15). Several brain nuclei have been implicated in stress- induced weight loss and food intake; however, it is still unknown which one could be the major initiator and/or regulator of the stress-induced responses. Lateral

intracerebroventricular injections of CRF induced weight loss in animals (18, 26), and antagonism of CRFRs in the third ventricle blocked the stress-induced weight loss and decrease in food intake of rats exposed to stress (17, 32). Because injections of CRF or CRFRs antagonists into the lateral and/or the third ventricle could potentially act on brain nuclei around the fourth ventricle, we can not exclude the brainstem as a potential mediator of changes in food intake and body weight. In addition, Grill et al (10, 11) have demonstrated the importance of the brainstem in regulating food intake and body weight in different conditions, including stress. Injections of Ucn into the fourth ventricle induced a decreased food intake in rats (12). Therefore, we hypothesize a role for areas adjacent to the fourth ventricle in regulating body weight and food intake during acute stress. Experiments described here used two types of acute stress. Repeated restraint stress which can be considered a severe stress, in which the animal tends to lose a significant amount of weight (16); and injection of saline and movement to a novel cage, which can be considered a mild stress. Both stressors cause weight loss and an inhibition of food intake but the effect is smaller with mild stress than with repeated restraint (14). Because previous studies using third or lateral injections of CRF agonists or antagonists could have potentially acted in areas around the fourth ventricle, we can not exclude this area as a potential mediator of stress responses. Three sets of Experiments were performed. The first tested the long-term effects both CRFRs agonists and antagonists infused into the fourth ventricle on food intake and body weight in 12-hour food-deprived rats. We hypothesized that CRF infusions into the fourth ventricle would inhibit food intake in rats that had been food-deprived overnight, and that CRFRs antagonist would prevent this decrease in food intake. We also analyzed food intake in rats infused with selective CRF agonists. The second set of Experiments tested the effects of different CRFRs antagonists in the fourth ventricle during

either restraint or mild stress. We hypothesized that antagonism of CRFRs in brain nuclei adjacent to the fourth ventricle would prevent the stress-induced responses in body weight and food intake. The third set of Experiments analyzed activation of brain nuclei in the brainstem of rats exposed to MS after antagonism of CRFRs by the immunohistochemistry (c-Fos) method. Previous studies have shown that areas regulating peripheral and central signals, such as the nucleus of tract solitarius and the raphe nuclei, are connected to the forebrain, specifically the hypothalamus, by CRF-containing neurons (5). We hypothesized that antagonism of CRFRs in areas adjacent to the fourth ventricle before stress would prevent activation of those brain nuclei, and also prevent further activation of the hypothalamus.

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CHAPTER 2

LITERATURE REVIEW

Stress Definition

Stress (106) is defined as a physiological event to maintain homeostasis during a life-threatening situation (127). The stress response is usually generated by a stressor, which includes several different factors such as tension, anxiety or trauma (45). Hubbard (45) describes a stressor as any circumstance that causes a disruption of homeostasis (101), and Dallman (21) defines stressors as "the stimuli that induce physiological stress; and stress responses are changes in the brain and body that happen with intermittent stress". The physiological response to a stressful situation that tries keep homeostasis, first described by Sterling (119) is known as allostasis. The allostatic mechanism involves the neural, endocrine, autonomic, and immunologic systems (69, 70). Occasionally, the allostatic systems might not respond appropriately to stress, leading to an allostatic load (70). Allostatic load is thus defined as a failure to adapt to a stressful situation (70), it usually occurs when stress is constant or exaggerated, and it can lead to different negative responses in the body and consequent pathologies (52).

CRF Receptors and Brain

The neuropeptide corticotrophin-releasing factor (CRF), a 41-amino acid peptide, discovered in 1981 (116), has been identified as the initiator of behavioral and physiological responses to stress. CRF is released in response to stress, such as pain, fear, anxiety or an

emotional signal arriving at different brain nuclei, including the paraventricular nucleus (PVN) of the hypothalamus (103). CRF can also be activated by angiotensin II, neuropeptide Y (NPY), serotonin, acetylcholine, interleukin–1 and interleukin–6 (103) and it can be inhibited by gamma-aminobutyric acid (GABA), substance P, atrial natriuretic peptide (ANP), endogenous opioids and 1-arginine (101, 103).

CRF has two G-protein coupled receptor types: CRFR1 and CRFR2 (15, 87, 100). CRFR2 presents two variants (CRFR2β and CRFR2α), which are differently located in the body. CRFR1 and the variant CRFR2α prevails in neural tissue (62), whereas CRFR2β is expressed in areas such as heart, gastrointestinal tract, arterioles and muscles (50, 62).

Besides CRF (87), rodents also express the variants of CRF (87): urocortin (Ucn) (123), Ucn II (91) and Ucn III (124), which have similar function as CRF. The affinity of CRF and its variants to bind with each of the CRFRs varies (87). CRF has a higher affinity for CRFR1 than for CRFR2, whereas Ucn I has a similar affinity for both receptors (123). Ucn II and III appear to be selective ligands for CRFR2 (59, 123).

Several antagonists for CRFRs have been described, and they vary according to specificity and affinity for the receptors. Some non-selective CRFR antagonists include, $\alpha hCRF_{(9-41)}$ (94), and astressin (AST) (37). The CRFR antagonist, $\alpha hCRF_{(9-41)}$, appears to have a partial agonist effect both in vitro (112) and in vivo (73), whereas AST shows a very weak partial agonist effect (74) and a better antagonist effect (67). Moreover, AST showed a higher affinity for binding to CRFRs than $\alpha hCRF_{(9-41)}$ (37). Antisauvagine-30 (ASV30) is a competitive selective CRFR2 antagonist (97) that has been shown to be more potent and have better solubility in the cerebrospinal fluid than AST (12). In our study, we tested the effects of the CRFRs antagonists,

αhCRF$_{(9-41)}$, AST, and ASV30 infused into the fourth ventricle before the response to restraint or mild stress in rats.

The Systemic Response to Stress

The endocrine response to stress includes hormones under regulation of the pituitary gland (anterior and posterior), the autonomic nervous system, and endogenous opioid release (38, 103). The immunologic response consists of cytokine and eicosanoid production and activation of the kallikrein-kinin system (103). The metabolic responses are characterized by changes in whole body energy balance, lipid metabolism, and protein and amino acid metabolism (71, 103). The intensity of response may vary accordingly to: a) the potency to the stressor; b) the nature of exposure to the stressor, which can be continuous or intermittent; c) the stage of response, being acute (the first hours after exposure) or chronic (days or months); and d) individual differences, influenced by personal perception and interpretation of the stressor, physical condition, and previous experiences or early developmental events (38, 71, 103).

A)-The Hypothalamic-Pituitary-Adrenal (HPA) axis

The most important endocrine responses to conditions that may threaten homeostasis are corticoid and catecholamine released by activation of the HPA axis (114) and the autonomic nervous system (21, 24, 55, 103).

CRF released from cells of the PVN of the hypothalamus, enters the primary capillaries in the tubuloinfundibular tract, and passes through the hypophyseal-portal veins to reach the secondary capillary plexus in the adenohypophysis, where it stimulates both the synthesis and release of the adrenocorticotropic hormone (ACTH) from the corticotrope cells (71, 103, 116).

ACTH, a large polypeptide molecule, composed of a 39-amino acid sequence (116), is synthesized from its precursor, proopiomelanocortin (POMC) (101, 103). The anterior pituitary

also releases other secretagogs such as vasopressin (AVP) (32), oxytocin and catecholamines (101), which then stimulate the adrenal gland to release both glucocorticoids (GCs) and catecholamines (103). ACTH is also the ligand for melanocortin receptor 2 and activates Gs-protein and adenylcyclase in the fasciculata cells located in the adrenocortex (103). The second messenger cyclic AMP activates the enzyme desmolase causing the conversion of cholesterol to pregnenolone, which is the initial reaction in corticosteroid synthesis (103). Under normal circumstances, glucocorticoid (GC) (105) secretion is controlled mainly by ACTH, produced by the pituitary gland (38, 71, 103). The production and secretion of ACTH are controlled by hypothalamic CRF and GCs, and to a lesser extent by vasopressin, angiotensin II, cholecystokinin, oxytocin, proinflammatory cytokines, vasoactive intestinal polypeptide (VIP) and catecholamines (103).

Free GCs crossing the brain-blood barrier are a powerful inhibitor of CRF in the hypothalamus and of ACTH in the pituitary gland (103), down-regulating synthesis and release of both hormones. Adrenalectomy, the removal of the adrenals, inhibits the down-regulation of the feedback of the HPA axis. This results in an augmentation of CRF mRNA transcription, which can be reversed with exogenous doses of synthetic GCs such as dexamethasone or prednisolone (2, 103). In unstressed individuals, glucocorticoid (GC) secretion maintains a circadian rhythm, controlled by the suprachiasmatic nucleus (1, 24). In humans, the secretory rates of GCs rise at dawn, and they decline to a low level in the late evening (38, 71, 103). In rodents, which are nocturnal, the rhythm is reversed with a peak in the evening and a nadir in the early morning (1).

Glucocorticoids

GCs (cortisol in humans, corticosterone in rodents) have a significant effect on metabolism. They modify carbohydrate metabolism by stimulating gluconeogenesis by the liver, decreasing cellular glucose utilization and increasing circulating concentrations of glucose (2, 38). GCs induce amino acid mobilization from the muscle, decrease synthesis of proteins and increase catabolism of proteins, thus, supplying proteins to the liver for energy (2, 38). GCs also mobilize fatty acids from adipose tissue provide an energy substrate for the liver (38).

The GCs also affect the anti-inflammatory system, by suppressing synthesis and mobilization of white blood cells such as eosinophils and lymphocytes, and decreasing immune function (38). Changes in the cardiovascular and pulmonary systems are reflected as an increase in resistance and blood pressure, which delivers more oxygen and glucose to the organs (2, 38). GCs can also lead to changes in the endocrine and reproductive systems (32), by inhibiting growth hormone (5, 39), thyroid hormone (5), and reproductive hormones (102).

In summary, the glucocorticoids can block energy storage, while mobilizing energy from fat and proteins. They increase blood flow and inhibit anabolic conditions such as growth, reproduction, inflammation and immunity (2, 24, 38). Thus, they can optimize the amount of energy available for responding to a stressor.

B)- Opioid Release

Following ACTH release from the pituitary gland, other hormones that are derived from the ACTH precursor POMC, such as α- and β-melanocyte-stimulating hormone, β-lipotropin and β-endorphin are also secreted. Their secretion does not appear to play a role in behavioral responses or pain under normal conditions, but in the stressful conditions, when ACTH levels are high, the opioids can be involved (38, 103). β-endorphin has an opiate effect in the nervous

system, and can inhibit pain perception (38, 103). In addition, its activation in stress response causes a decrease in food intake due to a reduction of gastrointestinal peristalsis and inhibition of fluid secretion (113).

C)- Autonomic System

The spinal cord, brainstem and the hypothalamus are responsible for controlling activity of the autonomic nervous system (38). The sympathetic nervous system is activated by stress while the parasympathetic nervous system is inhibited (38). Catecholamines play an important role in the physiologic reaction to stress and injury (38, 103). The release of catecholamines (nor-epinephrine and epinephrine) into the blood stream from the adrenal gland is due to activation of the sympathetic nervous system. These hormones increase arterial pressure, blood flow to target organs (such as heart, muscle, kidneys, brain and eyes), rate of blood coagulation, cellular catabolism, mental activity and muscle strength (38).

Acute versus Chronic Stress

It is critical that we understand the differences between acute and chronic stress, as they can initiate different biological responses in the body. Acute stress is a short period of exposure to a stressor while chronic stress is a continuous exposure to a stressor (38, 103). The GCs play a role in response to both acute and chronic stress. CRF mRNA expression in the hypothalamus and ACTH release from the pituitary are inhibited during acute stress by free GCs arriving in the brain from the blood stream (22, 23, 121, 126). In contrast, during chronic stress, CRF mRNA expression in the brain and activity of the HPA axis are not down-regulated (22, 23). Recently, Dallman et al (23) suggested that other hormones, such as insulin might play a major role in the inhibition of the down-regulation of the HPA axis during chronic stress, whereas corticosterone might play a minor role in this mechanism (22). The authors suggest that chronically elevated

GCs can act either indirectly or directly in the body. GCs can stimulate food consumption, especially fat and sweets, through central mechanisms regulating feeding behavior (84), and, GCs can also stimulate release of insulin, leading to increase of fat stores in the body (22, 83).

If the body is unable to adapt to a stressful situation, the increased levels of glucocorticoids will lead the body to a catabolic condition (24, 55) and the results can be fatal. In chronic stress, however, the consequences can be life-threatening due to development of chronic disease in different systems such as the cardiovascular system, endocrine abnormalities and to development of psychiatric disorders (depression, drug abuse, anxiety) (22-24, 101). These diseases and disorders develop due to the actions of sustained elevated levels of glucocorticoids and catecholamines in the body (70, 80).

In addition, some studies have shown that rats previously exposed to a stress present a hyper-responsiveness of the HPA axis when submitted to a novel stress (4, 40, 65). Those animals showed exaggerated levels of ACTH and corticosterone levels during the second novel stress (31, 33, 40). This might suggest a sensitization of some receptors, such as CRFRs in the brain during the second exposure to stress, as some suggested a sustained increase in CRF expression in brain nuclei such as the PVN (46). In Experiments described here, we tested whether the antagonism of CRFRs during restraint would prevent the subsequent hypersensitivity to a second novel exposure.

Models of Acute Stress

Pare et al described several different types of stress models used in animal research [For review:(81)]. Repeated restraint stress (RRS) is one model of acute stress that can induce a change in the body weight of rats (81). Our RRS model consists of placing the rats in plastic restraining tubes for 3 hours for 3 consecutive days (43). The immobilization leads to an acute

increase in ACTH and glucocorticoid levels by activation of the HPA axis (29, 81). These hormones have been implicated in stress induced changes in food intake and body weight (81). Mild stress (MS) is another model of acute stress used in our Experiments, consisting of a 2 ml intra-peritoneal injection of saline and moving the rat to a different room for 2 hours. MS also induces a decrease in food intake and body weight, and an increase in GCs levels during stress but to a lesser extent than RRS (40). In our studies, we used both methods to induce stress.

The Effects of Acute Stress on Body Weight and Food Intake

Several investigators have demonstrated a decrease in body weight of rats exposed to acute stress (22, 98, 110) or in response to activation of CRFRs in the brain (54). We have previously demonstrated that RRS can induce a decrease in body weight and food intake during the stress period (43). Moreover, after the stress, rats maintain a decrease in body weight, and do not compensate for their body weight loss by overeating (42). These results might suggest that the stressed rats adjust the level at which they regulate their body weight. Rats submitted to MS can also show a decrease in food intake; however it is a smaller effect than with RRS (40). This might be explained by activation of dopamine, opiates (77), or serotonin release (86, 88). Harris et al (40) also showed that rats submitted to a second novel stress after exposure to RRS showed hypophagia and increased levels of GCs compared with rats that had not been previously stressed, suggesting a hyper-responsiveness of CRF receptors in the brain.

Acute stress changes both body fat and lean body mass. The decrease in body weight during acute stress in rats (23, 110, 121) is associated with a decrease in lean tissue (water and protein) rather than a decrease in fat tissue (130) during the stress period. During the post-stress period, however, both fat and lean mass are diminished by the same percentage (41, 43, 129, 130), suggesting that this effect in the post-stress period is due to the change in fatty acid

metabolism since adipocytes in restrained rats show an increased number of beta-adrenergic receptors and increased fatty acid oxidation (130).

Humans exposed to severe stress may show either an increase or a decrease in body weight (22). Wardle et al (126) showed that food intake can either increase or decrease in adults according to the levels of stress. This also may be associated with the type of stress; acute traumatic events cause weight loss whereas chronic stress tends to cause weight gain.

Although CRF plays an important role in regulating food intake and body weight, others hormones and neurotransmitters can also participate in the stress-induced inhibition of food intake and body weight regulation. Energy balance is maintained by different centers in the brain, and the hypothalamus appears to play a primary role in maintaining homeostasis through anorexigenic and orexigenic peptides, arriving either from within the brain or from the periphery. Anorexigenic peptides such as CRF, cholecystokinin, cocaine- and amphetamine-regulated transcript, alpha-melanocyte-stimulating hormone, vasopressin and leptin inhibit feeding, whereas orexigenic peptides such as neuropeptide Y (NPY), galanin, agouti-related protein, melanin-concentrating hormone, and ghrelin increase feeding [For review: (58)]. CRF appears to play an important role centrally, in regulating different peptides such as in the serotonergic pathway (88, 89); and also in the periphery, by its own inhibitory action on gastrointestinal motility (113). Therefore, we hypothesized that CRF and CRFRs are important initiators of stress-induced changes in body weight and food intake. Our objective was to test whether CRFRs located in the brainstem were involved in producing changes in food intake and body weight of stressed rats.

CRF and the Brain

Several investigators have demonstrated that injection of CRF in the lateral ventricle inhibits food intake in different species, such as rats (54), rabbits (79), sheep (96), pigs (82), fish (25), and birds (26, 92). A similar effect on food intake was also observed in rats injected with CRFR agonists, Ucn (20) and Ucn II (20).

Other studies have shown that CRFR antagonists could block different types of stress responses. Lateral ventricle injections of CRFR antagonists before RRS partially blocked the stress-induced decrease in food intake (53, 110), prevented the effects of subsequent injections of CRF into the lateral ventricle in food deprived rats (48), and also attenuated the anxiogenic responses in food intake in mice (85). Smagin et al (110) showed that $\alpha hCRF_{(9-41)}$ injected into the third ventricle before each day of restraint blocked the stress related changes in food intake and body weight of rats exposed to RRS. However, it could not stop the stress-induced activation of HPA axis (110), indicating that the area responsible for hypophagia is in, or near, the hypothalamus and functions independently of pathways that activate the HPA axis.

In addition to the lateral and third ventricle injections of CRF and CRFR antagonists, Grill et al suggested that areas adjacent to the fourth ventricle are important in maintaining energy balance responses to stress (34, 35). Injections of Ucn in the fourth ventricle inhibited food intake in rats (36), although the degree of inhibition was less than that caused by Ucn infusion into the lateral ventricle. It is possible that injection of either CRF or CRF antagonists into the lateral or third ventricle could have potentially acted in brain nuclei around the fourth ventricle. Consequently, we can not exclude the brainstem as a mediator of changes in food intake and body weight of stressed rats. In our study, we tested the effects of the antagonism of CRFRs in the fourth ventricle on food intake, body weight, and GC release in rats exposed to RRS or MS.

In addition to the decrease in food intake, CRF plays an important role in behavioral responses to stress. CRF injections into the lateral ventricle increased some behaviors, such as grooming (76) whereas CRF into the third ventricle inhibited exploratory behavior of rats (115). CRFRs antagonists in the lateral ventricle also blocked anxiogenic behavior such as food intake changes in food-deprived rats (48), anxiogenic effects of CRF in acoustic startle reflex in mice (93) and sexual behavior in hamsters (49). Therefore, CRF also appears to promote changes in behavior of unstressed animals.

Localization of CRFRs in the Brain

The different types of response often seen in stress are usually due to activation of either CRFR1 or CRFR2, as they seem to play different roles during stress according to their localization in the brain [For Review: (8, 27)]. CRFR1 has been implicated in mediating anxiogenic-like (51), and endocrine responses (122). This could be because CRFR1 is mainly localized in brain areas responsible for emotions and vigilance, such as the neocortex, the hippocampus, and the limbic system (15); and also in the hypothalamus and pituitary, responsible in regulating hormonal release in stress (122). Also, studies have shown that mice lacking CRFR1 exhibit an attenuated anxiety and hormonal response to stress (18). These changes in behavior have also been observed in studies using antisense oligonucleotides to CRFR1 in rats (61).

CRFR2, is predominantly found in brain areas such as the lateral septal nucleus, the ventromedial hypothalamic nucleus (VMH), the PVN, the choroid plexus, the amygdala, the raphe nuclei (RN), the nucleus of tract solitarius (NTS), and the area postrema (AP) (15). The anxiogenic and anxiolytic effects observed during stress may be regulated by activation of CRFR2 (44). Bale et al showed that CRFR2 knockout mice are hypersensitive to stress, and

present an exaggerated anxiety-like behavior (7), which includes food intake and appetite behavior (20, 28, 85). Therefore, CRFR2 might play an important role in regulating homeostasis and energy balance (6). Studies have shown that CRFR2 play a major role in anorexia-induced response to stress (111), for example, food intake was decreased in CRFR1 knockout mice infused with Ucn (11). The decrease in food intake was also observed in rats receiving the selective CRFR2 agonist, UcnII (47). This effect in feeding was blocked when the selective CRFR2 antagonist ASV-30 was injected into the lateral ventricle (85), supporting the idea that CRFR2 might be the major regulator in feeding-responses to stress. However, some studies show that both receptors might influence food intake (104), as CRFR1 has been implicated in the early feeding response to stress (11) whereas CRFR2 appears to regulate the late phase of food intake during stress (19).

The hypothalamus appears to be the main brain region in integrating information from other brain nuclei and different stress responses, such as endocrine, autonomic, and behavioral. Dallman et al (9), showed that the PVN plays an important role in regulating food intake, body weight, and energy balance during stress. The VMH appears to influence the feeding response by regulating the circadian rhythm of GCs (64) in the suprachiasmatic nucleus (17). Extra-hypothalamic areas also express CRFRs. The amygdala has been identified as a center that initiates anxiety-like behavior as a response to stress (90, 109) and drug dependence (72), by activation of both subtypes of CRFR. The bed nucleus of the stria terminalis is also involved in behavioral responses, such as fear and anxiety (57), and also in drug dependence (30) together with the hippocampus and amygdala (16, 30). The Edinger-Westphal nucleus (E-WN) is known to regulate oculo-motor function, but it also appears to play some role during stress, as E-WN is the nucleus that has the highest concentration of Ucn-expression (10).

Brown et al (13) first suggested an integration between the brainstem and forebrain in energy balance by activation of CRFRs. Since then, attention has been given to the brainstem as a regulator in energy homeostasis during stress. The brainstem has been shown to connect and integrate peripheral signals to the forebrain [For Review: (34, 35)]. Central (78) or fourth ventricle (113) infusions of CRF in rats induce a delayed gastric emptying, leading to a similar inhibition of feeding as is produced by stress. In addition, peripheral infusion of CRF and Urocortins, and activation of CRFRs decrease gastric motility and emptying, down-regulating food intake [For Review: (68)].

It is accepted that different types of stress, including restraint, increase CRF expression in the brain (14, 107). One way to identify activation of specific brain areas is by c-Fos immunohistochemistry, which has been widely used for identifying cell nuclear cell activation in the brain [For Review: (99)]. The c-Fos, a proto-oncogene, is usually activated by some type of stimulation, and it plays a role in regulating early gene transcription although its function is not yet very clear [For Review: (99, 108)]. Investigators have shown that icv or peripheral injections of CRF and/or Ucn can elicit Fos expression in several different brain nuclei, including the PVN, NTS, and the RN (3, 56, 125). In addition, Wang et al (125) showed that peripheral injection of Ucn had a stronger effect than CRF in inducing Fos expression in the NTS and RN.

CRFRs, mainly CRFR2, and Ucn are highly expressed in brainstem areas, such as in the AP, the NTS, the RN, and locus coerulus (LC) (10, 122). The AP and NTS receive multiple inputs from the periphery (95, 128) and connect to areas, such as the PVN and the arcuate nuclei; therefore, regulating appetite. The LC presents a high concentration of catecholaminergic neurons that also connect to the PVN (60), and it appears to play a role in stress and opioid sensitivity thus, drug dependence (120). Studies have shown that the RN contains serotonergic

(5-HT) and CRF-neurons, and it is responsible for regulating serotonin release in the hypothalamus and for circadian rhythm in the SCN (89), by a neuronal pathway suggested previously by Morgan et al (75). RN-site-specific injections of CRFR2 agonist (Ucn II) can increase Fos expression of serotonergic neurons (118), and this effect is inhibited by previous injection of the CRFR2 antagonist, ASV30 (117, 118). Thus, CRF has been shown to regulate the serotonergic system in the RN by either activating or inhibiting neuronal activity (88) and subsequent release of serotonin in the hypothalamus through CRF connections (16). Therefore, the serotonergic system might play a role in acute stress-responses (66), such as anxiety-like behavior [For Review: (63)], and energy balance regulation. Those changes, however, are dependent on the level of stress (86).

The stress-induced changes in energy balance and behavior appear to be regulated by a complex pathway, involving visceral afferents, and several different nuclei in the brainstem and the forebrain. What is not yet clear is which of these nuclei or which CRFR subtype is responsible for the primary response that leads to weight loss and inhibition of food intake in rats exposed to stress. For this reason, we investigated the role of the brainstem in stress-induced changes in food intake and body weight. Our objective was to test whether agonism and/or antagonism of CRFRs located in the brainstem would modify the changes in food intake, body weight, corticosterone release, and c-Fos immunoreactivity induced by RRS or MS, and whether antagonism of CRFRs during restraint would prevent the subsequent hypersensitivity of RRS rats towards MS.

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CHAPTER 3

AGONISM/ANTAGONISM OF CRF RECEPTORS IN THE FOURTH VENTRICLE MODIFY FEEDING RESPONSES IN RATS

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ABSTRACT

The neuropeptide corticotrophin-releasing factor (CRF) is one of the first factors involved in the response to stress, and one of the initial responses is regulation of feeding behavior. CRF and its homologues, urocortin (Ucn), Ucn II and Ucn III, activate the two CRF receptor subtypes: CRFR1 and CRFR2. CRF has been shown to have a higher affinity for CRFR1 than for CRFR2; whereas Ucn I has a similar affinity for both receptors, and Ucn II and III are selective for CRFR2. Studies have shown that CRF also has hypophagic properties in unstressed animals. Experiments described here tested whether infusions of CRFR agonists and/or antagonists would change feeding behavior in food-deprived rats. In the first Experiment, we observed that infusions of a non-selective CRFR antagonist (αhCRF$_{(9-41)}$) into the fourth ventricle before infusions of 5 *u*g of CRF blocked a decrease in food intake; however, this CRFR antagonist did not change feeding patterns in hungry rats in Experiment 2. In Experiment 3, we found that CRF and Ucn II inhibited food intake, showing both an early and a late response. We conclude that CRF has a role in regulating feeding behavior in unstressed animals, and that the brainstem might be responsible for integrating these responses during food-deprivation.

Key words: brainstem, αhCRF$_{(9-41)}$, CRF, Ucn II, food-deprivation, rats

INTRODUCTION

The brain is critical for regulation of all systems in the body during stress (22). Stress can down- or up-regulate some neuropeptides, neurotransmitters, and receptors in specific brain nuclei (8, 25); and thus, induce emotional and behavioral responses. The neuropeptide corticotrophin-releasing factor (CRF), a 41-amino acid peptide, was discovered in 1981 (48) and more recently homologues of CRF have been identified (36): urocortin (Ucn) (52), Ucn II (38) and Ucn III (53), which have similar functions as CRF. CRF has two G-protein coupled receptor subtypes: CRFR1 and CRFR2, located in several different brain nuclei and in the body (3, 36, 42). The affinity of CRF and its homologues to bind with each of the CRFRs varies (36). CRF has a higher affinity for CRFR1 than for CRFR2, whereas Ucn I has a similar affinity for both receptors (52), but Ucn II and III appear to be selective ligands for CRFR2 (26, 52).

The different responses often seen in stress are usually due to activation of either CRFR1 or CRFR2, as they seem to play different roles during stress according to their localization in brain nuclei [For Review: (1, 11)]. CRFR1 has been implicated in mediating anxiogenic-like (19), and endocrine responses (51). CRFR1 is mainly localized in the neocortex, the hippocampus, and the limbic system (3); and also in the hypothalamus and pituitary gland (51).

In contrast, CRFR2 is predominantly found in brain areas such as the lateral septal nucleus, the ventromedial hypothalamic nucleus (VMH), the paraventricular nucleus of hypothalamus (PVN), the choroid plexus, the amygdala, the raphe nuclei (RN), the nucleus of tract solitarius (NTS), and the area postrema (AP) (3). The anxiogenic and anxiolytic effects observed during stress may be regulated by activation of CRFR2. Studies have shown that CRFR2 play a major role in anorexia-induced response to stress (45). Food intake was decreased in CRFR1 knockout mice infused with Ucn (2). Inhibition of food intake was also observed in rats receiving the

selective CRFR2 agonist, Ucn II (16), and it was blocked when a selective CRFR2 antagonist was used in the lateral ventricle (34). However, some studies show that both receptor subtypes might mediate stress-induced changes in food intake (43), as CRFR1 is implicated in the early feeding regulation (2) whereas CRFR2 appears to regulate the late phase of food intake during stress (5).

Investigators have demonstrated that lateral intracerebroventricular (icv) injection of CRF inhibits food intake in different species (9, 10, 21, 31, 33, 39, 41). A similar effect on food intake was also observed in rats injected into the lateral ventricle with the CRFR agonists, Ucn (6) and Ucn II (6). Several investigators have demonstrated the importance of CRFRs in areas adjacent to the ventricles; however, it is not clear which area is responsible for regulating or initiating food intake and body weight changes during stress. Lateral intracerebroventricular (icv) injection of CRF induces weight loss in rats and birds by inhibiting food intake (21, 39). Injections of a non-specific receptor antagonist (αhCRF$_{(9-41)}$) in the third ventricle before each day of restraint in rats subjected to repeated restraint, were able to block the body weight loss (44). And, fourth ventricle injections of Ucn inhibited food intake in rats (14), although the degree of inhibition was less than that caused by Ucn infusion into the lateral ventricle. Lateral (30) or fourth ventricle (47) infusions of CRF in rats delays gastric emptying, leading to a similar inhibition of feeding as is observed during stress. Therefore, we cannot exclude the possibility that CRF agonists and antagonists injected into the lateral and third ventricle might have acted in areas around the fourth ventricle.

We hypothesize that fourth ventricle infusions of CRF, CRFR agonists or antagonists would change food intake of food-deprived rats. Therefore, the objective of this study was to test whether activation of CRFRs located in the brainstem by CRF or Ucn II would modify food

intake of rats that had been food-deprived, and whether the antagonism of CRFRs before infusion of CRF would attenuate the inhibition of intake caused by CRF.

METHODS

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.

Exp 1: *Food intake of food-deprived rats receiving αhCRF$_{(9-41)}$ prior to CRF infusions in the fourth ventricle*

This experiment was designed to test whether infusions of a non-selective CRFR antagonist into the fourth ventricle before infusions of 5 *u*g of CRF would block an inhibition of food intake in hungry rats.

Twenty-eight male Sprague-Dawley rats (Harlan Sprague Dawley, Indianopolis, IN) weighing approximately 379g were included in this experiment. The rats were placed in hanging wire cages in a room with controlled temperature (22.7°C) and humidity (52%) and a 12:12h light-dark cycle (7am: 7pm). The rats had free access to water and standard rat chow (Purina rodent chow 5001: Purina Mills, MO) throughout the experiment. All rats were fitted with 26G guide cannulae (Plastics One, Roanoke, VA) aimed at the fourth ventricle. They were anaesthetized with ketamine/xylazine mix (90 mg/kg Ketamine, 10 mg/kg Xylazine) administered i.p.. They were positioned in a stereotaxic apparatus, an incision was made in the skin along the midline, and periosteum was scraped from the skull. The coordinates for placing the cannula in relation to the midline at the occipital suture were 2.5 mm anterior, 0 mm lateral, and 5.2 mm ventro-dorsal (Paxinos & Watson Brain Atlas) (32). Cannulas were fixed to the skull with jewelers' screws and dental cement. The rats were injected with

analgesic Ketophen (2mg/kg Ketoprofen) immediately after surgery and again the next day. The rats were allowed 1 week for a complete recovery before the cannula placement was confirmed. The position of the cannula in the fourth ventricle was confirmed by measuring hypoglycemia-induced gluconeogenesis. The 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 *u*g 5-thio-D-glucose (Sigma-Aldrich, St Louis, MO) in 2 *u*l sterile isotonic saline over one minute. Those rats that showed a doubling of blood glucose from the baseline 60 minutes after the infusion were included in the experiment. Rats were allowed one week before the beginning of the experiment.

The animals were handled daily before the beginning of the Experiment to decrease stress associated with handling. On the day of the Experiment, the rats were divided into 4 weight-matched groups: αhCRF$_{(9-41)}$/CRF, Saline/CRF, αhCRF$_{(9-41)}$/Saline, Saline/Saline. Rats were then food deprived overnight before the Experiment. The following morning, one hour after lights were on, all rats received a 2 *u*l infusion of saline or 5 *u*g (~1.3 nmol) of αhCRF$_{(9-41)}$ (Bachem Bioscience, King of Prussia, PA) into the fourth ventricle over one minute. Ten minutes later, all rats received a second infusion of saline or 5 *u*g of CRF (~1.0 nmol) into the fourth ventricle. All animals were kept in their home cages, and food was returned ten minutes after the end of all infusions. Food intake was recorded at 2, 4, and 6 hours after food was returned. Body weight was also recorded immediately before and the day after infusions.

Exp 2: *Food intake of rats after αhCRF$_{(9-41)}$* or *saline infusions into the fourth ventricle in 12h-food-deprived rats*

This Experiment was conducted because Experiment 1 showed that the CRFR antagonist, αhCRF$_{(9-41)}$, inhibited food intake of food-deprived rats.

Twenty-nine male Sprague-Dawley rats weighing approximately 335g were fitted with fourth ventricle cannula as described for Experiment 1. Seven days after testing cannula placement the rats were food-deprived overnight. The next morning, rats were divided into 2 weight-matched groups, and received a 2 *u*l infusion of either 5 *u*g of αhCRF$_{(9-41)}$ (~1.3 nmol) or of saline in the fourth ventricle. Rats were kept in their home cages throughout the experiment. Food intake was recorded at 2h, 4h, and 6h after infusion. Body weight was also measured immediately before and 24 hours after infusions.

Exp 3: *Food intake after infusions with CRF, Ucn II or saline into the fourth ventricle in 12h-food-deprived rats*

This experiment was conducted to test the effect of fourth ventricle CRF, Ucn II or saline on body weight and food intake in rats.

Twenty-nine male Sprague-Dawley rats weighing approximately 422g were fitted with fourth ventricle cannula as described for Experiment 1. Seven days after testing cannula placement, the rats were divided into 3 weight-matched groups, and they received a 2 *u*l infusion of CRF (~0.6 nmol) or Ucn II (~ 1.0 nmol) or saline in the fourth ventricle. Rats were kept in their home cages throughout the experiment. Food intake was recorded for one day before and at 2h, 4h, 6h, 12h, 24h, 48h, and 72h after infusion. Body weight was also measured on the day of infusion and 24, 48, and 72 hours after infusion.

Statistics:

Food intake and body weight data were analyzed by ANOVA repeated measures (Statistica, Stat Software, Tulsa, OK). Intervaled food intake was compared by two-way ANOVA. Duncan's Multiple Range Test was used for post hoc comparisons among groups. Differences were considered statistically significantly at $p < 0.05$.

RESULTS

Exp 1: *Food intake of food-deprived rats receiving αhCRF$_{(9-41)}$ previous to CRF infusions in the fourth ventricle*

In this Experiment, we did not find any effect of CRF infusion on food intake as intakes of Saline/CRF rats were the same as those of Saline/Saline rats at all time points. The CRFR antagonist αhCRF$_{(9-41)}$ stimulated food intake of αhCRF$_{(9-41)}$/Saline rats 2h after infusion (Fig 1A: second infusion: $p > 0.01$), compared with αhCRF$_{(9-41)}$/CRF ($p< 0.006$) or Saline/CRF ($p < 0.02$) rats, but intake was not different from that of Saline/Saline rats (Fig 1A: NS). A significant effect on food intake was also noted in cumulative food intake 0-6h after infusions (Fig 1B: second infusion: $p< 0.02$, first X second infusions: $p<0.04$), in which cumulative food intake from 0-6h was greater in αhCRF$_{(9-41)}$/Saline rats compared to αhCRF$_{(9-41)}$/CRF rats ($p<0.007$). There was no difference in food intakes of the groups at any other time after infusions (Fig 1A: NS) and no treatment effect on weight change during the next 24 hours (Fig 2: NS).

Exp 2: *Food intake of rats after αhCRF$_{(9-41)}$* or *saline infusions into the fourth ventricle in 12h-food-deprived rats*

There was no effect of αhCRF$_{(9-41)}$ on food intake of 12h food-deprived rats at 2h, 4h, 6h, and 24h (Fig 3A: NS), or on cumulative food intake 0-24h after infusion (Fig 3B: NS). Also, there was no effect on body weight of the rats (data not shown: NS).

Exp 3: *Food intake of rats after infusions with CRF, Ucn II or saline into the fourth ventricle in 12h-food-deprived rats*

There was a significant inhibition of food intake measured 4-6h after infusion in rats receiving CRF or Ucn II (Fig 4A: treatment: $p < 0.004$). Ucn II also inhibited food intake at 12-24h after infusion (Fig 4A: treatment: $p < 0.004$), with a significant decrease at 24h cumulative intake (Fig 4B: treatment: $p< 0.002$). There were no differences in food intake at 0-2h, 2-4h, or 6-12h after infusions. Ucn II-infused rats showed a significant decrease in cumulative food intake over 24 hours when compared to saline-infused rats (Fig 5: treatment: $p < 0.03$). Weight change following infusion was not different among the groups (Fig 6: NS).

DISCUSSION

Investigators have shown that CRF infused into the lateral ventricle reduces food intake in food-deprived animals once food is replaced (17, 18). Therefore, we suggest that CRF might play an important role in regulating feeding responses during food-deprivation.

Others have shown that, $\alpha hCRF_{(9-41)}$, a non-selective CRFR antagonist (40), inhibited the effects of a subsequent infusion of CRF, preventing a decrease in food intake of food-deprived rats, but $\alpha hCRF_{(9-41)}$ alone did not have the same effect (17, 20). In contrast, our results in Experiment 1, showed that $\alpha hCRF_{(9-41)}$/Saline rats increased food intake once food was replaced; however, we did not observe changes in food intake between $\alpha hCRF_{(9-41)}$/CRF and Saline/CRF rats. We suggest that endogenous CRF was already high due to food restriction and that infusions of $\alpha hCRF_{(9-41)}$ blocked CRF binding to CRFR, but the antagonist effect of $\alpha hCRF_{(9-41)}$ was inadequate for blocking elevated levels of CRF caused by both food deprivation and infusion of CRF.

Although αhCRF$_{(9-41)}$, a competitive non-selective CRFR antagonist (40), has been previously reported to have a partial agonist effect both in vitro (46) and in vivo (29), we did not find a decreased food intake in food-deprived αhCRF$_{(9-41)}$/CRF infused rats. Therefore, we suggest that CRFRs located around the fourth ventricle might play a role in regulating feeding responses during food-deprivation, and this could be blocked by infusions of a CRFRs antagonist. Interestingly, we were unable to show that fourth ventricle infusion of the CRFR antagonist, αhCRF$_{(9-41)}$, decreased food intake response after food replacement in food-deprived rats in Experiment 2, which is consistent with previous studies (17, 20).

Previous studies have shown that lateral ventricle injection of CRF in food-deprived animals decreases food intake once food is replaced (17, 18). Therefore, suggesting that areas controlling for appetite regulation could be located in the forebrain. However, the brainstem contains nuclei such as the area postrema (AP) and the nucleus of solitarius tract (NTS), which have been implicated in regulating food intake and appetite by inputs from the periphery and connections to areas, such as the hypothalamus (4). We tested whether the brainstem played an important role in inhibiting food intake of CRF-infused rats. This brain area has been shown to be important in energy balance, and in regulating food intake and appetite (12, 13). One way to characterize the importance of the brainstem in regulating food intake and appetite is by using decerebrate rats, in which the neural connections between forebrain and brainstem are surgically destroyed. Daniels et al (7) showed that decerebrate rats infused with Ucn into the fourth ventricle decreased food intake, and Fos expression was increased in the NTS; therefore, indicating that the NTS might be an important regulator in the feeding response by activation of CRFRs.

It is known that CRF has two receptor subtypes: CRFR1 and CRFR2, which are located in several different brain nuclei and in the body (3, 36, 42). And, that CRF has different affinities for each of them, as CRF has a higher affinity for CRFR1 than for CRFR2, whereas Ucn I has a similar affinity for both receptors (52), and, Ucn II and III appear to be selective ligands for the CRFR2. Location and functions of CRFRs can also vary as CRFR1 is mainly localized in the neocortex, the hippocampus, and the limbic system (3); and also in the hypothalamus and pituitary (51), whereas CRFR2 is predominantly found in brain areas involved in feeding (3), supporting the idea that CRFR2 might be the major regulator in feeding-responses when CRFRs are activated.

For this reason, we conducted Experiment 3, in which again we tested the feeding response in food-deprived animals receiving fourth ventricle infusion of either CRF or Ucn II. We observed a decrease in food intake of both CRF and Ucn II infused rats after food was replaced, supporting the idea that both CRFRs in the brainstem are involved in the feeding response (43). The Ucn II infused rats showed a decrease in both intervaled and cumulative food intake at 24 hours after infusion, suggesting a regulation of food intake by CRFR2. These results are consistent with previous studies in which CRFR2 appears to regulate the late phase of food intake (5, 7). In contrast with Experiment 1, we found that CRF infused in the fourth ventricle decreased food intake 4-6 hours after infusion in food-deprived rats in Experiment 3. This could be explained by the exogenous amount of CRF infused into the fourth ventricle. In Experiment 1, we infused 5 *u*g of CRF in the fourth ventricle in rats, whereas in Experiment 3, we infused 3 *u*g of CRF. Therefore, it is tempting to postulate that higher levels of exogenous CRF infused into the fourth ventricle leads to an exaggerated negative feedback to some areas in the midbrain through some well-defined CRF-connections (3, 4).

CRFR2 is highly expressed in brainstem areas such as the raphe nuclei (RN), the NTS, and the AP (3), and these receptors appear to regulate the anxiogenic and anxiolytic effects often observed during stress (15). RN has been implicated in some of these responses, by regulating neuronal activity and release of serotonin in the brain. Serotonin is involved in different responses such as feeding and body weight regulation [For Review: (24)]. Therefore, the RN has been extensively studied as a potential target for several different responses in behavior by activation of CRFRs. Studies have shown that RN-site specific injection of selective CRFR2 agonist (Ucn II) increased c-Fos expression (50), whereas infusion of a CRFR antagonist Antisauvagine-30 (ASV30) blocked the cell activation (49, 50). CRF may regulate the serotonergic system in the RN by either activating or inhibiting neuronal activity (37) and subsequent release of serotonin by the hypothalamus through CRF connections (4). Therefore, the serotonergic system in the RN might play a role in responses (28), such as anxiety-like behavior [For Review: (27)], and energy balance regulation. The high doses of Ucn II used in Experiment 3 could be compared with a severe type of stress (35), which could lead to activation of CRFR2 in the RN and further decrease in food intake, as previously reported (23). Therefore, we conclude that CRF infusions might play a role in food-deprivation by activation of CRFRs of some brainstem nuclei, and that $\alpha hCRF_{(9-41)}$ can block endogenous CRF-binding to receptor but not endogenous and exogenous CRF.

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LEGENDS

Figure 3.1. Food intake after first and second infusions in food-deprived rats in Experiment 1. Panel A is intervaled, whereas Panel B is cumulative food intake 0-6 hours after infusions. Data are means $\pm$ SEM for 7 rats. Superscripts indicate significant differences between treatment groups for a specific time interval.

Figure 3.2. Body weight change 24 after infusions in food-deprived rats after first and second infusions in Experiment 1. Data are means $\pm$ SEM for 7 rats.

Figure 3.3. Food intake after infusion of αhCRF$_{(9\text{-}41)}$ or saline in food-deprived rats in Experiment 2. Panel A is intervaled food intake, whereas Panel B is cumulative food intake after infusions. Data are means $\pm$ SEM for 19-20 rats.

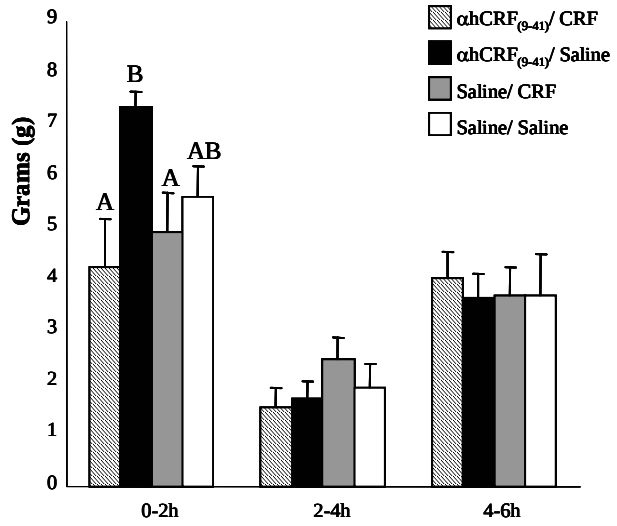
Figure 3.4. Food intake after infusion of saline, CRF, or Ucn II in food-deprived rats in Experiment 3. Panel A is intervaled food intake at different time points, whereas Panel B is cumulative food intake 2-24h after infusions. Data are means $\pm$ SEM for 9-11 rats. Superscripts indicate significant difference in food intake of treatment groups for a specific time interval.

Figure 3.5. Food intake data before and after saline, CRF, or Ucn II infusions in food-deprived rats at 24h, 48h, and 72h in Experiment 3. Data are means $\pm$ SEM for 9-11 rats. Superscripts indicate significant difference in cumulative food intake of treatment groups.

Figure 3.6. Body weight changes 24h, 48h, and 72h after infusions of saline, CRF, or Ucn II in food-deprived rats in Experiment 3. Data are means ± SEM for 9-11 rats.

Figure 3.1

A: *Intervaled food intake*



B: *Cumulative food intake*

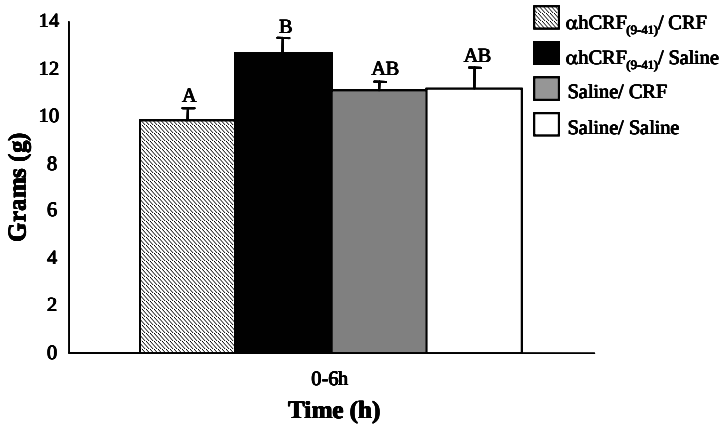


Figure 3.2

Body weight change

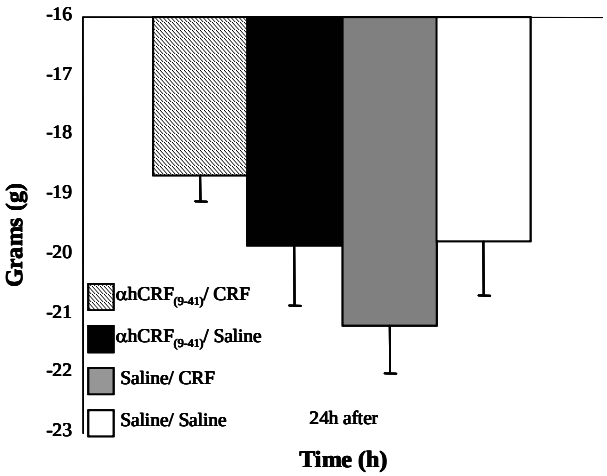


Figure 3.3

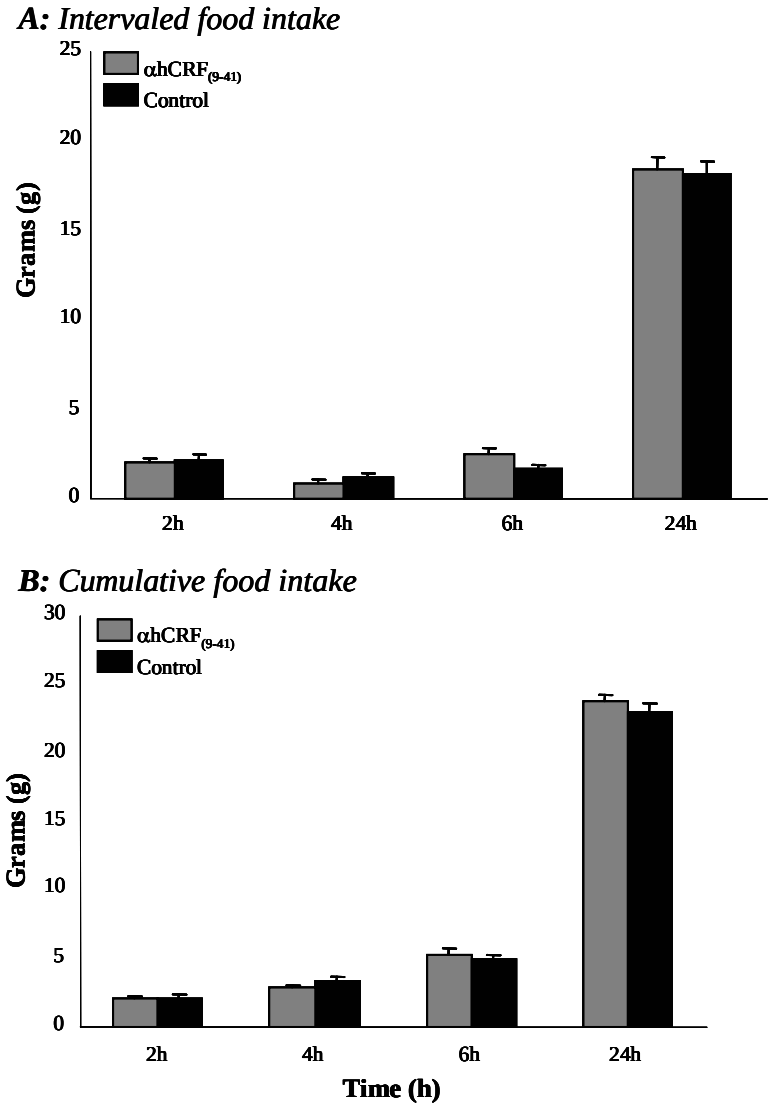

A: Intervaled food intake
αhCRF(9-41)
Control
Grams (g)
25
20
15
10
5
0
2h
4h
6h
24h
B: Cumulative food intake
αhCRF(9-41)
Control
Grams (g)
30
25
20
15
10
5
0
2h
4h
6h
24h
Time (h)

Figure 3.4

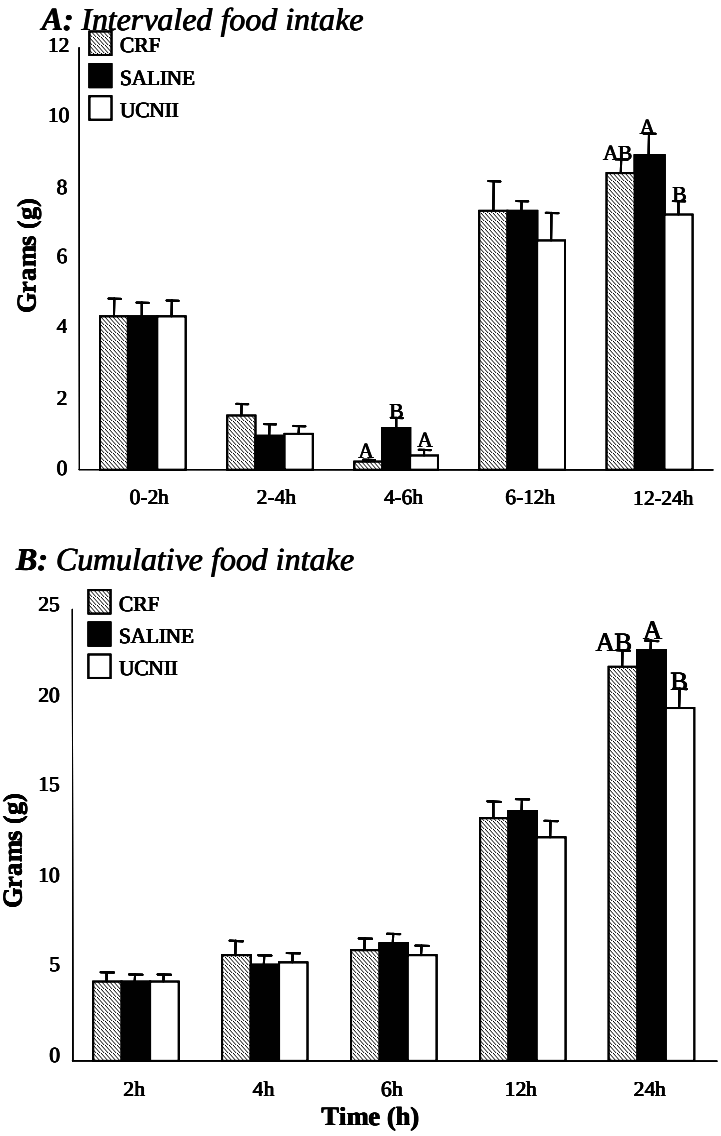

A: Intervaled food intake
CRF
SALINE
UCNII
Grams (g)
0
2
4
6
8
10
12
0-2h
2-4h
4-6h
6-12h
12-24h
A
B
A
AB
A
B
B: Cumulative food intake
CRF
SALINE
UCNII
Grams (g)
0
5
10
15
20
25
2h
4h
6h
12h
24h
Time (h)
AB
A
B

Figure 3.5

Cumulative food intake at 24h, 48h, and 72h

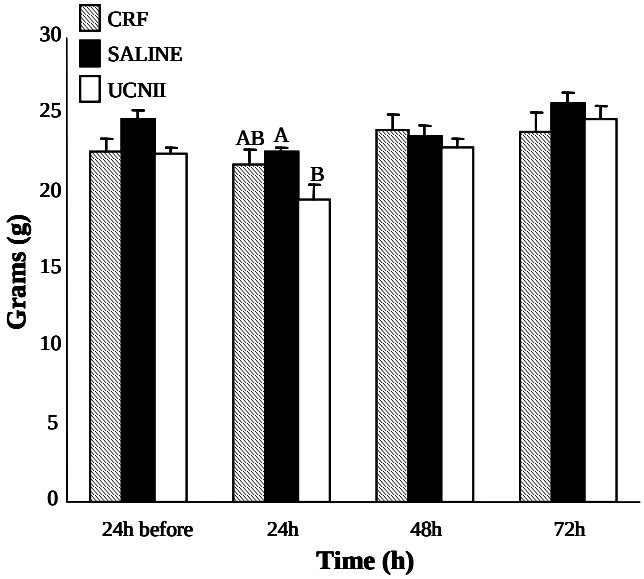
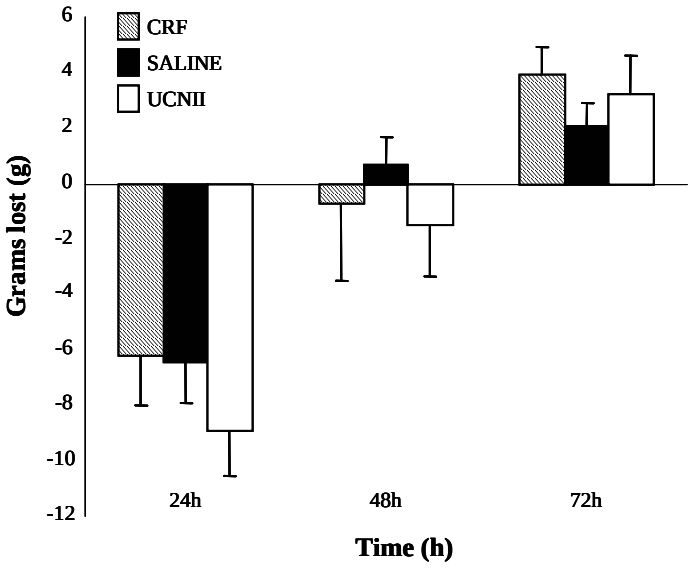


Figure 3.6

Body weight changes



CHAPTER 4

ANTAGONISM OF CORTICOTROPHIN-RELEASING FACTOR RECEPTORS IN THE FOURTH VENTRICLE MODIFIES RESPONSES TO MILD BUT NOT RESTRAINT STRESS

Miragaya, J., Harris, R.B. *To be submitted to the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.*

ABSTRACT

Stress is characterized by various endocrine, metabolic, and behavioral responses, and the neuropeptide corticotrophin-releasing factor (CRF) appears to be the initiator for the stress response. Two CRF receptor subtypes, CRFR1 and CRFR2, have been identified and they appear to mediate different types of stress responses. Repeated restraint stress (RRS) is one model of severe acute stress that inhibits food intake and decreases body weight whereas mild stress (MS) also inhibits food intake and body weight but to a lesser extent. We have also demonstrated an exaggerated glucocorticoid and behavioral response in animals that have been exposed to RR when submitted to a novel mild stress. Areas located adjacent to the third and/or fourth ventricle are known to be involved in the regulation of energy balance. Experiments here tested whether areas responsible for the down-regulation of body weight and the hyperresponsiveness of previously restrained rats. We infused antagonists to CRFR1, CRFR2, or all CRFR in the fourth ventricle immediately before either RRS or MS. The non-specific CRFR antagonist, $\alpha hCRF_{(9-41)}$, acted as a partial agonist, during RRS and MS, exaggerating the stress response. Astressin, another non-specific CRFR antagonist, had no effect on response to RRS or the hypersensitivity to a subsequent stress. In contrast, astressin did block the body weight loss and inhibition of food intake caused by MS. Antisauvagine-30, a CRFR2 antagonist produced a similar inhibition as astressin. This data indicate that the decrease in body weight and inhibition of food intake induced by RRS or by MS are mediated by different pathways, and that the brainstem might play an important role in regulating the response to MS.

Key words: brainstem, $\alpha hCRF_{(9-41)}$, astressin, antisauvagine-30, repeated restraint stress, mild stress, rats

INTRODUCTION

The neuropeptide corticotrophin-releasing factor (CRF) has been identified as an initiator of behavioral and physiological responses to stress. CRF, a 41-amino acid peptide, was discovered in 1981 (46) and has two G-protein coupled receptor subtypes: CRFR1 and CRFR2 (8, 40), and the CRFR2 presents two variants (CRFR2β and CRFR2α). The variant CRFR2α receptor prevails in neural tissue (30), whereas CRFR2β receptors are expressed in areas such as heart, gastrointestinal tract, arterioles and muscles (26, 30). Recently additional ligands that activate CRFRs have been identified. These are urocortin (Ucn), Ucn II and Ucn III. While Ucn II and III appear to be selective ligands for the CRFR2, CRF has a higher affinity for CRFR1 than CRFR2, and Ucn I has a similar affinity for both receptors (29, 48).

According to some studies, CRFR2 mediate behavioral responses (1, 35) to stress, including inhibition of food intake and body weight loss (14, 34, 35, 41), but CRFR2 do not seem to play a role in Hypothalamic-Pituitary-Adrenal (HPA) axis responses to stress (37, 43). Some studies have demonstrated the importance of CRFR2 in energy balance and body weight regulation by using knockout mice (2, 3). In addition, studies showed that the high density of CRFR2 in the PVN (4, 30, 50), and its connection to other areas of the hypothalamus, the brainstem and limbic structures, may play a role in suppressing food intake and causing weight loss in stressed rats (4). Many sites in the brain are involved in the initial response to a stressor. Nuclei, such as the dorsomedial nucleus of the hypothalamus (DMN), the arcuate nucleus (ARC), the paraventricular nucleus of the hypothalamus (PVN), the ventromedial hypothalamus (VMH), the area postrema (AP), the nucleus of the solitary tract (NTS), and the dorsal raphe nuclei (DRN) express CRFR2 (3, 13, 14, 41). The pituitary gland also (26) expresses CRFR2α (30).

Several investigators have demonstrated a decrease in body weight of rats exposed to acute stress (12, 42) or in response to activation of CRFRs in the PVN (28). Humans exposed to severe stress may show either an increase or a decrease in body weight (12). Similarly, Wardle et al (49) showed that food intake can either increase or decrease in adults according to the level of stress. This may be associated with the type of stress; acute traumatic events cause weight loss whereas chronic stress tends to cause weight gain. Dallman et al (12) hypothesized that the increase in body weight during chronic stress results from glucocorticoids enhancing the pleasantness of palatable foods, and other factors could be responsible for the weight gain, such as insulin, leading to a feedback inhibition of CRF. Also, a moderate type of acute stress can stimulate food intake due to a possible activation of opiates or dopamine in animals (31).

Pare et al described several types of stress used in different animals, and repeated restraint stress (RRS) is one model of stress that can induce a change in the body weight of rats (33). The immobilization leads to an acute increase in ACTH and corticosterone levels which tend to decrease with subsequent exposures (15, 33). These hormones have been implicated in stress induced changes in food intake and body weight (33). We have previously demonstrated that rats exposed to RRS for 3 hours for 3 consecutive days, showed a decrease in food intake and body weight on the days of stress (25). The rats do not compensate for the weight loss and do not show hyperphagia in the post-stress phase (25). Therefore, the weight of restrained rats remains lower than that of controls for extended periods of time (24). Others have reported that animals previously submitted to a stress showed exaggerated levels of adrenocorticotrophic hormone (ACTH) and corticosterone during a second novel stress (16, 18, 23). Moreover, Harris et al (23) showed that rats submitted to mild stress (MS) twelve days after exposure to RRS were hypophagic compared with rats exposed to MS that have not been previously stressed.

Investigators have demonstrated that lateral intracerebroventricular (icv) injection of CRF induces weight loss (28, 38). Injections of a non-specific receptor antagonist (αhCRF$_{(9-41)}$) in the third ventricle before each day of restraint in rats subjected to repeated restraint, were able to block the body weight loss (42), but could not stop stress-induced activation of HPA axis (42), indicating that the area responsible for hypophagia is in, or near, the hypothalamus (42) and functions independently of pathways that activate the HPA axis. Because injections of either CRF or CRFR antagonist into the third ventricle could potentially act on brain nuclei in the fourth ventricle, we can not exclude the brainstem as a mediator of changes in food intake and body weight of stressed rats. Grill et al demonstrated that areas adjacent the fourth ventricle also are involved in energy balance responses to stress (20, 21). Injections of Ucn in the fourth ventricle inhibited food intake in rats (22), although the degree of inhibition was less than that caused by Ucn infusion into the lateral ventricle. Therefore, in the study of Smagin et al (42), is possible that injection of αhCRF$_{(9-41)}$ in the third ventricle blocked receptors in the fourth ventricle. The objective of this study was to test whether CRFRs located in the brainstem would modify the changes in food intake, body weight, and coticosterone release that are induced by RRS or by a less severe stress, and whether antagonism of CRFRs during restraint would prevent the subsequent hypersensitivity of RRS rats towards mild stress.

METHODS

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.

Exp 1: *Stress responses in rats receiving αhCRF$_{(9-41)}$ infusion into the fourth ventricle*

This experiment was designed to test whether infusions of a non-selective CRFR antagonist into the fourth ventricle would block the body weight loss and inhibition of food intake caused by repeated restraint stress (RRS).

Ninety-five male Sprague-Dawley rats (Harlan Sprague Dawley, Indianopolis, IN) weighing approximately 340g were included in this experiment. Because of the large number of animals in this study, the experiment was done in three cohorts with treatment groups equally divided among cohorts. The rats were placed in hanging wire cages in a room with controlled temperature (22.7°C) and humidity (52%) and a 12:12h light-dark cycle. The rats had free access to water and standard rat chow (Purina rodent chow 5001: Purina Mills, MO) throughout the experiment. All rats were fitted with 26G guide cannulas (Plastics One, Roanoke, VA) aimed at the fourth ventricle. They were anaesthetized with ketamine/xylazine mix (90 mg/kg Ketamine, 10 mg/kg Xylazine) administered i.p. They were positioned in a stereotaxic apparatus, an incision was made in the skin along the midline, and periosteum was scraped from the skull. The coordinates for placing the cannula in relation to the midline at the occipital suture were 2.5 mm anterior, 0 mm lateral, and 5.2 mm ventro-dorsal, according to Paxinos & Watson Brain Atlas (32). Cannulas were fixed to the skull with jewelers screws and dental cement. The rats were injected with analgesic Ketophen (2mg/kg Ketoprofen) immediately after surgery and again the next day. The rats were allowed 1 week for a

complete recovery before the cannula placement was confirmed. The position of the cannula in the fourth ventricle was confirmed by measuring hypoglycemia-induced gluconeogenesis. The 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 *u*g 5-thio-D-glucose (Sigma-Aldrich, St Louis, MO) in 2 *u*l sterile isotonic saline over one minute. Those rats that showed a doubling of blood glucose from the baseline 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.

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: RRS/αhCRF$_{(9\text{-}41),}$ RRS/Saline, Control/αhCRF$_{(9\text{-}41),}$ Control/Saline. On each day of repeated restraint, 10 minutes before the beginning of each restraint, all rats received a 2 *u*l infusion of saline or 5 *u*g (~1.3 nmol) of αhCRF$_{(9\text{-}41)}$ (Bachem Bioscience, King of Prussia, PA) into the fourth ventricle over one minute. Restrained rats (RRS) 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. 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 tailbleeding. Twelve days after the restraint, half of the rats from each group were submitted to a mild stress, whereas the other half served as controls. Mild stress rats (MS) received a 2ml intraperitoneal injection of saline and were placed in new cages in a novel room for 2 hours. Control rats remained in their home cages. Both groups were food and water

deprived during the 2 hours of mild stress. Corticosterone levels were measured at 0, 15, 30, 60, 90, and 120 minutes after the start of MS in blood samples collected by tailbleeding.

Exp 2: *Effects of Mild Stress after αhCRF$_{(9-41)}$ infusion into the fourth ventricle*

This experiment was conducted to evaluate 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 male Sprague-Dawley rats weighing approximately 335g were fitted with fourth ventricle cannula as described for Experiment 1. Seven days after testing cannula placement, the rats were divided into 4 weight matched groups; Control/Saline, Control/αhCRF, MS/Saline, MS/αhCRF. 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. Rats received a 2 *u*l infusion of either αhCRF$_{(9-41)}$ (~1.3 nmol) or saline in the fourth ventricle 10 minutes before the beginning of MS.

Exp 3: *Astressin infusions into the fourth ventricle in rats exposed to RRS*

The results from Experiment 1, suggested that αhCRF$_{(9-41)}$ was acting as a partial agonist. For that reason, we repeated the experiment using a different non-selective CRFR antagonist, astressin.

Thirty-six male Sprague-Dawley rats weighing approximately 385 g were used. Experimental design was similar to Experiment 1, except that astressin (AST ~3 nmol; American Peptides Company, Sunnyvale, CA) was used as the CRFR antagonist. The number of animals is different from Experiment 1 because we did not have control animals during MS; instead, all rats were submitted to the stress. Corticosterone levels were measured at 0, 30, 60, and 120 minutes during MS twelve days after the end of RRS.

Exp 4: *The effects of Mild Stress after astressin infusion into the fourth ventricle.*

In Experiment 2, αhCRF$_{(9\text{-}41)}$ appeared to produce a partial agonist effect, exaggerating the response to MS. Therefore, the study was repeated using a different non-specific CRFR antagonist, astressin.

Thirty-four male Sprague-Dawley rats weighing approximately 359 g were used. Experimental design was similar to Experiment 2, except that astressin (AST ~3 nmol; American Peptides Company, Sunnyvale, CA) was used and food intake was measured at 2, 4, 6, 12, 24, and 48 hours after MS. Body weight was also measured before and 24, and 48 hours after MS.

Exp 5: *The effects of CRFR2 antagonism on the response to Mild Stress*

In Experiment 4, astressin infusion into the fourth ventricle blocked the response to MS. Because CRFR2 may mediate stress effects on food intake, Experiment 5 was conducted using a CRFR2 selective antagonist, Antisauvagine-30.

Thirty-three male Sprague-Dawley rats weighing approximately 404 g were used in this experiment. Experimental design was the same as Experiment 4, except that antisauvagine-30 (ASV30 ~3 nmol; Phoenix Pharmaceuticals, Belmont, CA) was used to selectively inhibit CRFR2 (7).

Statistics:

Animals tested in RRS experiments had their food intake and body weight analyzed by ANOVA repeated measures (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 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 different at $p < 0.05$.

RESULTS

Experiment 1 (RRS/αhCRF$_{(9-41)}$)

All RRS rats lost weight on the days of restraint (Fig 1A: stress: $p< 0.0001$, time: $p< .0001$, stress X time: $p< 0.03$, and stress X treatment: $p< 0.03$). Controls were not significantly different from each other, but both were different from RRS rats. RRS/αhCRF$_{(9-41)}$ rats lost significantly more weight than RRS/Saline rats ($p< 0.001$). In addition, all rats ate less on the days of stress, with intake returning to baseline levels after the end of stress (Fig 1B: stress: $p< 0.001$, time $p< 0.0001$, and stress X treatment $p< 0.001$). Cumulative food intake showed that RRS/αhCRF$_{(9-41)}$ rats ate less than any other group during the 3 days of restraint (Fig 1B: stress: $p< 0.0001$, stress X treatment: $p< 0.02$). Corticosterone levels were measured by tailbleeding at 0 and 60 minutes on the second day of RRS. Both RRS had significantly high levels of corticosterone at 60 minutes when compared to control groups but no treatment effect was noted (Fig 2A: stress: $p< 0.0001$). Rats were then divided into MS or Control groups twelve days after RRS. There was no significant effect of either αhCRF$_{(9-41)}$ or of RRS on food intake or body weight measured 24 hours after MS twelve days after the end of RRS (data not shown). All rats were tailbled at 0, 15, 30, 60, 90, and 120 minutes during MS. MS caused a significant increase in corticosterone levels 15 minutes after the start of stress (Fig 3A: MS: $p< 0.0001$) for groups exposed to MS. Previous exposure to RRS did not produce any significant exaggeration of responses to MS. A significant effect was also observed at 30 minutes during MS (Fig 3A: MS: $p< 0.002$), in which the Restraint/Saline/Control rats had significantly lower corticosterone levels

compared with Control/Saline/MS ($p< 0.02$), Restraint/$\alpha hCRF_{(9-41)}$/MS ($p< 0.02$), and Restraint/Saline/MS rats ($p< 0.01$). When grouping rats infused with either saline or $\alpha hCRF_{(9-41)}$ together, a significant effect was observed in rats submitted to MS at 15 (Fig 3B: MS: $p< 0.001$) and at 30 minutes (Fig 3B: MS: $p< 0.003$).

Experiment 2 (MS/$\alpha hCRF_{(9-41)}$)

Neither MS nor $\alpha hCRF_{(9-41)}$ had any effect on the amount of weight gained by rats during the 24 hours after MS in Experiment 2 (Fig 4A: NS). Interval food intake at 0-2h was decreased in MS/$\alpha hCRF_{(9-41)}$ rats when compared to MS/Saline rats on post hoc comparisons (Fig 5A: $p< 0.04$). No significant overall effect was noted between groups at 4h, but post hoc comparisons showed a significant difference between MS/$\alpha hCRF_{(9-41)}$ and Control/$\alpha hCRF_{(9-41)}$ rats (Fig 5A: $p< 0.03$). A significant effect on food intake was also noted at 6h (Fig 5A: treatment: $p< 0.04$), but no significant effect was observed during post hoc comparisons. Also, cumulative food intake of MS/$\alpha hCRF_{(9-41)}$ was significantly lower than for Control/$\alpha hCRF_{(9-41)}$ rats, and all other groups at 6h (Fig 6A: $p< 0.03$) during post hoc comparisons. There was no difference in food intakes of the groups 6-24h after MS (Fig 5B: NS).

Experiment 3 (RRS/AST)

Restrained rats lost weight during the stress period (Fig 7A: stress: $p< 0.0002$, time: $p< 0.0001$), and regained weight after the restraint at the same rate as the control rats; however, RRS rats never returned to weight of the control rats, maintaining a significantly reduced body weight to the end of the experiment. Astressin 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, and the effect was greater for both restraint groups compared with controls, but there was no difference between RRS/Saline and RRS/AST rats (Fig 7B: time: $p< 0.0001$, stress X

time: $p < 0.003$). The rats then were submitted to MS twelve days after RRS, and food intake and body weight were decreased in all animals with no significant differences between the groups 24 hours after MS (data not shown). There was no effect of AST on corticosterone concentrations of MS or of Control rats; therefore these two treatment groups were combined. Rats previously exposed to RRS showed higher levels of corticosterone 60 minutes after the start of MS when compared with animals that had not been previously restrained on post hoc comparisons(Fig 8A: stress: $p < 0.02$).

Experiment 4 (MS/AST)

All Control and MS rats lost weight 24h and 48h after MS. The loss of weight was significantly greater in MS/Saline rats than in any other group. MS caused significant weight loss in rats infused with saline at both 24 (Fig 4B: treatment: $p < 0.01$, stress X treatment: $p < 0.01$) and 48 hours after stress (Fig 4C: treatment: $p < .04$, stress X treatment: $p < 0.01$). Astressin also blocked a stress-induced inhibition of food intake at 2-4 hours (Fig 5C: stress X treatment: $p < 0.01$), and 48 h (Fig 5E: stress X treatment: $p < 0.03$) after MS. Overall, AST significantly increased food intake between 12 and 24 hours after MS (Fig 5D: treatment: $p < 0.02$) but, posthoc analysis found no differences between specific groups. Astressin increased cumulative food intake of both control and MS rats compared with their saline infused counter-parts at 12h (Fig 6B: stress X treatment: $p < 0.02$) and 48h (Fig 6B: stress X treatment: $p < 0.03$) after MS. Cumulative food intake was also significantly different 24 hours after MS (Fig 6B: stress X treatment: $p < 0.03$), however post hoc analysis showed no difference between specific groups.

Experiment 5 (MS/ ASV30)

There was no effect of MS or ASV30 on body weight change of rats during the 24 hours after MS in Experiment 5 (Fig 4D: NS). ASV30 did prevent a MS-induced weight loss measured 48 hours after stress (Fig 4E: stress X treatment: $p< 0.04$). MS/Saline rats ate significantly less than MS/ASV30 rats 12 hours after MS (Fig 5G: stress X treatment: $p< 0.04$), but there were no significant differences in food intake among groups at 2h, 4h, 6h, 24h, or 48h. Mild stress significantly inhibited cumulative food intake of saline-infused rats at 12h (Fig 6C: stress X treatment: $p< 0.01$), and 24 h after stress (Fig 6C: stress X treatment: $p< 0.001$). Control/AST rats ate more than Control/Saline rats 24h after stress (Fig 6C: $p< 0.009$). There was no effect of MS on cumulative food intake of ASV infused rats.

DISCUSSION

We have previously demonstrated that repeated restraint stress can induce a decrease in body weight and food intake during the stress period (25). Moreover, after the end of stress, rats maintain a sustained decrease in body weight, and they do not compensate for their body weight loss by overeating once stress has ended (24). These results suggest that the stressed rats adjust the level at which they regulate body weight. Other investigators have demonstrated a decrease in body weight of rats exposed to acute stress (12, 39, 42) or in response to activation of CRF receptors in the brain (28), whereas chronic stress has been reported to cause weight gain in rats (12). In contrast, Wardle et al (49) showed that humans can either decrease or increase their food intake according to the type of stress. Stress can be either acute or chronic; acute traumatic events cause weight loss whereas chronic stress tends to cause weight gain (12). It is critical that we understand the differences between acute and chronic stress, since they can initiate different biological responses. Acute stress is a short period of exposure to a stressor while chronic stress

is a continuous exposure to a stressor. Glucocorticoids (GCs) are hormones released from the cortex of the adrenals, and can play an important role in homeostasis and stress. In humans, the main GC released is cortisol whereas in rodents it is corticosterone. Their secretion is stimulated by ACTH released from the anterior pituitary, which is stimulated by the CRF released from the hypothalamus. Under physiologic conditions, the hypothalamus-pituitary-adrenals axis (HPA) acts in a circadian rhythm, whereas this pathway can be deregulated during stress. CRFR mRNA expression in the hypothalamus and ACTH release from the pituitary are inhibited during acute stress by glucocorticoids (11), whereas during chronic stress, CRFR mRNA expression in the brain and the HPA axis are not down regulated (11). Recently, Dallman et al (12) have hypothesized that hormones such as insulin may be responsible for the weight gain during chronic stress, whereas corticosterone might play a minor role on this mechanism.

Previously, we have reported that injection of αhCRF$_{(9\text{-}41),}$ a non selective antagonist for the CRFRs, in the third ventricle blocked weight loss in rats exposed to RRS (42). The Experiments described here were conducted to test whether the inhibition of changes in body weight and food intake were due to antagonism of CRFRs around the fourth ventricle because it is possible that αhCRF$_{(9\text{-}41)}$ diffused into the fourth ventricle following injection. Others have suggested that the fourth ventricle may play a role in the behavioral response to stress because infusion of Ucn in this ventricle led to weight loss and a decrease in food intake of rats (22), similar to that produced by lateral ventricle infusions. These results could imply that brain areas located in areas near the third and/or the fourth ventricle are involved in the mediation of stress-induced body weight loss.

The primary objective of the studies described here was to test whether infusions of a CRFR antagonist into the fourth ventricle would block the weight loss and the decrease in food

intake of rats exposed to stress. Unexpectedly, in Experiment 1, RRS/αhCRF$_{(9-41)}$ rats showed an exaggeration in response to stress when compared to RRS/Saline rats. These results are surprising, since αhCRF$_{(9-41)}$ is a CRF receptor antagonist, and would be expected to inhibit behavioral and endocrine changes that are induced by stress. We conducted Experiment 1 in three equally divided cohorts and found the same outcome each time.

There were two possible explanations for the results of Experiment 1. The first is that antagonism of CRFR in the brainstem could lead to an exaggerated negative feedback in some areas of the midbrain, such as the hypothalamus. The CRF connections between the hypothalamus and the brainstem have already been described (5, 9), and this connection may play a role in suppressing food intake and causing weight loss in stressed animals. Therefore, these CRF containing neurons could have been responsible for the exaggeration in decreasing food intake and body weight during stress (9).

The second explanation is that αhCRF$_{(9-41)}$ has a partial agonist effect (44). If this was the case, we would also expect the Control/αhCRF$_{(9-41)}$ rats to show decrease in body weight and food intake during the period of the infusions. However, Control/αhCRF$_{(9-41)}$ rats 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 (17, 19), handling the animals can be a mild form of stress, and it is possible that αhCRF$_{(9-41)}$ infusions in control rats might have blocked this mild form of stress. In contrast, restrained animals have an exaggerated response to stress when receiving αhCRF$_{(9-41)}$ infusions into the fourth ventricle. This effect could be due to the partial agonist effect of αhCRF$_{(9-41)}$, which could have exacerbated the response to restraint. Because Control/αhCRF$_{(9-41)}$ did not lose weight but αhCRF$_{(9-41)}$ exaggerated the stress responses of restrained rats, this relationship is not as simple as αhCRF$_{(9-}$

$_{41)}$ being a partial agonist. We suggest then, that the partial agonist effect might be specific and may represent an interaction with other stress related systems. For example, others have reported that an acute severe stress can down-regulate serotonin activation, whereas a mild form of stress can up-regulate serotonin release (47). Since the infusions were into the fourth ventricle, it is possible that the partial agonist effect of αhCRF$_{(9\text{-}41),}$ could have represented an increased down-regulation of the serotonergic neurons that colocalize CRFRs, leading to an exaggerated response.

Although the measurements made in this study did not identify the mechanisms responsible for the exaggerated response in αhCRF$_{(9\text{-}41)}$–treated rats, the results from Experiment 3, in which we used astressin clearly showed that the effect was a specific effect of the antagonist rather than interference with feedback regulation. We then conducted Experiment 3, in which we tested the effects of astressin during RRS. Astressin is also a non-selective CRFR antagonist (7), but it does not have a partial agonist effect like αhCRF$_{(9\text{-}41)}$ (44). Once again, we were able to show that restraint stress caused a sustained decrease in body weight and food intake during stress. The body weight and food intake of RRS/AST rats, however, were not significantly different from those of RRS/Saline rats, suggesting that CRFR in the brainstem were unlikely to be responsible for inducing weight loss and hypophagia in restrained rats.

Because the HPA axis is regulated via the hypothalamus, we would not expect fourth ventricle antagonists to inhibit the endocrine response in stress. This was confirmed by the significant rise in corticosterone concentrations in the stress groups when compared to control groups and there was no significant treatment effect. Therefore, we conclude that the brainstem does not play a role in exaggerating the HPA axis by a possible feedback connection to the hypothalamus in RRS animals (9). Previous studies demonstrated that exposure to repeated

restraint can induce an exaggeration of HPA axis activation in rats submitted to MS (23). In both Experiments 1 and 3, we tested the long-term effects of RRS in rats exposed to MS twelve days after restraint stress. The rats did not receive a second infusion of CRFR antagonist during the second novel stress, as our objective was to observe long-term effects in body weight, food intake, and endocrine changes in rats that received infusion of a CRFR antagonist at the time of restraint. We did not see any significant effect of receptor antagonists although rats that had previously been exposed to RRS had higher concentrations than non-restrained rats when they were exposed to MS. Interestingly, αhCRF$_{(9-41)}$ did not act as a stressor during RRS and thus, no partial agonist effect was noted. Experiment 1 showed a MS effect whereas Experiment 3 showed a hyperactivity of the HPA axis in previously restrained rats. Therefore, result from Experiment 3 supports the concept that previously restrained rats are sensitive to a second novel stress.

The data from Experiments 1 and 3 indicated that CRFR in the brainstem were not involved in the initiation of energetic responses to repeated restraint stress. Those results were different from expected, since others have shown that injections of Ucn into the fourth ventricle inhibited food intake and body weight of rats (22, 45). It is possible that injections of Ucn into the fourth ventricle worked as mild stress, therefore, we tested whether even though the brainstem appears to not regulate the body weight and food intake in response to severe stress, it might mediate some aspects of the mild stress response.

In Experiment 2, we found that αhCRF$_{(9-41)}$ also had an agonist effect in rats exposed to MS and so we tested whether astressin would possibly block the responses to MS. Astressin blocked the effects of MS on food intake and body weight, supporting our previous hypothesis of a possible regulation of energy balance through the brainstem. Others have also shown that the

brainstem plays an important role in this energy balance regulation by its connection with the forebrain (21). In addition, the effects of MS on body weight and food intake are similar to Grill's findings, where Ucn injections into the fourth ventricle decreased food intake and body weight, with the biggest effect at 24 hours (22). Results from Experiment 4 described here confirm that CRFRs mediate this response because antagonism of CRFRs adjacent to the fourth ventricle prevented both weight loss and inhibition of food intake in rats exposed to MS.

It is possible that the brainstem might integrate a feedback to other brain nuclei according to the level of stress, as others have shown that its connection to other areas of the hypothalamus, may play a role in suppressing food intake and causing weight loss in stressed rats (4). The results from Experiment 5 indicate that CRFR2 are critical to the MS response, consistent with reports that CRFR2 play an important role in energy balance and body weight response to stress (2, 3). Others have shown that CRFR2 regulates the long-term effect on food intake whereas CRFR1 appears to regulate early food intake responses (6, 10). In Experiment 2, we observed an early response in food intake in MS/αhCRF$_{(9\text{-}41)}$–infused animals. Therefore, it is possible that αhCRF$_{(9\text{-}41)}$ acted as an agonist in CRFR1 during stress, and had either an agonist or no effect on CRFR2 in response to MS.

In Experiment 5, we found that antagonism of the CRFR2 prevented the stress-induced inhibition of food intake in rats exposed to MS. MS/ASV30–infused animals did not show a late response in food intake at 12h and 24h. Additionally, MS rats infused with ASV30 did not decrease body weight at 24 and 48 hours after stress, supporting the idea that the brainstem may regulate body weight in response to stress through CRFR2. However, we cannot conclude from these results that CRFR2 is an important regulator of mild stress in the brainstem alone. Since the infusions were into the fourth ventricle, it is possible that the CRFR antagonists acted in

some brain areas, such as the raphe nuclei. The raphe nucleus appears to colocalize CRFR2 and serotonin neurons, and studies have shown that stress might activate serotonergic neurons, according to the type of stress applied (47) and thus might decrease or increase the responses to stress. CRFR containing neurons from the brainstem connect to the hypothalamus (9), and depending on the type of stress, CRFR2 can down-regulate neuronal activation from the raphe nuclei to the midbrain and subsequent release of serotonin (27, 36, 47). Therefore, it is possible that an attenuation of responses to mild stress by antagonism of CRFR neurons occurred through a pathway that linked the brainstem to the hypothalamus. It is also possible that inhibition of CRFR2 in the fourth ventricle with ASV30 prevented stimulation of serotonergic neurons in the raphe nuclei, preventing release of serotonin in the hypothalamus. However, we did not measure or analyze serotonin in response to MS, but futures studies should look at this possible association.

In conclusion, results from the studies described here suggest that changes in food intake and body weight induced by RRS and by MS are mediated by different pathways. It appears that the brainstem may play an important role in regulating responses to mild stress but not to restraint stress. It is possible that CRFR2 in the brainstem regulates mild stress responses through feedback to other areas in the brain, such as the hypothalamus. Future studies should investigate areas in the brainstem that could be responsible for responses to stress. In Experiments 1 and 2 we found that αhCRF$_{(9\text{-}41)}$ acted as a partial agonist in stressed but not control rats when infused into the fourth ventricle. This implies that αhCRF$_{(9\text{-}41)}$ required a stress induction for the agonist effects.

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LEGENDS

Figure 4.1. Daily body weight of rats in Experiment 1 from 7 days before RRS to day 7 of the experiment period (A) and cumulative food intake for 3 consecutive days before, during and after RRS (B). Values are means ± SEM for 15-16 rats. Asterisks indicate a significant difference in body weight of RRS/αhCRF$_{(9-41)}$ and RRS/Saline rats ($p < 0.0001$), and symbols indicate a significant effect in stress groups compared to control at $p < 0.05$. Superscripts indicate significant difference in cumulative food intake of treatment groups ($p < 0.002$).

Figure 4.2. Serum corticosterone concentration measured before and 60 minutes after the start of RRS. Data are means ± SEM for 13-15 rats. Symbol indicates significant difference in rats exposed to stress at $p < 0.05$. No treatment effect was observed between groups.

Figure 4.3. Serum corticosterone concentration measured before, and during MS in animals previously exposed to RRS. Symbols indicate a significant MS effect at $p < 0.05$. Data are means ± SEM for 4-6 rats. Figure A represents all eight groups of animals, and Figure B represents animals divided by previous exposure to RRS and a second novel MS.

Figure 4.4. Body weight change 24 and 48 hours after MS in animals infused with different CRFR antagonists into the fourth ventricle. Panel A is body weight change for rats infused with αhCRF$_{(9-41)}$ in Experiment 2. Panel B and C are body weight changes in rats infused with AST in Experiment 4. Panel D and E are body weight changes in rats infused with ASV30 in Experiment 5. Data are means ± SEM for 8-9 rats. Superscripts indicate significant differences between treatment groups for a specific time interval.

Figure 4.5. Intervaled food intake after MS of rats infused with different CRF receptors antagonists into the fourth ventricle. Panels A and B are intakes of rats infused with $\alpha hCRF_{(9-41)}$ in Experiment 2. Panels C to E are intakes of rats infused with AST in Experiment 4. Panels F to H are intakes of rats infused with ASV30 in Experiment 5. Data are means $\pm$ SEM for 8-9 rats. Superscripts indicate significant differences between treatment groups for a specific time interval.

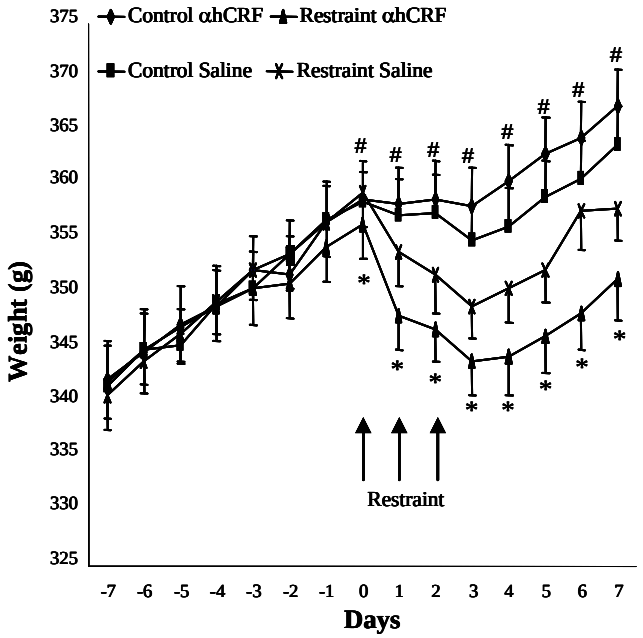
Figure 4.6. Cumulative food intake of rats after exposure to MS with different CRF receptors antagonists infused into the fourth ventricle. In Experiment 2, asterisk and symbol show significant difference in food intake between MS and Control $\alpha hCRF_{(9-41)}$ infused rats at 4h ($p< 0.03$), and at 6h ($p< 0.02$) between MS/$\alpha hCRF_{(9-41)}$ and all groups (A). In Experiment 4, symbols show a stress effect in food intake at 12h ($p< 0.02$), and asterisks indicate treatment effect between controls at 12h ($p< 0.02$), and treatment effect between MS rats at 48h ($p< 0.03$) (B). In Experiment 5, symbol and asterisk indicate stress and treatment effect in MS/Saline rats compared to Control/ASV30 rats at 12h ($p< 0.01$), and asterisks indicate treatment effect between controls at 24 h ($p<0.009$), symbol indicates stress effect at 24h ($p< 0.009$). Values are means $\pm$ SEM for 4 groups of 8-9 rats.

Figure 4.7. Daily body weight of rats in Experiment 3 from 3 days before RRS to day 6 of experiment period (A) and cumulative food intake for 3 consecutive days before, during, and after RRS (B). Values are means $\pm$ SEM for 7-9 rats. Symbols indicate significant difference between RRS and Control groups ($p< 0.002$). Superscripts indicate significant difference in cumulative food intakes of the treatments groups ($p< 0.006$).

Figure 4.8. Serum corticosterone concentration measured before, and during MS in animals previously exposed to RRS. No significant effect of AST was found. Groups of saline and AST-infused rats were then analyzed together according to previous exposure to RRS and the second novel MS. Symbol indicates stress effect at $p < 0.05$. Data are means $\pm$ SEM for 5–9 rats.

Figure 4.1

***A:** Daily body weights*



***B:** Cumulative food intake*

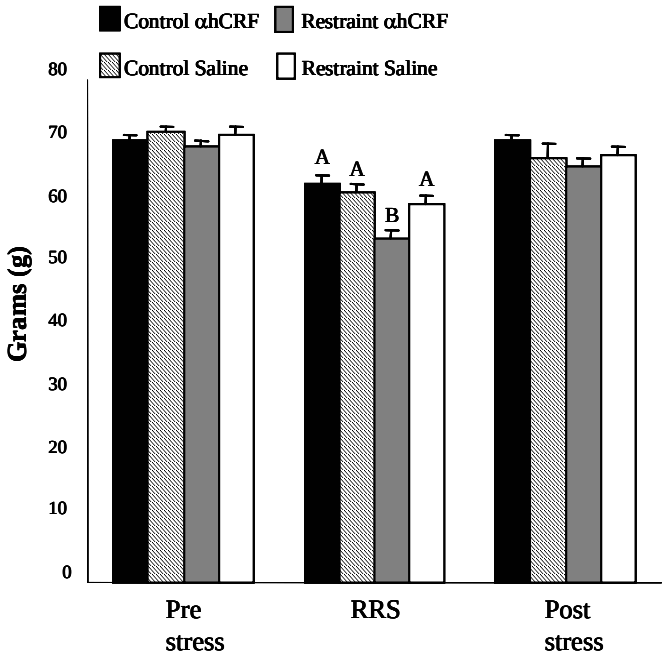


Figure 4.2

Corticosterone levels

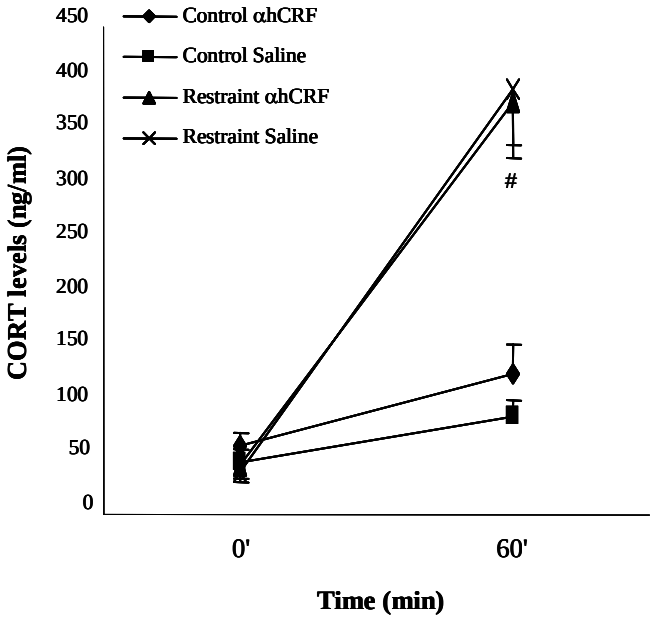
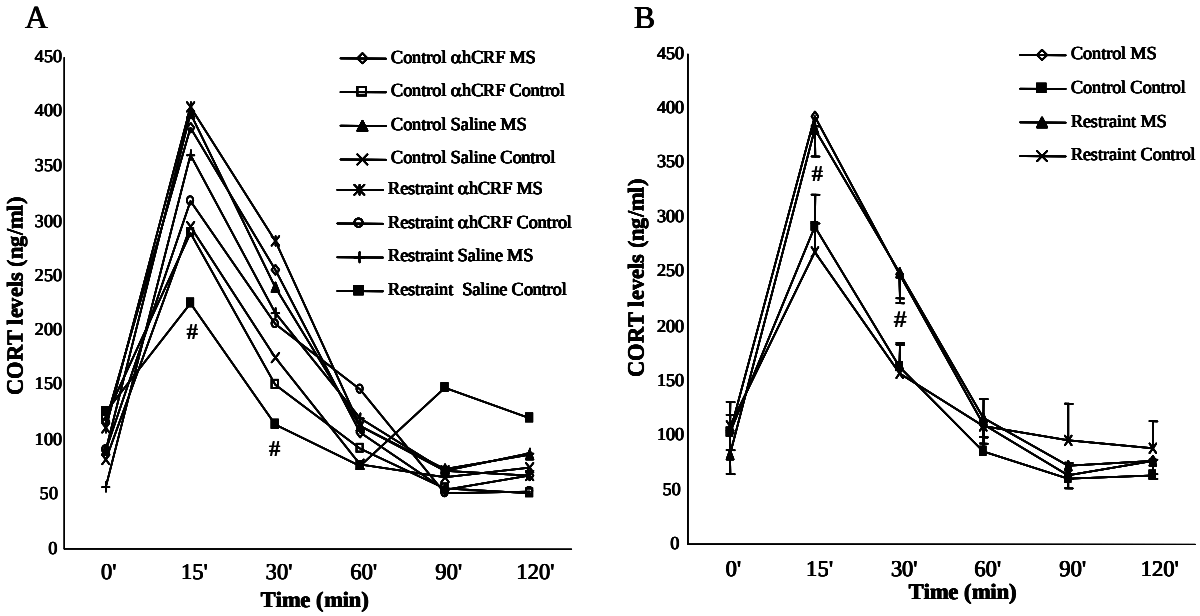
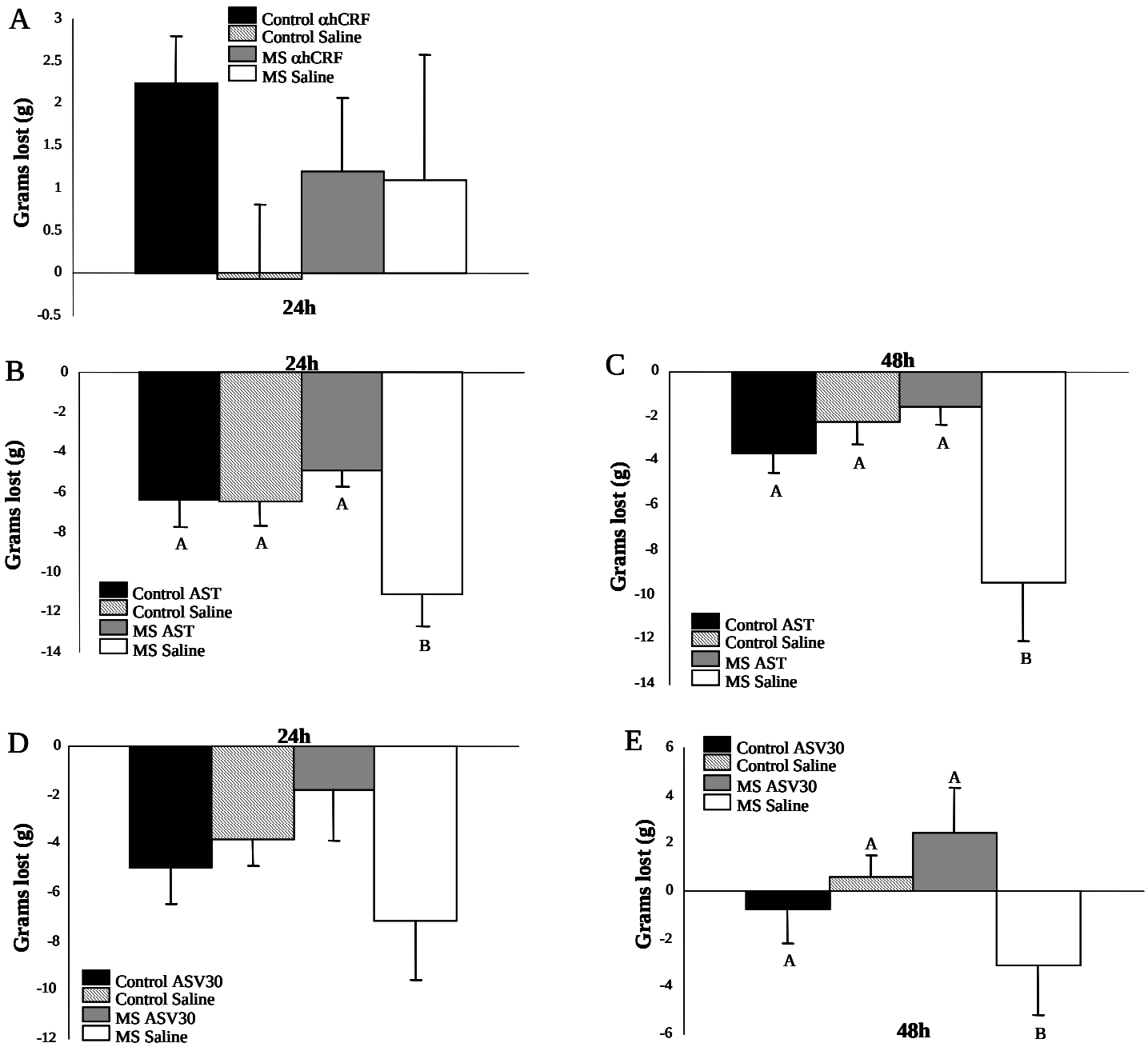


Figure 4.3

Corticosterone levels during MS



Body weight changes



Intervaled food intake

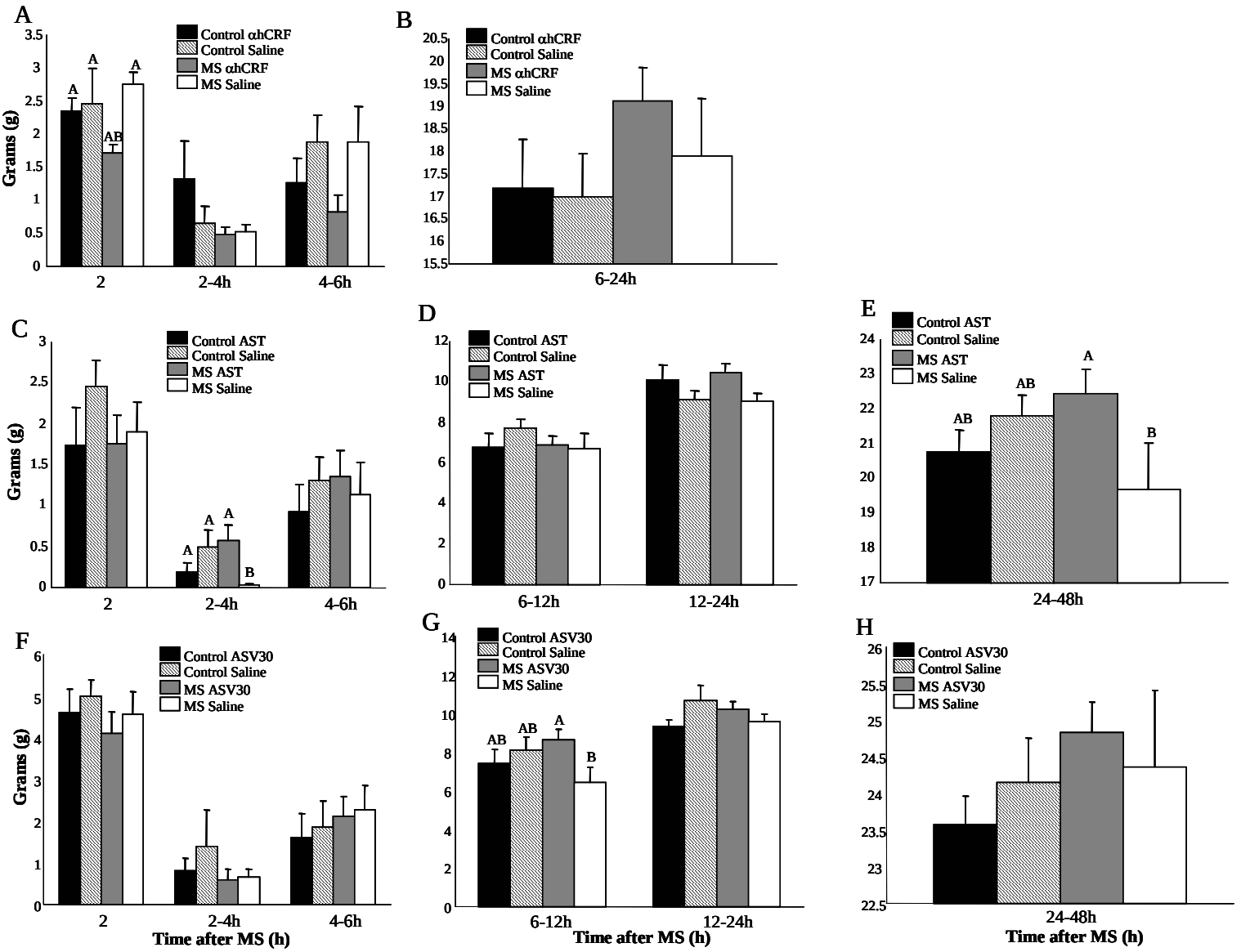


Figure 4.6

Cumulative food intake

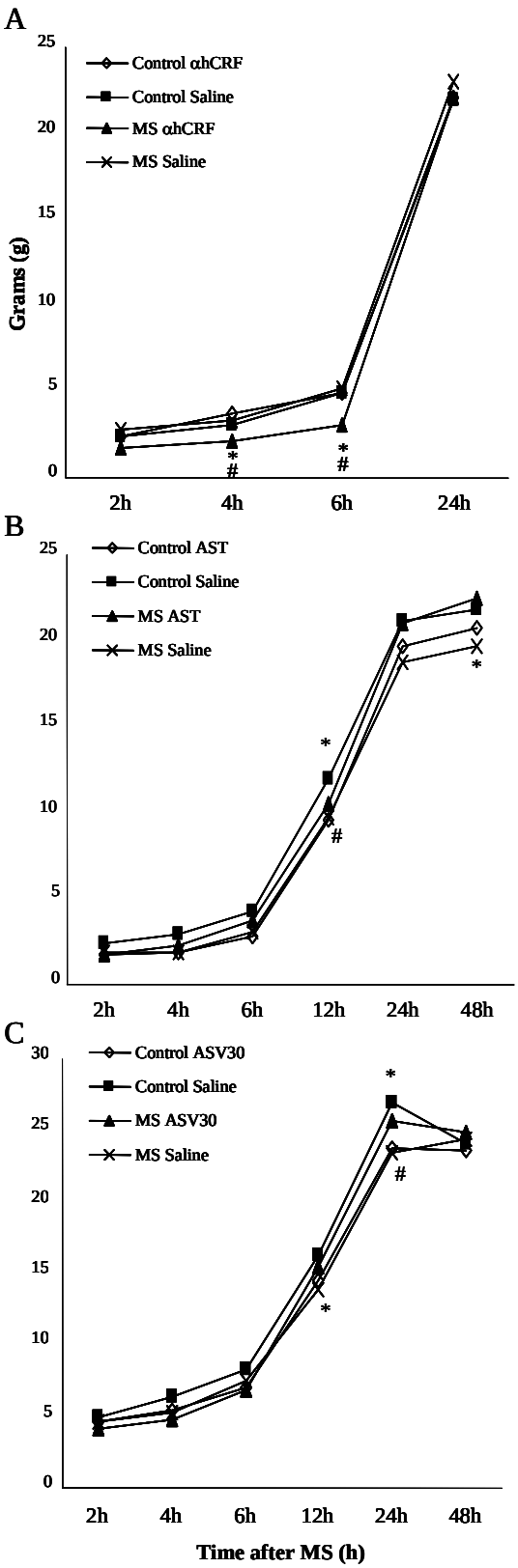
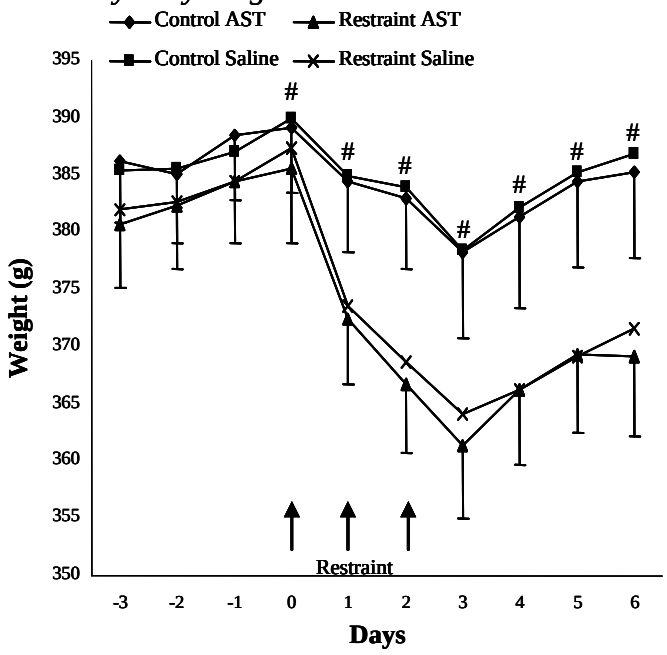


Figure 4.7

***A:** Daily body weight*



***B:** Cumulative food intake*

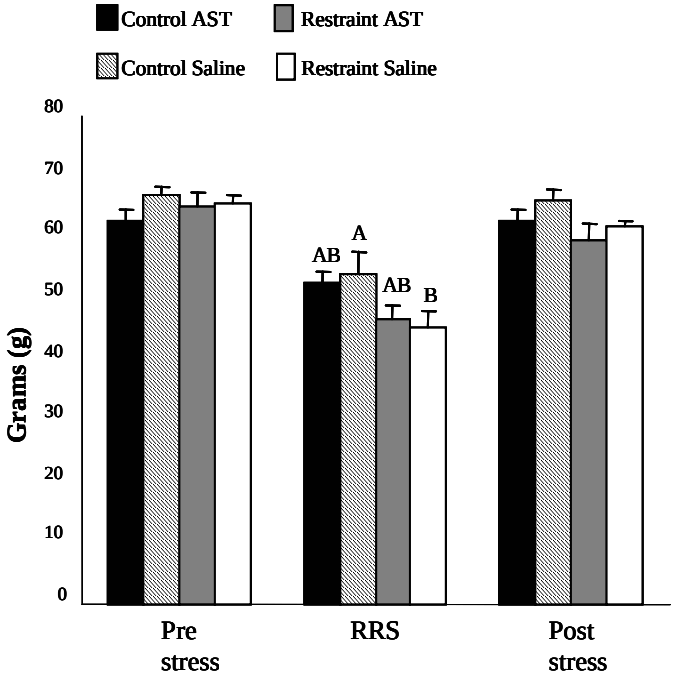
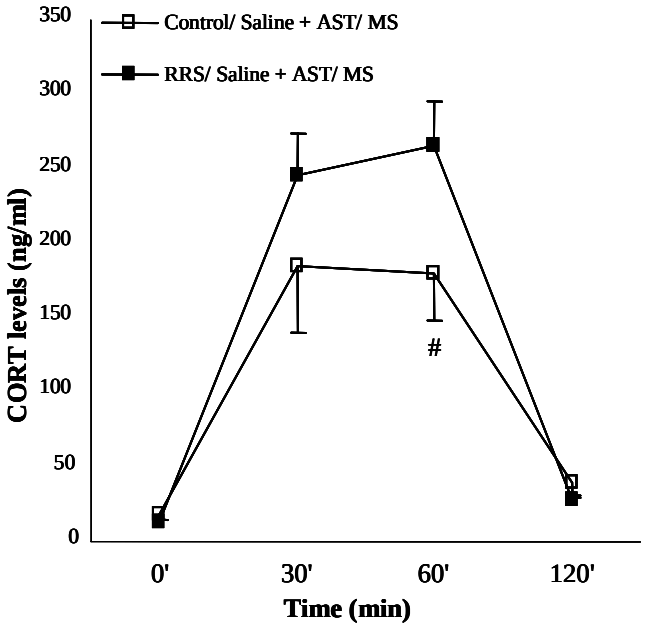


Figure 4.8

Corticosterone levels



CHAPTER 5

CHANGES IN FOS LIKE IMMUNORREACTIVITY IN BRAINSTEM NUCLEI AFTER FOURTH VENTRICLE INFUSION OF A CRF RECEPTOR ANTAGONIST DURING STRESS

Miragaya, J., Harris, R.B. *To be submitted to the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.*

ABSTRACT

Stress is characterized by various endocrine, metabolic, and behavioral responses, and the neuropeptide corticotrophin-releasing factor (CRF) appears to be a primary initiator of these response. Two CRF Receptor subtypes, CRFR1 and CRFR2, have been identified and they appear to mediate different types of stress responses. It is known that stress, CRF, and CRF Receptor agonists can increase the expression of the immediate early gene, Fos, in different areas in the brain, including the brainstem. Several brain nuclei in the brainstem area have been implicated in regulating feeding responses by either efferent input connections from the periphery or output to the forebrain. These nuclei include the nucleus of solitarius tract (NTS) and the raphe nuclei (RN). We have previously noted that antagonism of CRFRs in the fourth ventricle prevents the decrease of food intake that is usually seen during stress. The objective of this study was to identify the anatomical location of CRFR that are inhibited by fourth ventricle CRFR antagonists as an indication of which nuclei may mediate a stress-induced inhibition of food intake. We infused a non-selective CRFR antagonist, astressin, in the fourth ventricle immediately before rats were exposed to mild stress. Astressin partially inhibited Fos like immunoreactivity in the NTS and totally inhibited Fos expression in the RN of stressed rats. Based on these data, we conclude that the brainstem might play an important role in regulating neuronal activation during stress.

Key words: brainstem, astressin, mild stress, rats, c-Fos.

INTRODUCTION

The neuropeptide corticotrophin-releasing factor (CRF), a 41-amino acid peptide, discovered in 1981 (57), is released in response to exposures to stressors, such as pain, fear, anxiety or emotion (50), and it has been identified as the initiator of behavioral and physiological responses to stress. Its two receptors are CRFR1 and CRFR2 (8, 41, 49), which can exert different functions during stress according to their location in the brain. CRFR1 and the variant CRFR2α prevail in neural tissue (31), whereas CRFR2β are expressed in areas such as heart, gastrointestinal tract, arterioles and muscles (24, 31).

Rodents also present the CRFR agonists (41): urocortin (Ucn) (62), Ucn II (44) and Ucn III (63), which have similar function as CRF. The non-selective CRFRs antagonist astressin (AST) (22) has been widely used and has advantages compared to another non-selective CRFRs antagonist, $\alpha hCRF_{(9-41)}$ (45). $\alpha hCRF_{(9-41)}$, has been shown to have a partial agonist effect both in vitro (55) and in vivo (35), whereas AST shows a very weak partial agonist effect (36), and has a higher affinity for CRFRs than $\alpha hCRF_{(9-41)}$ (22).

Activation of either CRFR1 or CRFR2 leads to different responses, as they seem to play different roles during stress according to their localization in the brain [For Review: (3, 14)]. CRFR1 has been implicated in mediating anxiogenic-like (27), and endocrine responses (61). CRFR1 is mainly localized in brain areas responsible for emotions and vigilance, such as the neocortex, the hippocampus, and the limbic system (8); and areas responsible in regulating hormonal release in stress such as the hypothalamus and pituitary (61). CRFR2, are predominant in brain areas such as the lateral septal nucleus, the ventromedial hypothalamic nucleus (VMH), the paraventricular nucleus of hypothalamus (PVN), the choroid plexus, the amygdala, the raphe nuclei (RN), the nucleus of tract solitarius (NTS), and the area postrema (AP) (8). In addition,

some studies show that both receptors might play a role in the regulation of food intake during stress (51), as CRFR1 appears to inhibit early feeding responses (5) whereas CRFR2 appears to regulate the late phase of food intake in stressed rats (11).

Brown et al (6) first suggested an integration between the brainstem and forebrain in energy balance through CRFRs. The brainstem has been shown to connect and mediate peripheral signals to the forebrain; by several different receptors and peptides involved in the appetite regulation [For Review: (17, 18)]. Therefore, the stress-induced responses in energy balance and behavior appears to be regulated by a complex pathway, involving visceral afferents, and several different brain nuclei in the brainstem and the forebrain. The AP and NTS receive multiple inputs from the periphery and connect to areas, such as the PVN and the arcuate nuclei (9); therefore, regulating appetite through different peptides, including CRF [For Review: (18, 65)].

Similar to humans, rats show a decrease in body weight during acute stress (12, 47, 54). In a previous study, we found that antagonism of CRFR2 in the fourth ventricle prevented the inhibition of food intake produced by mild stress. However, it is not yet clear which brainstem nuclei is responsible for the primary response that lead to stress-induced inhibition of food intake in rats exposed to mild stress. Therefore, our objective was to identify which nuclei in the brainstem of rats were activated by exposure to mild stress and to determine whether the activation was inhibited by fourth ventricle infusion of the non-selective antagonism of CRFR.

METHODS

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. This experiment was designed to test whether infusions of the non-selective CRFR antagonist, astressin (AST), into the fourth ventricle would block activation of Fos immunoreactivity of brainstem nuclei that express CRFRs.

Twenty-four male Sprague-Dawley rats (Harlan Sprague Dawley, Indianopolis, IN) weighing approximately 360g were placed in hanging wire cages in a room with controlled temperature (22.7°C) and humidity (52%) and a 12:12h light-dark cycle. The rats had free access to water and standard rat chow (Purina rodent chow 5001: Purina Mills, MO) throughout the experiment. All rats were fitted with 26G guide cannulas (Plastics One, Roanoke, VA) aimed at the fourth ventricle. They were anaesthetized with ketamine/xylazine mix (90 mg/kg Ketamine, 10 mg/kg Xylazine) administered i.p. They were positioned in a stereotaxic apparatus, an incision was made in the skin along the midline, and periosteum was scraped from the skull. The coordinates for placing the cannula in relation to the midline at the occipital suture were 2.5 mm anterior, 0 mm lateral, and 5.2 mm ventro-dorsal, according to Paxinos & Watson Brain Atlas (39). Cannulae were fixed to the skull with jewelers screws and dental cement. The rats were injected subcutaneously with analgesic Ketophen (2mg/kg Ketoprofen) immediately after surgery and again the next day. The rats were allowed 1 week for a complete recovery before the cannula placement was confirmed. The position of the cannula in the fourth ventricle was confirmed by measuring hypoglycemia-induced gluconeogenesis. 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 *u*g 5-thio-D-glucose (Sigma-Aldrich, St Louis, MO) in 2 *u*l sterile isotonic saline over one minute. Those rats that showed a doubling of blood glucose 60 minutes after the infusion were included in the experiment. Rats were allowed one week of recovery before the beginning of the experiment.

Body weight was recorded on the day of perfusion. The rats were divided into 4 weight-matched groups: MS/AST, MS/Saline, Control/AST, and Control/Saline. On the day of experiment, 10 minutes before the beginning of stress, all rats received a 2 *u*l infusion of saline or 10*u*g (AST ~3 nmol; American Peptides Company, Sunnyvale, CA) into the fourth ventricle over one minute. Mild stress rats (MS) received a 2ml intraperitoneal injection of saline and were placed in new cages in a novel room for 1 hour. Control rats remained in their home cages. Exactly sixty minutes after the beginning of MS, the animals were anaesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine and then perfused intracardially with 75 ml ice-cold saline followed by 200ml 4% paraformaldehyde solution. The brains were collected and postfixed in 4% paraformaldehyde solution for 24 hours at 4°C, and were then placed in sucrose azide for 36 hours (or until brains had sunk) at 4°C. The brains were stored at - 80°C until the day of slicing. Brain slices were made at 25 *u*m using a cryostat, and sliced in this order: one slice for slide for anatomy purposes, one slice for immunohistochemistry, and eight slices were stored at -20°C in cold sterile cryoprotectant solution (50 mM phosphate buffer, 30% ethylene glycol, 20% glycerol).

Immunohistochemistry was performed for Fos-like immunoreactivity (FLI) on free-floating rat brain slices. The brain slices were then rinsed with 0.3% H_2O_2 in 0.2M PBS for 60 minutes, then placed in 4% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) for 2 hours. The slices were then incubated with a rabbit anti-c-Fos polyclonal antibody (1:10,000;

PC38, Ab-5, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.3% Triton X-100 in a cold room for 48-72 hours. To confirm specificity of the binding, some slides were incubated in 1% NGS with no primary antibody. The slices were washed 3 times in PBS with 0.3% Triton X-100, 20 minutes each time, and were then incubated with a biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 60 minutes. They were washed again 3 times in PBS with 0.1% Triton X-100, 20 minutes each time, and incubated with avidin-biotin complex (ABC Kit, Vector Laboratories, Burlingame, CA) in PBS with 0.3% Triton X-100 (dilution of reagents was made 30 minutes before use) for 60 minutes. The brain slices were washed one time with PBS with Triton X-100, and twice with 0.2M PBS for 20 minutes each. For visualization of FLI, the slices were developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Burlingame, CA) in dH_2O for approximately 2-4 minutes, and were placed in PBS until mounting. The brain slices were mounted on clean-subbed Superfrost microscope slides (Fisher Scientific), and allowed to dry overnight in the slide warmer. They were then washed twice in dH_2O for 4 minutes each, dehydrated in ethanol at 70%, 95%, and 100% for 4 minutes each, and placed in xylene twice for 8 minutes, and once for 15 minutes. The slices were mounted with coverslip and permount solution (Fisher Scientific).

Analysis of c-Fos immunostaining was done by counting the neurons activated in the NTS and the RN. The anatomical levels and semi quantitative analysis of c-Fos labeled nuclei were based in the Paxinos & Watson Brain Atlas (39). Brain regions were counted bilaterally in six rostrocaudal levels for the NTS and three levels for the RN. Image was projected from a Nikon microscope (Nikon E400, Nikon Instruments, Melville, NY) to a computer using Q-capture video camera (Qimaging, Burnaby, Canada) and Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). Pictures were taken, and each slice was analyzed separately

using a 10x10 grid in Adobe Photoshop 5.0 software (Adobe Systems Incorporated, San Jose, CA) designed to match the 27mm reticule 1mm square 10x10 grid (Nikon Instruments, Melville, NY) in the microscope. Based on this technique, the investigator was able to compare the picture in the computer to the picture in the microscope, and only count Fos immunopositive cells. Data was then pooled, and the sum of the number of immunopositive particles in a specific brain region was analyzed by two-way analysis of variance (ANOVA) using Statistica software (Statistica, Stat Software, Tulsa, OK). Duncan's Multiple Range Test was used for post hoc comparisons among all groups. Differences were considered statistically different at $p < 0.05$.

RESULTS

Fourth ventricle infusion of AST decreased FLI in the NTS of rats exposed to MS (Fig 2: stress: $p < 0.0001$, treatment: $p < 0.006$). Stress increased FLI in both MS/AST and MS/Saline groups compared to Control/Saline ($p < 0.0002$ and $p < 0.00006$, respectively), and Control/AST groups ($p < 0.005$ and $p < 0.0001$, respectively), but FLI was significant lower in MS/AST rats compared to MS/Saline rats ($p < 0.01$).

FLI in the RN was significantly higher in both MS groups compared to Controls (Fig 4: stress: $p < 0.001$, stress x treatment: $p < 0.01$), but AST decreased FLI in rats exposed to stress, and FLI was significantly lower in MS/AST rats compared to MS/Saline rats ($p < 0.004$). Moreover, FLI was not statistically different between Controls and MS/AST rats.

In summary, AST infused into the fourth before stress totally prevented Fos expression in the RN, and partially blocked FLI in the NTS.

DISCUSSION

We have previously suggested that injection of either CRF or CRF agonists/antagonists into the lateral or third ventricle could have potentially acted in brain nuclei around the fourth ventricle (not published). Grill et al suggested that areas adjacent the fourth ventricle are important in maintaining energy balance responses to stress (17, 18). Previously we tested whether the brainstem played an important role in regulating responses to mild stress, and demonstrated that CRFRs antagonists infused into the fourth ventricle blocked the stress-induced inhibition of food intake and body weight in rats exposed to mild stress, but not to the more severe stress of repeated restraint.

Some studies have shown that different types of stress increase CRF expression in the brain (7, 52). C-Fos immunohistochemistry has been widely used for identification purposes of nuclear cell activation in the brain (48). Fos, a proto-oncogene, is usually activated by some type of stimulation, and it plays a role in regulating early gene transcription although its function is not yet very clear [For Review: (48, 53)]. Investigators have shown that i.c.v. or peripheral injections of CRF and/or Ucn can elicit FLI in several different brain nuclei, including the NTS, and the RN (1, 28, 64). In addition, Wang et al (64) showed that peripheral injection of Ucn had a stronger effect than CRF in inducing FLI in the NTS and RN. In addition, these nuclei have been implicated in regulating feeding by inputs from the periphery [For Review: (66)] or activation of other peptides (28). Therefore, supporting the idea that brainstem might play a role in feeding behavior by activation of CRF-containing neurons.

CRFRs, mainly CRFR2, and Ucn are highly expressed in brainstem areas, such as the AP, the NTS, and the RN (4, 61). In this Experiment, we observed that although FLI was higher in both MS groups, AST decreased activation of cells in the NTS, implying that FLI was due in part

to activation of CRFR. Because AST did not totally block Fos in the NTS, this implies that some of the activation of the NTS was due to a stress-induced peptide that was not a ligand for CRFR. It is known that the NTS plays an important role in regulating feeding responses and appetite. The NTS receives several inputs from the periphery and send efferent fibers to areas, such as the PVN and the arcuate nuclei (9), which, then could regulate appetite by activation of different peptides and neurotransmitters, including CRF [For Review: (18, 65)]. Fourth ventricle injections of CRF or Ucns can decrease food intake by direct activation of brain nuclei responsible for regulating appetite (19, 56); or peripheral injections of Ucn, by decreasing gastric emptying and motility [For Review: (34)]. This central effect might be due to connections from the NTS to the forebrain areas responsible for food intake regulation [For Review: (26, 30)]; whereas the peripheral effect (26) could be due to either a direct activation of the NTS by activation of CRFRs and/or sympathetic nervous system (15, 38, 46). Several investigators have shown the importance of the brainstem in regulating appetite, and decerebrate rats, a well-known model (20, 21), have became an important tool in evaluating the role of the caudal brainstem in regulating feeding responses (25). In addition, Daniels et al (13) showed that an intact connection between the forebrain and brainstem is not essential in feeding behavior, as fourth ventricle infusion of Ucn in decerebrate rats induced Fos expression in the NTS, a brain nuclei that has been implicated as a primary regulator of feeding response.

In contrast to the NTS, we found that FLI in the RN was totally inhibited in AST infused rats exposed to MS, as count levels were not significantly different from controls, implying that all of the stress-induced FLI was due to activation of CRFR. Recently, studies have shown that the RN expresses serotonergic (5-HT) and CRF-neurons (60), and it may be the main brain nuclei responsible for regulating serotonin release in the hypothalamus (43). Serotonin has been

implicated in appetite regulation by suppressing food intake and decreasing body weight [For Review: (29)], and in psychiatric diseases involving stress and CRF (2, 10), and severe stress tends to increase serotonin levels in the brain (37). Interestingly, some forebrain nuclei involved in the anxiety-like behavior and feeding responses, such as the neocortex, amygdala, hypothalamus, hippocampus, and the periaqueductal grey matter [For Review: (16, 23)] also contain CRF-rich neurons. It is tempting to suggest that AST prevented stress-induced FLI induced by stress in the RN, and further neuronal activity of areas in the forebrain that plays a role in regulating appetite and anxiety-like behavior; however, we did not measure serotonin levels or FLI in the forebrain.

Additionally, studies have shown Fos expression changes in neurons located in the RN after RN-site specific injection of selective CRFR2 agonist (Ucn II) (59) and/or antagonist (ASV30), and they were accentuated in the dorsal raphe nucleus (DRN) (58, 59). Thus, CRF has been shown to regulate the serotonergic system in the RN by either activating or inhibiting neuronal activity (42) and subsequent release of serotonin in the hypothalamus through CRF connections (9). Therefore, the serotonergic system might play a role in the acute stress-responses (33), such as anxiety-like behavior [For Review: (32)], and energy balance regulation; however, those changes are dependent on the level of stress (40). It has been shown that low doses of Ucn II does not alter 5-HT neuronal activity, whereas higher does of Ucn II and severe stress can increase 5-HT activity (40, 42).

Based on this present study and previous studies, we conclude that the brainstem might play an important role in regulating feeding responses to mild stress by either direct regulation of brain nuclei responsible for appetite, such as the NTS. It is also tempting to conclude the importance of the brainstem in regulating appetite during stress by CRF-connections to the

forebrain or activation of other neuropeptides. Therefore, future studies should analyze neuronal connections from the brainstem to some brain nuclei in the forebrain that have been implicated in stress-induced responses in food intake.

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LEGENDS

Figure 5.1. Photomicrographs showing internal control brain sections in Control rats infused with either Saline (A) or AST (B), and a rat exposed to MS after either Saline (C) or AST (D) infusions. Brains slices were not incubated with primary antibody to show specificity of the binding.

Figure 5.2. Histogram showing total number of Fos-activated cells in the NTS after 60 minutes of stress in rats. Panel shows the four groups: Control/Saline, Control/AST, MS/Saline, and MS/AST. Data are means for 5-6 rats. Superscripts indicate significant differences between treatment groups.

Figure 5.3. Photomicrographs showing Fos-activated cells in the NTS at several levels in Control rats infused with either Saline (A) or AST (B), and a rat exposed to MS after either Saline (C) or AST (D) infusions. Number of Fos-activated cells were increased in MS groups compared to Controls, but MS/Saline showed higher number of Fos expression in the NTS.

Figure 5.4. Histogram showing total number of Fos-activated cells in the RN after 60 minutes exposure to MS in rats. Panel shows both control and MS groups infused with either Saline or AST into the fourth ventricle before stress. Data are means for 5-6 rats. Superscripts indicate differences between treatment groups.

Figure 5.5. Photomicrographs showing Fos-activated cells in the RN at different levels in Control rats infused with either Saline (A) or AST (B), and a rat exposed to MS after either Saline (C) or AST (D) infusions. Number of Fos-activated cells were increased in MS groups compared to Controls, but MS/Saline showed higher number of Fos expression in the RN.

Figure 5.1

Internal control brain sections

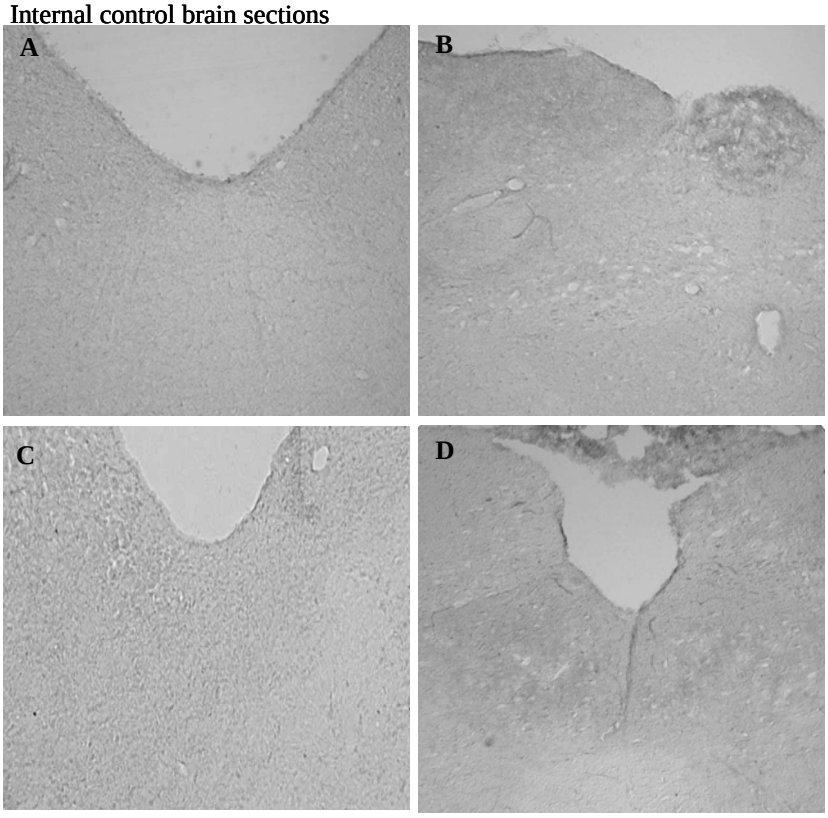


Figure 5.2

Total number of Fos-activated cells in the NTS

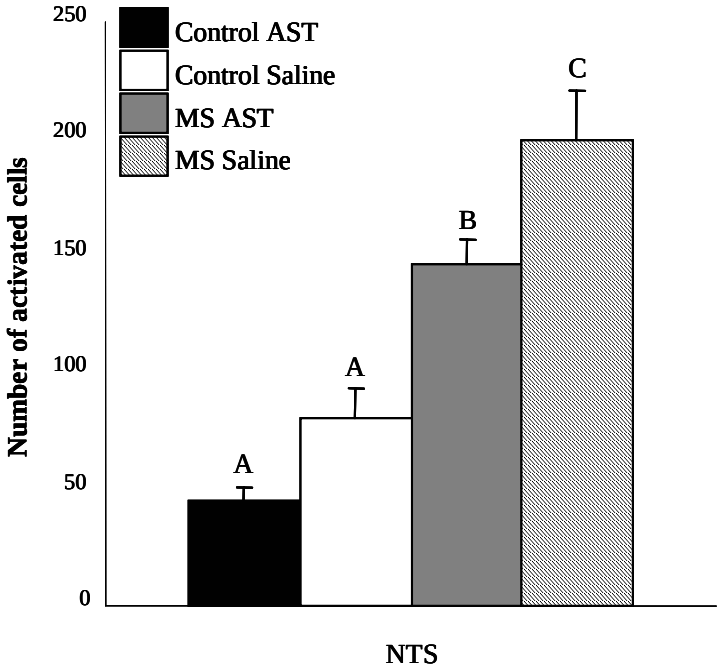
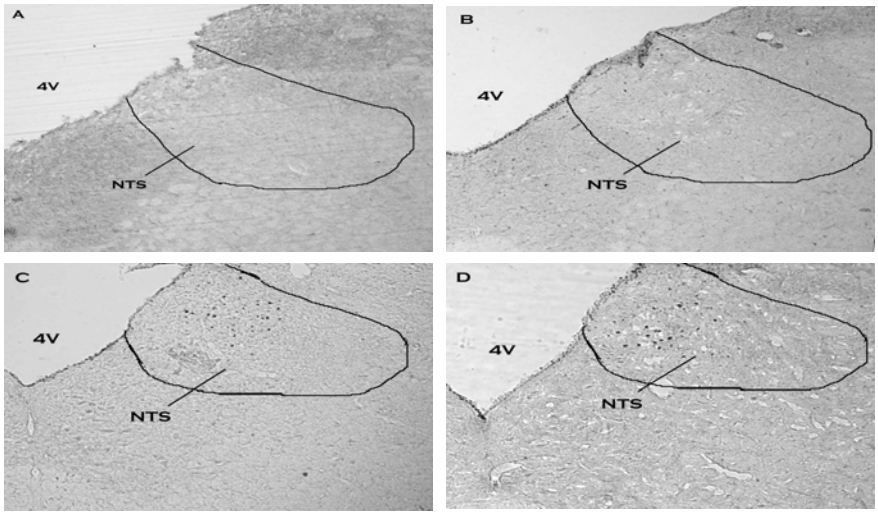


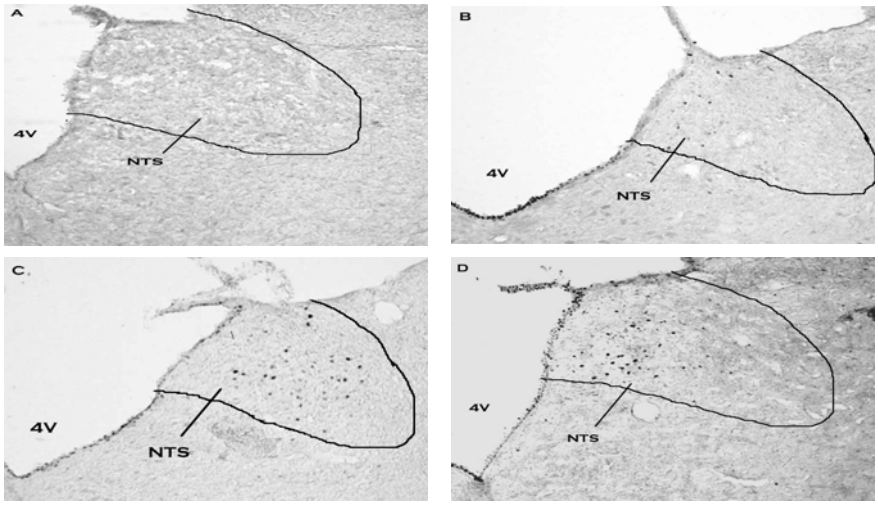
Figure 5.3

Brain section pictures of the NTS at several levels

Picture 1:



Picture 2:



Picture 3:

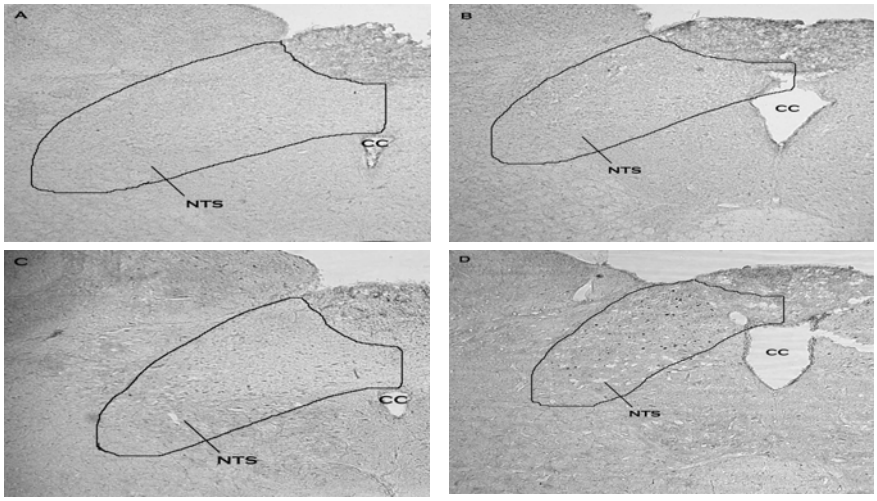


Figure 5.4

Total number of Fos- activated cells in the RN

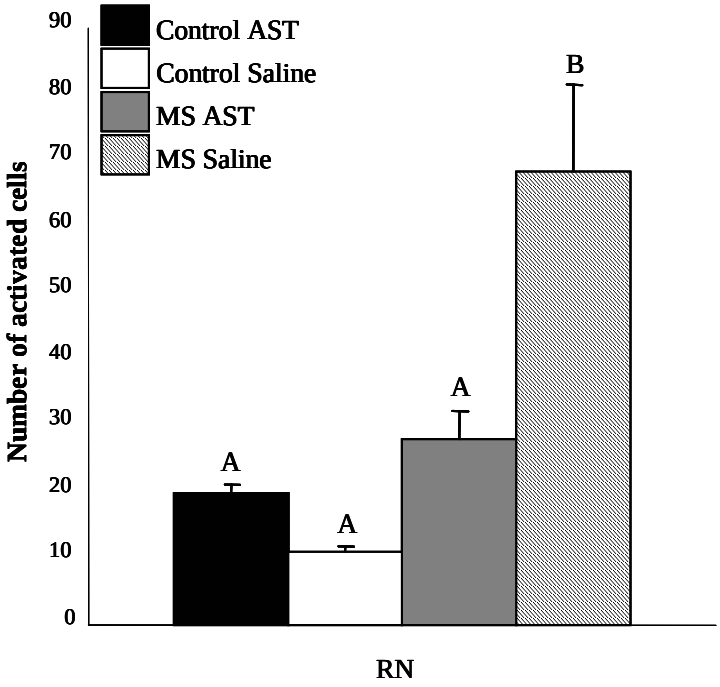
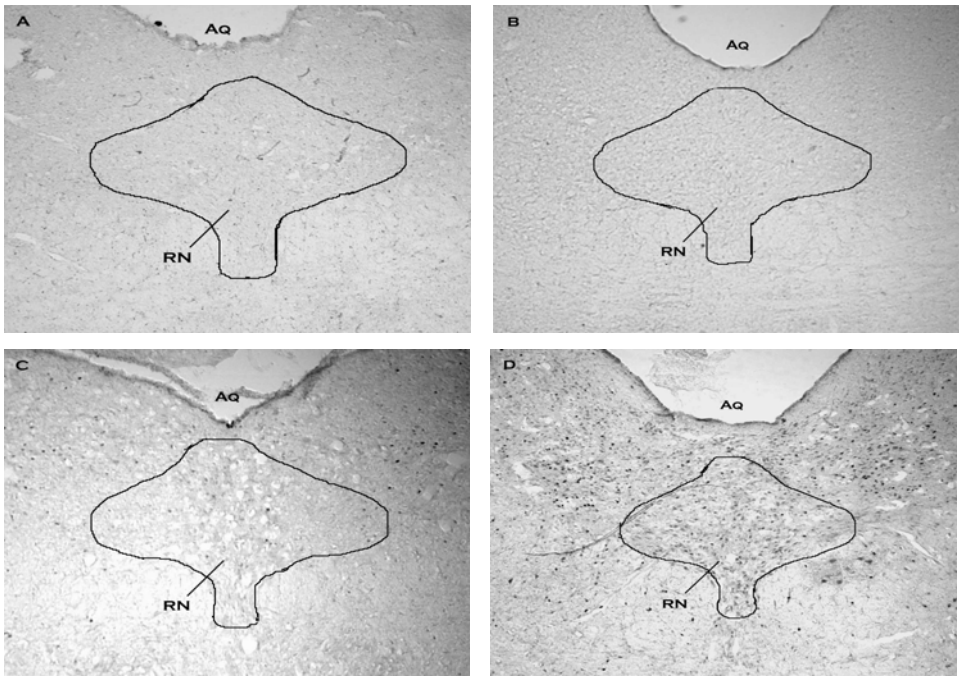


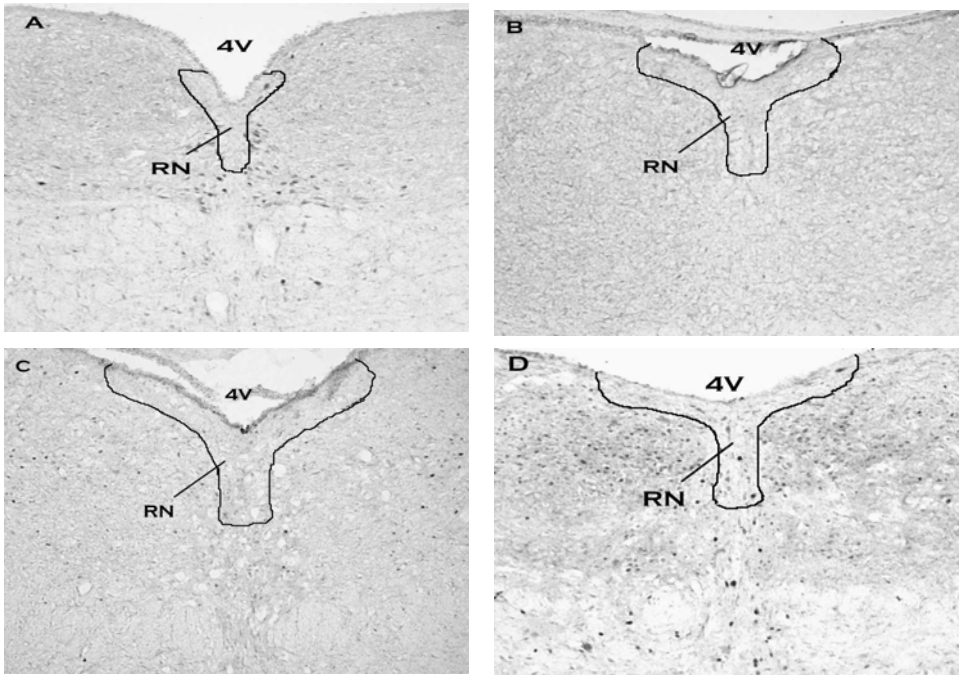
Figure 5.5

Brain section pictures of the RN at several levels

Picture 1:



Picture 2:



CHAPTER 6

SUMMARY AND CONCLUSION

In 1936, Seyle defined the concept of stress (19), and by that time, he had already predicted the important role that complex word means and can play in our lives (18). Since then, stress has been extensively studied because of its impact on health status, as it regulates several different responses in the body, including endocrine, behavioral, immune, and metabolic. These conjunctions of changes appear to be mediated or initiated by one organ, the brain, and previous studies have shown the importance of several brain nuclei in the stress-response. Stress can down- or up-regulate some neuropeptides, neurotransmitters, and receptors in specific brain nuclei (3, 12); thus, inducing emotional and behavioral diseases.

Although different hormones, receptors, and neurotransmitters can play a role in regulating the stress response, corticotrophin-releasing factor (CRF) (22) appears to be the initiator of this response. The complex pathway that CRF can regulate in the brain and in the body is yet not clear; however, it is known that CRF can be regulated by different stress responses, and it can be activated by other hormones as well (17). According to the subtype of CRF Receptors (2, 14, 16) and their localization in the brain (1, 4) and body, different responses might occur. Stress, and thus, CRF can also regulate the behavioral response to stress, such as anxiogenic and anxiolytic effects (20). Those effects, though, are dependent on the type of stress, as acute stress induced weight loss and chronic stress induces weight gain. Previous studies showed that rats submitted to acute stress showed body weight decrease and inhibition of

food intake during stress (13). Furthermore, we have demonstrated that stressed rats do not tend to compensate for the weight loss and hyperphagia (9).

Although several brain nuclei have been implicated in stress-induced weight loss and food intake, it is still unknown which one could be the major site regulating, or even initiating the stress-induced responses in body weight and food intake. Previous studies showed that lateral (11, 15) and/or third ventricle (10, 21) injections of either CRF agonist or antagonist can change the feeding pattern, usually observe in stress. However, we have suggested that this response might be due to activation of CRFRs in areas around the fourth ventricle. And, based on Grill's studies (7), we have hypothesized that areas around the fourth ventricle might be the major initiator in feeding responses to stress. It is known that the brainstem plays an important role in the appetite (5, 6), as it receives input connections from the periphery, and sends efferent signal to the midbrain.

The series of Experiments in this dissertation investigated whether CRFRs in the fourth ventricle might initiate and/or regulate stress-induced responses in body weight and food intake. In the first set of Experiments, we investigated whether activation and/or blockage of CRFRs in the fourth ventricle would change feeding behavior of hungry rats. We have suggested then, that CRFRs around the fourth ventricle might be responsible for regulating this decrease in food intake. In the second set of Experiments, we used two types of acute stress; repeated restraint stress and mild stress. Both stressors cause weight loss and an inhibition of food intake but the effect is smaller with mild stress than repeated restraint (8). From this Experiment, we concluded that antagonism of CRFRs in brain nuclei adjacent to the fourth ventricle might play a role in regulating food intake during a mild, but not a severe stress. Based on these previous results, the objective of the last Experiment was to identify activation of some brain nuclei in the

brainstem of rats exposed of mild stress after antagonism of CRFRs. We hypothesized that antagonism of CRFRs in areas adjacent to the fourth ventricle before stress would prevent activation of those brain nuclei, and prevent further activation of the hypothalamus. We then, concluded that CRFR antagonists infused in the fourth ventricle decreased Fos expression in some brain nuclei responsible for feeding and behavior responses, such as the NTS and the RN.

Therefore, based on results from these Experiments, we conclude that the brainstem can be an important regulator and/or initiator of changes in food intake and body weight induced by acute stress by activation of CRFRs. Based on these results, we can also conclude that (1) CRF can play a role in regulating feeding behavior in unstressed animals, (2) that the decrease in body weight and inhibition of food intake induced by stress are mediated by different pathways, depending upon the level of stress, (3) the brainstem might play an important role in regulating the response to stress, (4) that some brain nuclei in the brainstem might be responsible for some of the stress-induced responses, (5) and that the CRFR2 might be responsible for the down-regulation of food intake in stressed animals.

This study focused in the effects of acute stress in body weight, food intake, hyperactivity of the HPA, and Fos expression in rats infused with CRFRs agonists and/or antagonists in the fourth ventricle. And, we presumed that these changes were due to activation of CRFRs in some brainstem nuclei only. Our Experiments were based only in fourth ventricle infusions; therefore, we also cannot exclude other brain nuclei that could be involved in the stress responses that are not adjacent to the ventricles.

Based on these results, we suggest that future studies should investigate the importance of the brainstem in initiating and regulating changes in food intake induced by acute stress. We also suggest that future studies should analyze the role of some brainstem nuclei that co-express

both CRFRs and other neuropeptides involved in feeding behavior. In addition, these results will lead us to a better understanding of the role of the brainstem in acute stress, and lead future investigation of those brain nuclei.

Future studies will investigate the role of the brainstem in the stress responsiveness and its effect on food intake and body weight. We have previously demonstrated that antagonism of CRFR2 in the brainstem blocked the mild stress effects on food intake and body weight. Therefore, studies will initially use a selective CRFR2 antagonist in the fourth ventricle before mild stress, and then analyze brain nuclei activation in the brainstem area by c-Fos immunohistochemistry method. This would not only limit brain areas that would be involved in the stress response by activation of the CRFR2 in the brainstem but also prove that CRFR2 is involved in the mild stress response mediated by the brainstem. Further analysis of these brain nuclei will be conducted by the use of site-specific injections of CRFRs antagonists during stress. And, this study will reveal the importance of these brain nuclei, which will lead us to a following study that will investigate the neural pathways between the brainstem and the mid and/or the forebrain. For this study, identification of the CRF-connections from the brainstem to the mid or forebrain would be based on viral or gold retrograde tracing studies. After limiting brain areas and neuronal connections, we propose further investigation of other neuropeptides, including serotonin that might also be expressed and/or activated in those nuclei during stress, by double-labeling immunohistochemistry. Completion of these studies would give us a better understanding of the interaction between CRF system during mild stress in the brainstem and mid/forebrain.

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