THE EFFECTS OF ANTAGONISM OF CRF RECEPTORS IN AREAS ADJACENT TO THE THIRD VENTRICLE DURING REPEATED RESTRAINT STRESS

by

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(Under the Direction of Ruth B.S. Harris)

ABSTRACT

Corticotropin-releasing factor (CRF) initiates many of the immunologic, endocrine, autonomic, metabolic and behavioral responses to stress through activation of central and peripheral CRF receptors (CRFR). The two types of CRFR, CRFR1 and CRFR2, are differentially distributed throughout the brain and mediate different aspects of the stress response. Exposure to repeated restraint (RR), a model of acute stress, induces chronic changes such as a sustained reduction in body weight and hyperreactivity of the endocrine response to mild stressors applied in the post-RR period. In the first Experiment, we found that mice exposed to RR exhibit increased anxiety-like behavior in the post-RR period. In the second set of Experiments we found that antagonism CRFR1 during RR results in attenuation of the sustained decrease in body weight while antagonism of CRFR2 prevents stress-induced hypophagia. These results imply that activation of CRFR1 is necessary to induce the chronic effects of RR on body weight.

INDEX WORDS: restraint stress, CRF, CRF receptors, behavior, corticosterone

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CHAPTER 1

INTRODUCTION

An estimated 66% of adults and 17% of children in the US may be classified as either overweight or obese (22) and a recent study reported that approximately 85% of those who lose weight are not successful at maintaining their weight loss (1). While many experimentally-induced models of weight loss are reversed when the intervention that caused the weight loss is removed (11, 21), repeated restraint (RR) is a model of acute stress in which animals exhibit a sustained reduction in body weight as many as 80 days after the end of the intervention (14). Rats exposed to RR, 3 hours of restraint stress on each of 3 consecutive days, reduce food intake and increase energy expenditure on the days of restraint, resulting in weight loss. Mice exposed to 2 hours of restraint stress on each of 3 consecutive days exhibit similar changes in body weight and food intake (16). In the days following RR, food intake and energy expenditure return to the level of non-restrained controls, however, RR animals fail to compensate for the weight lost during RR and never return to the weight of controls (12, 15). Elucidation of the mechanisms behind the sustained weight loss induced by RR as well as further exploration of additional chronic effects of RR will provide new information on the regulation of body weight and may eventually provide solutions to those struggling to maintain a healthy weight.

In addition to a sustained reduction in body weight, RR animals exhibit exaggerated corticosterone release in response to novel mild stressors applied in the post-RR period (10). Although hyperresponsive to mild stressors, RR animals exhibit normal basal circulating levels of corticosterone (17). The increased sensitivity of the endocrine response to novel stressors in

the post-stress period has been associated with the impaired ability of the glucocorticoids to suppress HPA axis activity (6) and a lowering of the threshold for the initiation of endocrine responses to stress (10). Studies have shown that RR animals exhibit increased anxiety-like behavior in three well-established behavior tests, the elevated plus maze (EPM), the defensive withdrawal paradigm, and the light-dark box when behavior is measured immediately after the end of the RR (13, 16, 19, 20, 23). The first study described here tested whether RR induced a long-term increase in anxiety-like behavior in addition to the sustained changes in body weight and HPA axis hyperreactivity We hypothesized that mice exposed to RR would exhibit an increase in measures of behaviors indicative of anxiety in the EPM and light-dark box when compared to non-restrained controls.

Although regulated by a number of neurotransmitters, hormones and receptors, the primary initiator of the body's multiple responses to stress appears to be corticotropin-releasing factor (CRF) which mediates responses to stress through activation of CRF receptors (CRFR) (4, 28). The two types of CRFR, CRFR1 and CRFR2, are differentially distributed throughout the brain and the body. The effects of stress are dependent upon the type and location of the CRFR activated (4). While CRFR1 activation has been implicated in promotion of the hypothalamic-pituitary-adrenal (HPA) axis response and increases in behaviors indicative of anxiety (7, 26, 27), CRFR2 are believed to mediate stress-induced hypophagia and increases in energy expenditure (8, 9). CRFR2 may also facilitate the return to homeostasis following activation of the endocrine response to stress, however, further exploration of this role is necessary (2, 3).

Centrally infused CRFR antagonists have been shown to attenuate or reverse CRF- and restraint-induced changes in body weight, food intake, HPA axis activation, and anxiety-like behavior (5, 8, 18, 24, 29). Antagonism of all CRFR in areas adjacent to the third ventricle

immediately before RR prevented RR-induced weight loss in rats (25). The second set of Experiments described here tested whether specific antagonism of CRFR1 or CRFR2 in areas adjacent to the third ventricle would attenuate the response to RR. We hypothesized that central antagonism of CRFR1 would prevent RR-induced weight loss as well as the hyperresponsiveness of the HPA axis in the post-RR period while central CRFR2 antagonism would attenuate RR-induced hypophagia. We hypothesized that antagonism of all CRFR in areas adjacent to the third ventricle during RR would completely attenuate RR-induced decreases in food intake, body weight, and the hyperreactivity of the endocrine response in the post-RR period.

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CHAPTER 2

LITERATURE REVIEW

According to the National Health and Nutrition Examination Survey of 2002-2004, an estimated 66.3% of adults and 17.1% of children in the US are overweight or obese (67). Further complicating this issue, a recent study suggested that approximately 85% of people who lose weight are not successful in maintaining their new, reduced weight (6). Similarly, weight loss in many animal models is rapidly reversed as soon as the intervention which caused the weight loss is terminated (37, 65). The model of repeated restraint (RR) is unique in that animals exposed to RR lose weight and do not compensate for the weight that they have lost once RR ends (40). This sustained reduction in body weight has been recorded as many as 80 days after restraint has ended (40). Elucidation of the mechanism behind the effects of RR will provide new information on the regulation of body weight and may help to provide solutions for individuals trying to maintain a healthy weight.

Definition of Stress

The word stress is used to describe the state of an organism while under the influence of external or internal forces that threaten to alter its homeostasis (57). A stressor, in turn, may be any condition which endangers, or is perceived to endanger, an individual's homeostasis (90). Exposure to a stressor such as RR results in a myriad of responses, of which activation of the hypothalamic-pituitary-adrenal axis (HPA axis), an increase in energy expenditure, an exaggeration of behaviors indicative of anxiety, and a decrease in feeding and appetite are only a few (22, 40, 41, 50, 55, 89). The sympathetic and parasympathetic nervous systems are also

activated during stress exposure, resulting in an increase in renal nerve activity and plasma catecholamine levels as well as an increase in blood pressure and redirection of blood flow towards skeletal muscle (58). Stress-induced activation of the central nervous system results in a suppression of immune function and modulation of the release of growth factors and reproductive hormones (29). These modifications of endocrine, sympathetic, metabolic, immunologic, and behavioral functions during stress are essential for protecting the body from damage and enabling the body to return to homeostasis or to adapt to a new situation (60, 61). The magnitude and duration of the response to stress is dependent upon the perceived severity of the stressor and the amount of time the organism is exposed to the stressor (75).

The HPA axis

Corticotropin-releasing factor (CRF), a 41-amino acid neuropeptide, has been identified as the primary initiator of behavioral and physiologic responses to stress (91). Although predominantly produced by the parvocellular cells of the paraventricular nucleus (PVN), CRF can also be found in extrahypothalamic structures such as the hippocampus, locus ceruleus (LC), the parabrachial area, and the olfactory bulb (49). Hypothalamic CRF content, as evidenced by CRF immunoreactivity (CRF-ir), is significantly increased in response to stress exposure (35). Exposure to stress resulted in an increase in CRF-ir content in the LC, an area of the brain believed to mediate behavioral responses to stress through an interaction between CRF and norepinephrine (20, 49, 92). The same researchers also reported stress-induced increases in CRF-ir in the PVN, a brain nucleus responsible for stress-induced suppression of food intake (20, 49). In addition to increased expression in response to stress, central injection of CRF mimics the effects of stress on the body, resulting in activation of the HPA axis, activation of the sympathetic nervous system and subsequent increases in serum catecholamine concentration,

inhibition of gastric motility, enhancement of anxiety-like behavior, and inhibition of feeding behavior (15, 34, 51, 69, 76, 78).

The body's primary endocrine response to stress is mediated through activation of the HPA axis. CRF released into the circulation from the hypothalamus acts on the corticotrope cells of the anterior pituitary to stimulate the release of adrenocorticotrophic hormone (ACTH) and β-endorphins, both products of pro-opiomelanocortin (POMC). Release of ACTH, in turn, initiates the synthesis and release of glucocorticoids (GCs) from the adrenal cortex while feeding back to down-regulate the synthesis and release of hypothalamic CRF (18). GCs, which include cortisol in humans and corticosterone in rodents, are the final product of HPA axis activation. GCs increase the availability of circulating concentrations of glucose by reducing cellular glucose utilization and increasing hepatic gluconeogenesis (3, 18). GCs also alter metabolism by increasing catabolism of protein from muscle and mobilizing fatty acids from fat stores (3). In rodents, basal levels of corticosterone release vary in a diurnal rhythm with peak GC release occurring at the onset of the dark phase and the nadir occurring at the onset of the light phase (5).

Although primarily under the control of catecholamines; serotonin, GABA and acetylcholine are also thought to play a role in mediating CRF release (28, 33, 73). Arginine vasopressin (AVP), is co-expressed and co-secreted with CRF in response to stress and has been shown to act concurrently with CRF to trigger the release of ACTH through activation of AVP 1b receptors in the pituitary (47, 48, 52). In non-stressed rats, CRF and AVP are co-expressed in approximately 50% of all CRF-expressing neurons in the PVN (100). Repeated exposure to the same stressor, or chronic stress, results in a substantial increase in the number of neurons co-expressing CRF and AVP and this is associated with increased ACTH release in response to

subsequent novel stress exposure (26). Research suggests that during periods of chronic stress primary control of ACTH secretion shifts from CRF to AVP (12, 56, 66).

The endocrine response to stress is terminated through a negative feedback mechanism. Elevated circulating GCs bind to hippocampal mineralocorticoid receptors and act at the level of both the pituitary and hypothalamus to inhibit further production and release of CRF and ACTH (11, 31, 90). Peak GC release occurs within 20 or 30 minutes of HPA axis activation (30). GCs may enhance sensitivity of the HPA axis to negative feedback, as evidenced by the decrease in the amount of time necessary for GC release to peak with each exposure to the same stressor (59, 63). Chronic elevation of GCs has been implicated in inhibition of thyroid function and reproductive behaviors (3, 4, 18). Chronically elevated circulating levels of GCs and the subsequent dysfunction in HPA axis regulation have also been linked to the development of cardiovascular disease, depression and central adiposity (27, 62).

CRF receptors and their ligands

HPA axis activation and other CRF-mediated responses to stress are initiated through the binding of CRF to G protein-coupled CRF receptors (CRFR). Other ligands for CRFR include the CRF variants urocortin I (Ucn I), Ucn II, and Ucn III. The affinity of CRF and its variants for the two types of CRFR, CRFR1 and CRFR2, varies. CRF has a 10-fold higher affinity for CRFR1 than CRFR2 while Ucn I binds with equal affinity to both CRFR1 and CRFR2 (72). When compared to CRF, icv infusion of Ucn I produces similar, although slightly less potent, increases in anxiety-like behavior (87). In contrast to CRF and Ucn I, Ucn II and Ucn III appear to selectively bind CRFR2 (53, 96, 97).

The two types of CRFR are differentially distributed at sites in the brain and throughout the body, lending support to the theory that CRFR1 and CRFR2 play different roles in

modulating the body's response to stress. Central CRFR1 are primarily located in areas of the brain associated with emotion, memory, and HPA axis activation such as the neocortex, the hippocampus, the limbic system, the hypothalamus and the pituitary (19, 87, 88). CRFR2 are less prevalent in the brain than CRFR1, located primarily in subcortical structures such as the PVN and the ventromedial and arcuate nuclei of the hypothalamus (19, 95). A low density distribution of CRFR2 are found in the lobes of the pituitary, in contrast to CRFR1 which are expressed in the pituitary in relatively large numbers (80). The selective distribution of CRF2 receptor mRNA in the ventromedial nucleus of the hypothalamus and in the PVN suggest a role for CRFR2 receptors in stress-induced changes in food intake and metabolism (68, 84). Icv infusion of Ucn I is a much more effective appetite suppressor than CRF, particularly in the PVN (25, 87, 98). Additionally, a recent study performed by Inoue et al. found that icv Ucn II, which selectively binds CRFR2, inhibits food and water intake in rats (45).

The role that each subtype of CRFR plays during the stress response has been further elucidated through studies involving CRFR antagonists and mice genetically engineered to lack the CRFR gene (KO mice). Several labs have independently produced mice deficient in CRFR1, CRFR2, or all CRFR (8, 10, 23, 74, 86, 88). It is now widely accepted that activation of CRFR1 during exposure to stress is necessary to activate the HPA axis (11). CRFR1 are also believed to mediate stress-induced increases in behaviors indicative of anxiety. Mice deficient for CRFR1 exhibit basal corticosterone and ACTH levels similar to their wild-type (WT) littermates. When challenged by a stressor, however, both corticosterone and ACTH release are notably blunted in CRFR1 KO mice (86, 88). These mice also display decreased anxiety-like behavior in the light-dark box, a behavioral paradigm often used to assess anxiety (88). Rat studies involving peripheral application of antalarmin, a CRFR1-specific antagonist, further support these theories

by showing a reduction in behaviors indicative of anxiety in the elevated plus maze, a second validated behavioral paradigm, and an attenuation of the rise of ACTH associated with exposure to a brief period of restraint (85, 99). Interestingly, CRFR1 KO mice display elevated basal levels of AVP protein and mRNA in the PVN, indicative of the compensatory role AVP and its receptors play in HPA axis regulation in the absence of normal regulation through the CRFR1 (66). Although CRFR1 activation is thought to be important for HPA axis activation and induction of anxiety-like behavior, activation of CRFR1 does not appear to influence stress-induced alterations in food intake. When centrally injected or administered orally, NBI 27914, another CRFR1 antagonist, had no effect on CRF- or Ucn I-induced hypophagia (71, 83). Changes in feeding behavior in response to stress are believed to lie under the control of CRFR2 (11).

While there is consensus that activation of CRFR2 results in the hypophagia commonly observed during exposure to stress, other aspects of the stress response that are mediated by CRFR2 are unclear. Concurrent lateral ventricular administration of antisauvagine-30, a selective CRFR2 antagonist, with Ucn I or CRF completely reversed the decrease in food intake and body weight observed when the stress peptides were infused alone, supporting a role for CRFR2 in the control of feeding responses to stress (24). Unlike CRFR1 KO mice, CRFR2 KO mice exhibit heightened HPA axis activity in response to stress. Despite normal basal ACTH and corticosterone levels, CRFR2 KO mice exposed to restraint stress show a rapid, exaggerated release of ACTH compared to WT littermates (8, 23). Researchers found that an elevation in serum corticosterone could be detected 2 minutes after the onset of restraint stress in CRFR2 KO compared to 5 minutes after the onset of restraint in WT littermates. Additionally, corticosterone levels were still significantly elevated 90 minutes after a 5-minute restraint in CRFR2 KO

compared to WT animals (23). Results from these studies provide evidence that CRFR2 may function to dampen or reverse the effects of CRFR1 activation during exposure to stressors. This may occur through more rapid termination of ACTH secretion and/or corticosterone following the termination of stress leading to a more rapid restoration of homeostasis. Heightened anxiety-like behavior in the elevated plus maze and open field have also been observed in CRFR2 KO mice (7). Bale et al. have reported that these CRFR2 KO mice exhibit increased CRF mRNA levels in the central nucleus of the amygdala. This is significant as the central nucleus of the amygdala is an important component of the limbic system which contributes to changes in behavior such as responses to aversive stimuli and appetitive behavior (9, 77, 93).

Mice deficient for both subtypes of CRFR (CRFR1/2 KO) have also been developed (10, 74). Absence of both types of CRFR affect mice differently depending upon the sex of the mouse. In comparison with WT mice, female CRFR1/2 KO mice display decreased anxiety-like behavior in the light-dark box and elevated plus maze (10, 21). Male CRFR1/2 KO mice, in contrast, display increased anxiety-like behavior in the same paradigms. The results of non-specific CRFR antagonist studies in rats support the data produced by the CRFR1/2 KO females. Central injection of α-helicalCRF(9-41) (αhCRF), a nonspecific CRFR antagonist, attenuates immobilization- and CRF-induced anxiety-like behavior in rats (14, 46, 81). Similarly, astressin, a second non-specific CRFR antagonist, injected into the lateral ventricle decreases CRF-induced anxiety-like behavior in a familiar environment (46). Jones et al. also reported that central astressin injection attenuated CRF-induced weight loss and hypophagia in hungry rats. Finally, although basal levels of ACTH and corticosterone were similar to WT mice, both male and female CRFR1/2 KO did not increase release of ACTH or corticosterone following 10 min of restraint stress (87) and males showed no HPA response to social defeat (87).

Repeated Restraint

Rats exposed to RR (3 hours of restraint on each of 3 consecutive days) experience an inhibition of food intake and a loss of body weight during the days of restraint (43). While RR rats exhibit hypophagia during the period of RR, food intake rapidly returns to pre-stress levels once stress ends. In the post-stress period, RR rats gain weight at the same rate as non-stressed controls, however, they fail to compensate for the increase in energy expenditure and decrease in food intake during restraint and do not return to the weight of controls (40). Although the initial weight loss during restraint is comprised of lean tissue, in the post-stress period body composition is altered so that the percent composition of lean and fat mass in RR rats is similar to control rats (101). This sustained weight loss has been noted as many as 80 days after the end of restraint and is in stark contrast to other methods of experimentally-induced weight loss, such as food restriction, where weight loss is rapidly reversed once restriction has ended (37, 39). Rats exposed to RR experience neither a chronic elevation of circulating corticosterone nor a permanent alteration in the expression of corticotropin-releasing factor receptors (CRFR) or their ligands (40). Although activated by RR, the HPA axis of RR rats quickly recovers, with serum corticosterone returning to baseline by the second hour of the 3 hour restraint (44). When measured immediately following the second day of 3 hour restraint, mRNA expression of UCN in the Edinger Westphal nucleus and CRFR1 in the PVN are significantly increased in rats exposed to RR, however, this increase in stress peptides is lost when measured 40 days after RR (39). Although primarily studied in rats, mice responds similarly when exposed to 2 hours of restraint on each of 3 consecutive days (36, 42).

Hyperreactivity in the post-RR period

A sustained reduction of body weight is not the only chronic effect of RR. Although baseline serum corticosterone levels and the daily nadir of corticosterone release remain normal, RR animals exhibit enhanced secretion of glucocorticoids when challenged by a novel mild stressor in the post-stress period (36, 44). This effect is not unique to RR, as others have reported increased sensitivity to subsequent stressors following an acute stressor. For example, rats exposed to one or three daily sessions of footshock show an exaggerated corticosterone response to a stress applied 10 days later (17, 32, 79). The increased sensitivity of the endocrine response to novel stressors in the post-stress period has been associated with the impaired ability of the glucocorticoids to suppress HPA axis activity (16) and a lowering of the threshold for the initiation of endocrine responses to stress (36). It is important to note that, although RR animals exhibit a chronic response to acute stress, they are not chronically stressed. Chronically stressed animals exhibit a similar exaggerated endocrine response to novel mild stressors (2, 13). Additionally, chronically stressed animals have enlarged adrenal glands, small thymus glands, and elevated baseline corticosterone levels (2). In contrast, baseline corticosterone levels of RR animals are not elevated when measured 12 days after RR (36) and the weight of the thymus and adrenals do not differ from those of controls (43).

In addition to enhanced endocrine sensitivity, studies have shown that RR animals exhibit increased anxiety-like behavior immediately after the end of the restraint stress in three well-established behavior tests, the elevated plus maze (EPM), the defensive withdrawal paradigm, and the light-dark box (38, 42, 54, 64, 82). We have also shown that mice exhibit enhanced anxiety-like behavior in the elevated plus maze (EPM) and the light-dark box, as many as 12 and 20 days, respectively, after RR. Behaviors indicative of anxiety in these paradigms are based on

the natural tendency of the rodent to explore a novel environment and avoid brightly illuminated, open spaces (70). A marked preference for the dark, enclosed areas in both of these arenas is indicative of enhanced behavioral anxiety.

This is not the only report of long-lasting modification of anxiety-like behavior in stressed animals. Enhanced levels of anxiety-type behavior in the open field apparatus have been recorded in rats as many as 14 days after a single session of inescapable foot shock (94). Also, one 5 minute exposure to a cat resulted in reduced exploratory behavior in rats tested in the EPM 21 days later (1). However, these observations are rare, and no comparable periods of time have been observed in response to restraint stress.

In summary, exposure to a stressor results in the modification of various endocrine, sympathetic, metabolic, immunologic and behavioral systems. Activation of CRFR mediate many of the responses to stress exposure. The aspect of behavior modified is dependent upon the type of CRFR activated. CRFR1 receptors, activated mainly by CRF and Ucn I, are involved in the initiation of HPA axis activation and play a role in the induction of anxiety-like behavior. CRFR2, partially activated by CRF but predominantly by Ucn I, II, and III, mediate stress-induced feeding behavior and may facilitate recovery of the HPA axis after the termination of stress.

RR, an acute stress, results in several chronic conditions including a sustained reduction in body weight and an exaggerated endocrine response to subsequent novel mild stressors. CRFR antagonists infused icv have been shown to attenuate or reverse CRF-, Ucn I- and restraint-induced changes in body weight, food intake, HPA axis activation, and anxiety-like behavior. Therefore, we hypothesize that CRFR in areas of the brain adjacent to the third ventricle play a role in mediating RR-induced weight loss as well as endocrine and behavioral

hyperresponsiveness during the post-stress period. However, we do not know which CRFR subtype mediates the different components of the long-term effects of RR. The studies described here tested whether infusion of CRFR antagonists into the third ventricle immediately before RR attenuated RR-induced weight loss and the subsequent increases in endocrine responsiveness in the post-stress period in RR rats. The objective of these studies was to determine which CRFR subtype is responsible for the chronic effects of RR in rats.

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CHAPTER 3

INCREASED ANXIETY-LIKE BEHAVIOR DURING THE POST-STRESS PERIOD IN MICE EXPOSED TO REPEATED RESTRAINT STRESS[1]

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ABSTRACT

Mice exposed to repeated restraint (2h of restraint on each of 3 consecutive days) lose weight and do not return to the weight of non-stressed controls after restraint ends. These mice also exhibit an exaggerated endocrine response to mild stressors in the post-stress period. To determine if other aspects of the stress response are altered NIH Swiss mice were repeatedly restrained then evaluated for anxiety-like behavior in various behavioral tests. Restrained mice exposed to the defensive withdrawal apparatus and the marble burying test 6 and 17 days, respectively, after restraint displayed no increase in anxiety-like behavior. Twelve days after the end of repeated restraint half of the control and the restrained mice were subjected to the mild stress of an intraperitoneal injection of saline before placement in an elevated plus maze. Restrained mice not subjected to mild stress showed the same level of anxiety as the control mice exposed to mild stress. Placement in a light-dark box 20 days after restraint also resulted in an increase in anxiety-like behavior in mice that had been exposed to repeated restraint. Restrained mice released more corticosterone than non-restrained controls exposed to defensive withdrawal or EPM apparatus although baseline corticosterone remained at control levels. These results suggest that repeated restraint induces an exaggeration of both endocrine and behavioral responses to subsequent mild stressors. This post-stress hypersensitivity to mild stress may contribute to the sustained reduction in the body weight of restrained animals.

Key words: elevated plus maze, light-dark box, defensive withdrawal, marble-burying, corticosterone

INTRODUCTION

Mice exposed to 2 hours of restraint each morning for 3 consecutive days (repeated restraint, RR) lose weight during the period of restraint (Harris, et al., 2001). It has been shown that this initial weight loss is due to an increase in energy expenditure and a decrease in food intake on the days of restraint (Harris, et al., 2006). Following RR, food intake and energy expenditure return to pre-stress levels and rats gain weight at the same rate as controls, however, they do not return to the weight of the controls (Harris et al., 2001). Although baseline serum corticosterone levels and the daily nadir of corticosterone release remain normal, RR animals exhibit an exaggerated corticosterone response to novel mild stressors in the post-stress period (Harris, et al., 2004). Although much of the work with this model has been carried out with rats, mice exposed to RR also show the same sustained down-regulation of body weight and hyper-responsiveness to subsequent mild stressors (Harris et al., 2004; Harris et al., 2001). Others have reported similar increased sensitivity to subsequent stressors in animals that have been acutely stressed. For example, rats exposed to one or three daily sessions of footshock show an exaggerated corticosterone response to a stress applied 10 days later (Caggiula, et al., 1989; Gomez, et al., 2002; Servatius, et al., 1994). The increased sensitivity of the endocrine response to stressors in the post-stress period has been associated with the impaired ability of the glucocorticoids to suppress hypothalamic-pituitary-adrenal (HPA) axis activity and a lowering of the threshold for the initiation of endocrine responses to stress (Buwalda, et al., 1997; Harris et al., 2004).

Exposure to stress results in a myriad of other responses, of which activation of the HPA axis (Cook, 2004), an increase in energy expenditure (Harris et al., 2006), and a decrease in feeding and appetite (Harris, et al., 2002b) are only a few. Exposure to stress also results in

behavioral changes. For example, a period of acute restraint stress has been shown to increase anxiety-like behavior in three well-established behavior tests, the elevated plus maze (EPM), the defensive withdrawal paradigm, and the light-dark box (Harris, et al., 2002a; Harris et al., 2001; MacNeil, et al., 1997; Morilak, et al., 2003; Smagin, et al., 1996). These behavioral tests rely on the conflict between the rodent's natural aversion to illuminated, open spaces and a tendency to explore novel areas. In addition to the sustained sensitivity of the endocrine response, there is evidence that exposure to an acute stressor can result in sustained increases in anxiety-like behavior. In 1992, van Dijken et al. reported that rats exposed to a single session of inescapable foot shock demonstrated enhanced anxiety-like behavior in the open field apparatus(Van Dijken, et al., 1992). Adamec and Shallow (1993) found that a single, 5 minute exposure to a predator caused exaggerated anxiety-like behavior in the EPM in rats. Therefore, this study was performed in order to determine whether RR induces long-lasting changes in anxiety-like behavior in various tests during the post-stress period in addition to causing the long-lasting hyper-reactivity of the HPA axis.

METHODS

Animals and Repeated Restraint: 32 male NIH Swiss mice (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 24g were housed in a humidity- and temperature-controlled room on a 12:12 hour light:dark cycle with free access to mouse chow (PMI International, Brentwood, MO) and water. They were housed in individual cages with grid floors to facilitate accurate measurement of food intake. After 7 days of baseline food intake and body weight measurements, the mice were divided into two weight-matched groups, non-restrained controls and restrained mice. Restrained mice were exposed to a repeated restraint

(RR) consisting of 2 hours of restraint on each of 3 consecutive days (*days 1-3*) in Perspex restraining tubes (9.5 X 2.5 cm, Plas-Labs, Lansing, MI) in an experimental room. Control mice were placed in shoebox cages in the same experimental room during the period of restraint. Both groups were deprived of food and water during the 2 hours of restraint. After restraint, the mice were returned to their home cages and given food and water *ad libitum*. Food intake and body weight were recorded for 7 days following RR.

Behavioral testing: All behavioral testing occurred between 9AM and 1PM on the designated day of experimentation. In an effort to confine testing to this time interval on any one day, the mice were divided into two subgroups containing an approximately equal number of restrained and control mice and each behavior test was conducted over 2 days. All tests, except for the marble-burying test, were conducted in a separate, experimental room. Behavior in the defensive withdrawal apparatus, EPM, and light-dark chamber was recorded and scored using the Ethovision Video Tracking, Motion Analysis and Behavior Recognition System (Version 2.3, Noldus Information Technology Inc., Leesburg, VA). Activity in the EPM was measured after the mice had been exposed to a mild stress, as described below. All other behaviors were measured in non-stressed animals.

Defensive Withdrawal: The defensive withdrawal apparatus consisted of a brightly lit, 76.2 X 76.2 cm chamber with a white floor and 23 cm high black walls. A 12 X 7 cm diameter black, cylindrical chamber with one open end was secured on one wall, approximately 30 cm from the corner. The mice were divided into two groups and tested 5 or 6 days (*day 8* or *9)* after RR. A mouse was placed headfirst inside the chamber and activity was recorded for 10 minutes.

Behavior was scored for latency to exit the dark chamber, number of entries into the dark chamber and middle zone of the apparatus, and the duration of time in the dark chamber and middle zone. The mouse was then returned to its home cage. Twenty-five minutes after removal from the defensive withdrawal apparatus approximately 30 μL of blood was collected from the tail for measurement of serum corticosterone concentration (Corticosterone RIA, MP Biomedicals, Irvine, CA).

Elevated Plus Maze: The EPM consisted of brightly illuminated 45 X 10 cm arms elevated 50 cm above the ground. Two opposite arms of the maze were painted black and enclosed by black walls measuring 40 cm high. The other two opposing arms were painted white and had no walls. Mice were tested in the EPM 9 or 12 days after RR (*day 12* or *15*). Previous studies have shown that rats exposed to severe stress exhibit more robust increases in anxiety-like behavior on the second exposure to the EPM (File and Gonzalez, 1996; Hogg, 1996), therefore, each mouse was allowed a 10 minute acclimation period in the EPM a day prior to behavioral testing. On the experimental day the mice were divided into 4 groups; control-MS, control-control, RR-MS, and RR-control. MS mice were given an intraperitoneal injection of 0.2mL saline immediately before being placed into the middle of the EPM facing a closed arm. Activity inside the maze was recorded for 10 minutes and scored for the frequency of entry into the arms and the time spent and distance moved within the arms. Mice were returned to their home cages after behavioral testing. Immediately before MS and 25 minutes following removal from the maze approximately 20μL of blood was collected from the tail for measurement of serum corticosterone concentration.

Marble-burying: Mice were adapted to cages with corncob bedding for 3 days before testing. Seventeen days after RR (*day 20*), mice were removed from their home cages and 15 glass marbles, evenly arranged in 5 columns of 3, were placed in the cages. The mice were then returned to their home cages. After 30 minutes, the mice were removed and the number of marbles at least 2/3 buried in the bedding was recorded. The marbles were only handled with gloves.

Light-Dark Box: The light-dark apparatus consisted of a 25.4 X 25.4 cm chamber with 40 cm high clear acrylic walls and a black insert chamber (Coulbourn Instruments, Allentown, PA) occupying half of the area. Mice were allowed access to the dark area via a small hole cut into the side of the insert. The floor of the chamber not covered by the insert was painted white and illuminated by bright, direct lighting. Mice were exposed to the light-dark box either 19 or 20 days (*day 21* or *23*) after RR. On the day of testing mice were placed in the white chamber of the light-dark box facing the opening to the dark chamber and activity was recorded for 10 minutes. Behavior was scored for number of entries into the dark chamber, total time in either chamber, and distance moved in the light chamber. Immediately before placement in the light-dark box and 25 minutes after removal, a small amount of blood was collected from the tail for measurement of serum corticosterone concentration.

Statistical Analysis

Body weight and food intake were compared using repeated measure ANOVA (Statistica, Stat Soft, Tulsa OK). Baseline body weight and food intake were used as covariates to determine changes between groups. Two-way ANOVA was used to analyze data from the behavioral

observations as well as measurements of serum corticosterone. For corticosterone concentration, concentration at time 0 was used as a covariate. Duncan's Multiple Range test was used for *post hoc* comparisons between groups. Differences were considered significant at $P<0.05$.

RESULTS

RR mice lost weight on the days of restraint (Fig. 3.1; Stress: $P<0.00007$, Day: $P<0.0000001$). Following RR, RR mice gained weight at the same rate as controls, however, they had not returned to the weight of the controls 6 days after the end of restraint. RR mice exhibited a significant reduction in food intake only on the days of stress, with intake returning to pre-stress levels immediately after the end of restraint (Fig. 3.2; $P<0.004$). The intake of the control animals was stable throughout the experimental period.

A 10 minute exposure to the defensive withdrawal apparatus 5 or 6 days after RR revealed no significant difference between RR mice and controls in any of the anxiety-type behaviors measured (data not shown). In comparison with controls, serum corticosterone concentration of RR mice was significantly elevated 25 minutes after removal from the defensive withdrawal apparatus (Control: 100 ± 5 ng/ml, RR: 123 ± 9 ng/ml).

A 10 minute exposure to the EPM 9 or 12 days after RR revealed a significant decrease in the number of entries made into the open arms (Fig. 3.3: $P<0.03$) as well as a significant increase in the time spent in the closed arms (Fig. 3.4: $P<0.004$) in RR mice. A decrease in the distance moved and the total time spent in the open arm of the maze were observed, but the values did not reach significance ($P<0.08$, data not shown). MS immediately before placement in the maze significantly decreased the number of entries control-MS mice made into the open arm of the maze (Fig. 3.3: $P<0.03$).

There was a significant interaction between RR and MS on the time that mice spent in the closed arms of the maze (RR: $P<0.1$, MS: $P<0.2$, RR X MS: $P<0.04$). MS increased the amount of time that control animals spent in closed arms but had no effect on RR mice because the amount of time the RR-control mice spent in the arms was the same as that of contol-MS and RR-MS mice (Fig. 3.4). There were no differences in serum corticosterone levels of the different groups before they were exposed to the EPM but 25 minutes after being in the maze, corticosterone was significantly higher in both RR-control and RR-MS groups compared with control-control mice (Fig. 3.5: RR: $P<0.05$, MS: $P<0.4$, RR X MS: $P<0.3$).

Marble-burying behavior 17 days after RR revealed no significant difference in the number of marbles buried in 30 minutes by RR mice compared with controls. RR mice buried 8 ± 1 out of 15 marbles, whereas control mice buried 7 ± 1 marbles.

In the light-dark box, RR mice exhibited a significant increase in the number of entries made into (Fig. 3.6: $P<0.006$) and the duration of the time spent in (Fig. 3.7: $P<0.03$) the dark chamber of the box. Serum corticosterone concentrations was elevated in RR mice immediately before testing (Fig. 3.8: $P<0.05$) and was increased in all mice 25 minutes after exposure to the light-dark box, however, there was no significant difference between groups.

DISCUSSION

Previous studies have demonstrated that rats and mice exposed to RR show an exaggerated endocrine response to a subsequent novel MS administered 12 or 14 days after RR (Harris et al., 2004). Although RR animals exhibit no change in the daily nadir of corticosterone release, exposure to a novel MS in the post-stress period evokes an elevated release of corticosterone. The objective of this study was to determine whether RR induces long-lasting

changes in other aspects of the stress response, particularly those that can be determined through behavioral measures of anxiety. A limited amount of information exists on acute stressors affecting behavior for lengths of time comparable to the periods of time observed in this experiment. Enhanced anxiety-type behavior in the open field apparatus has been recorded in rats as many as 14 days after a single session of inescapable footshock (Adamec and Shallow, 1993) and a 5 minute exposure to a cat has been reported to result in reduced exploratory behavior in the EPM 21 days later (Van Dijken et al., 1992). These observations are rare, and no comparable periods of time have previously been tested following exposure to restraint stress.

Behaviors indicative of anxiety in the EPM, the light-dark box, and the defensive withdrawal apparatus are based on the natural tendency of the rodent to explore a novel environment and avoid brightly illuminated, open spaces (Onaivi and Martin, 1989). A marked preference for the dark, enclosed areas in these arenas is indicative of enhanced behavioral anxiety. EPM behaviors considered to correlate with anxiety are the number of entries into the open arms of the maze and the time spent on these arms (Hogg, 1996). RR mice exhibited a decrease in the number of entries into the open arms of the maze as well as an increase in the amount of time spent in the closed arms when compared to controls. These results are consistent with others who reported that a period of acute restraint increases measures of behavioral anxiety in the EPM (Harris et al., 2002a; Harris et al., 2001; Morilak et al., 2003). However, intervals between stress and behavior testing in these previous experiments ranged from immediately after removal from the restraining apparatus to 30 minutes following restraint, contrasting with the 9 days used in this experiment. Similarities in the exploratory behavior of RR-control and RR-MS mice suggest that exposure to the experimental arena alone functioned as stressor and was sufficient to elicit increased anxiety-like behavior in RR mice.

In the light-dark box, a decrease in transitions between the light and dark chambers without a decrease in spontaneous locomotion is considered to be indicative of increased anxiety-like behavior (Costall, et al., 1989; Dailly, et al., 2002; Jones, et al., 1988). In this experiment, RR mice made a significantly greater number of entries into the dark chamber of the box and spent more time within the dark chamber than their non-restrained controls. Previous studies have demonstrated that 12 and 30 minute periods of restraint enhance anxiety-like behaviors of mice in the light-dark box, corroborating the findings of this experiment (Harris et al., 2001; MacNeil et al., 1997). It should be noted that, unlike previous tests, the baseline corticosterone concentration of restrained mice was elevated in comparison with controls before testing in the light-dark box. Both the HPA axis and anxiety are mediated by corticotropin-releasing factor (CRF) 1 receptors (Deak, et al., 1999; Gutman, et al., 2003; Timpl, et al., 1998). Therefore, it is possible that the condition of HPA axis activation resulted in increased anxiety-like behavior in the light-dark box.

In contrast with results obtained in the EPM and the light-dark box, exposure to the defensive withdrawal apparatus did not result in a difference in the behavior of RR and control mice. We have reported that a single 15 or 30 minute session of restraint increased anxiety-like behaviors measured in the defensive withdrawal apparatus (Harris et al., 2001; Smagin et al., 1996). Because the premise behind the anxiety behaviors in the defensive withdrawal apparatus is similar to that of the EPM and light-dark box it is surprising that no differences in behavior of RR and control mice were observed. There are a number of possible explanations for these results. Previous studies reporting an exaggeration of anxiety-like behavior after restraint measured behavior immediately after the end of restraint (Harris et al., 2001; Smagin et al., 1996), whereas observations for this study were made 5 to 6 days after restraint and

measurements of behavior in the defensive withdrawal test may only be sensitive to enhanced anxiety within a certain amount of time in the post-stress period. In addition, in this study the mice were initially placed into the dark chamber of the apparatus whereas in the light-dark box, they were placed in the light chamber. Costall et al. (1989) found that mice initially placed in the dark chamber of the light-dark box made fewer transitions from the dark chamber to the light chamber and exhibited a longer latency upon first exit from the dark chamber (Costall et al., 1989). Therefore it is possible that the defensive withdrawal would have detected different behaviors in control and RR mice if the test had been initiated with the mice in the open area of the apparatus.

Although RR mice exhibited an enhanced endocrine response to defensive withdrawal testing in comparison with controls, behavioral measures in the apparatus did not reflect this aspect of increased responsiveness. This result is not entirely surprising, as the release of glucocorticoids is primarily mediated by HPA axis activation and behavioral anxiety is thought to be mediated by the noradrenergic nuclei, including the locus ceruleus (LC) (Carrasco and Van de Kar, 2003). CRF, acting as a neurotransmitter, is believed to mediate activation of the LC through synaptic connections between CRF-containing terminals and LC dendrites (Van Bockstaele, et al., 1996). This belief is supported by evidence that CRF administered directly into the LC increases LC firing rates (Curtis, et al., 1997) while CRF receptor antagonists injected into the LC attenuate CRF-induced LC activation (Curtis, et al., 1999). Recent studies have shown that endogenous opioids may antagonize noradrenergic regulation by CRF and play a role in stress termination (Curtis, et al., 2001; Valentino and Van Bockstaele, 2001). These endogenous opioids may be responsible for the down-regulation of LC activation and the attenuation of anxiety-type behavior in the defensive withdrawal apparatus. Therefore, it is

possible that exposure to the defensive withdrawal apparatus resulted in increased HPA axis activation in restrained mice without enhancing anxiety-like behaviors measured by the test.

RR mice and control mice buried approximately the same number of marbles during the marble-burying test. The discrepancy between the observation of anxiety-like behavior in the EPM and the light-dark box and lack of anxiety-like behavior in the marble-burying test is most likely due to a difference in the type of anxiety measured by the different tests. While the EPM and the light-dark box base measurements of anxiety on an aversion to illuminated areas, the marble-burying test measures fear of a novel, invasive object. Burial of both noxious (Koolhaas, et al., 1999; Pinel, et al., 1994) and harmless (De Boer and Koolhaas, 2003; Njung'e and Handley, 1991) materials represents a perceived reduction in the potential threat posed by the material. The neural pathway controlling the type of anxiety measured by the EPM and light-dark box is believed to be independent of the pathway controlling marble-burying behavior (Treit, et al., 1993). Thus, it is possible that RR influences certain aspects of anxiety behavior while not influencing others.

Alternatively, it has been suggested that burying behavior is indicative of individualized characteristics such as aggression level or compulsion, which are most likely not affected by RR. Koolhaus et al. (1999) reported a positive correlation between the level of aggression in rats, determined by a decrease in latency time to attack an intruding male in a resident-intruder test, and the amount of time spent burying a prod which delivered electric shocks (Koolhaas et al., 1999). The number of marbles buried during the marble-burying test has also been shown to be reduced by selective serotonin re-uptake inhibitors (Ichimaru, et al., 1995; Takeuchi, et al., 2002) which are commonly used for the treatment of obsessive compulsive disorder in humans (Fallon and Mathew, 2000). Additionally, Njung'e and Handley (1991) found no role for novelty or

anxiety in burying behavior. They reported that groups of mice, when presented with marbles for 5 consecutive days or 5 times in a single day, buried the same number of marbles at each presentation. This lack of habituation to marbles suggests that burying behavior is compulsive. Given this evidence, it is not surprising that the number of marbles buried did not differ between control and RR mice.

In addition to demonstrating enhanced anxiety-like behavior in RR mice, the study described here confirmed our previous observations that mice exposed to 2 hours of restraint for 3 consecutive days lost weight and reduced food intake during the 3 days of restraint (Harris et al., 2001). Following restraint, food intake returned to the level of controls. In contrast, RR mice gained weight at the same rate as controls but did not return to the weight of the controls during the 6 days on which body weight was measured. In addition we found that, compared with controls, RR mice experienced a significant elevation of corticosterone following exposure to some of the behavioral tests while no differences were found in baseline corticosterone concentrations. This suggests that the behavioral tests were stressful and that the RR mice were hyper-responsive to this stress. Although we found no differences in baseline corticosterone of control and RR mice at *day 15*, baseline serum corticosterone was significantly elevated in RR mice on *day 21*. Chronically stressed animals exhibit a continuous activation of the HPA axis (Vernikos, et al., 1982), suggesting that RR mice in this study became chronically stressed, as they were exposed to a series of novel stressors during the behavioral measures. It is important to note that non-restrained controls did not exhibit the same chronic elevation in glucocorticoid levels on *day 21*, which suggests that manipulations in this experiment may have been perceived by RR mice as unpredictable stressors, eventually resulting in a state of chronic endocrine activation.

In conclusion, the results of this experiment demonstrate that exposure to RR results in a sustained sensitivity to mild stressors in the post-restraint period. This exaggeration is evident in increases in measurements of endocrine response such as corticosterone concentration and in increases in anxiety-type behavior. Because enhanced anxiety-like behavior is observed as many as 20 days following the end of restraint these results indicate that exposure to acute stress can induce long-lasting changes in the responsiveness of a variety of neuroendocrine systems.

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FIGURES

Figures 3.1 and 3.2: Daily body weight from the day before RR to *day 8* (3.1) and average food intake for 3 consecutive days before, after, and during RR (3.2). Values are means ± S.E.M. for 16 mice. Astericks indicate significant differences between control and restrained animals ($P<0.05$) and food intake values in a specific group of days that do not share a common superscript are significantly different at $P<0.002$.

Figures 3.3-3.5: The number of entries into (3.3) and the duration of time in (3.4) the arms of the EPM after an i.p. injection of 0.2mL saline. Serum corticosterone measured immediately before placement into (0) and 25 minutes after (25) the end of a 10 minute exposure to the EPM (3.5). Mice were subjected to RR 9 or 12 days before the 10 minute exposure to the maze. Data for the open arms and the closed arms were analyzed independently of one another. Values for a specific behavior segment that do not share a common superscript are significantly different at $P<0.05$. Data are means ± S.E.M. for 4 groups of 8 mice.

Figure 3.6-3.8: The number of entries into the dark chamber (3.6) and the total amount of time spent in either chamber (3.7) of the light-dark box during a 10 minute exposure. Behavior in the light-dark box was measured 19 or 20 days after RR. Serum corticosterone measured immediately before placement into (0) and 25 minutes after removal from (25) the light-dark box (3.8). Values for the two chambers were analyzed independently of one another. Behavior values within a specific segment that do not share a common superscript are significantly different at $P<0.006$. Corticosterone values that do not share a common superscript are significantly different at $P<0.05$. Data are means ± S.E.M. for 16 mice.

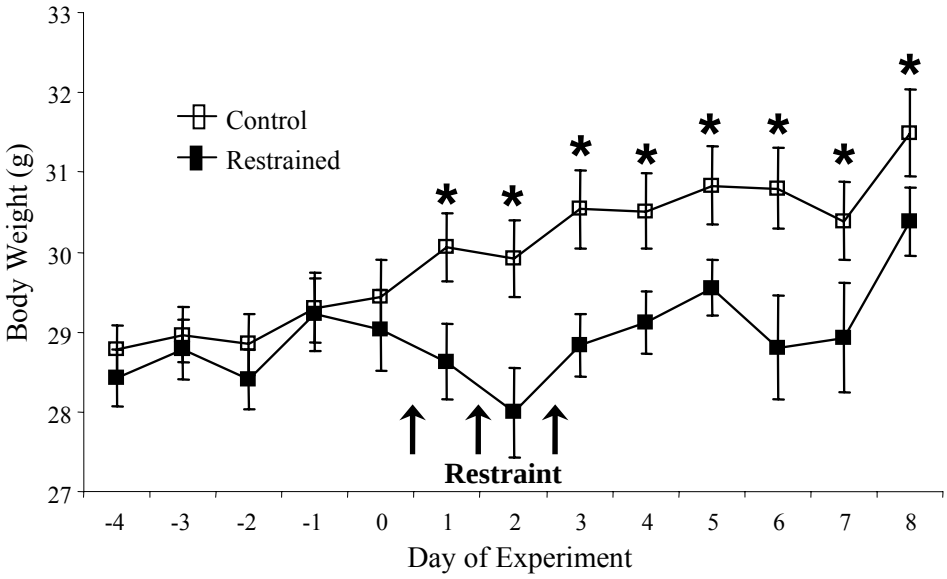

Figure 3.1: Daily body weight from the day before RR to *day 8*

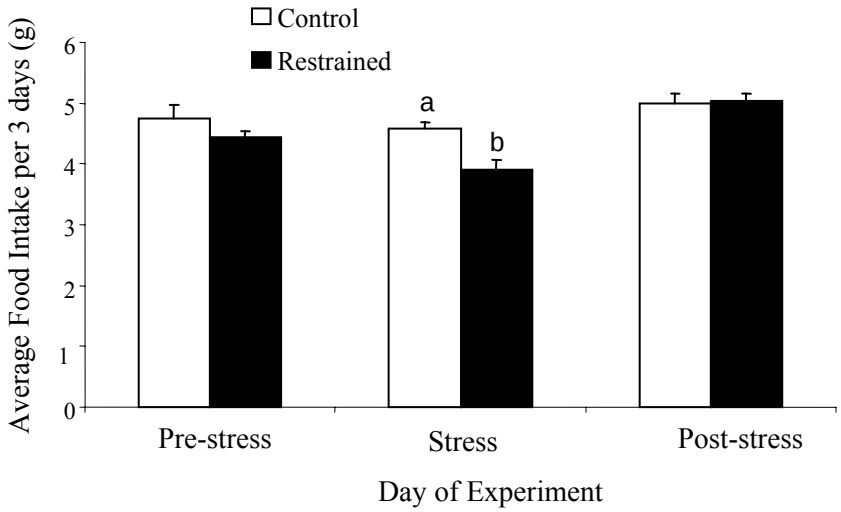

Figure 3.2: Average food intake for 3 consecutive days before, after, and during RR

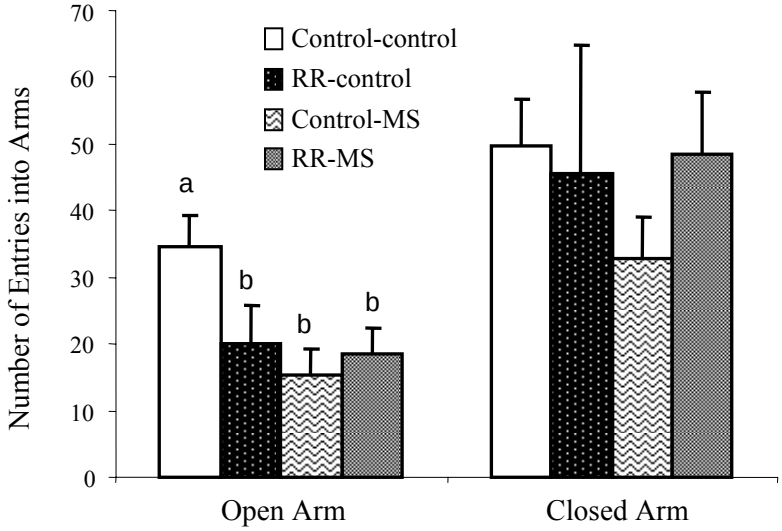

Figure 3.3: The number of entries into the arms of the EPM after an i.p. injection of 0.2mL saline

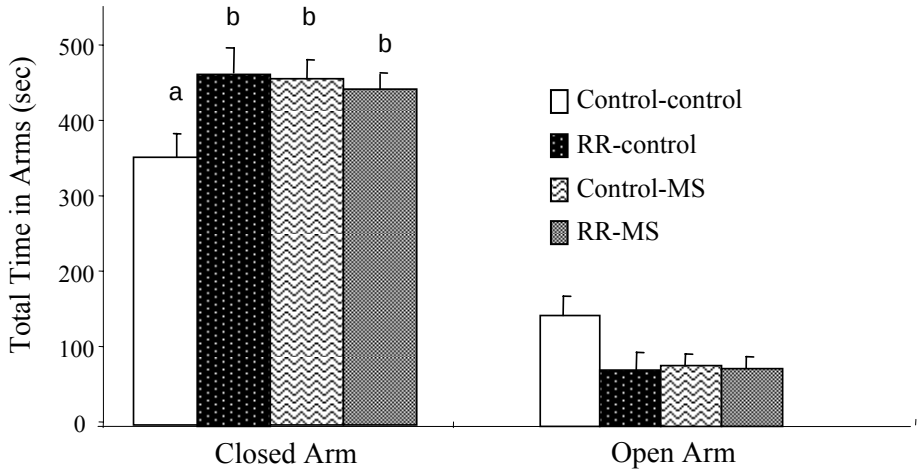

Figure 3.4: The duration of time in the arms of the EPM after an injection of 0.2mL saline

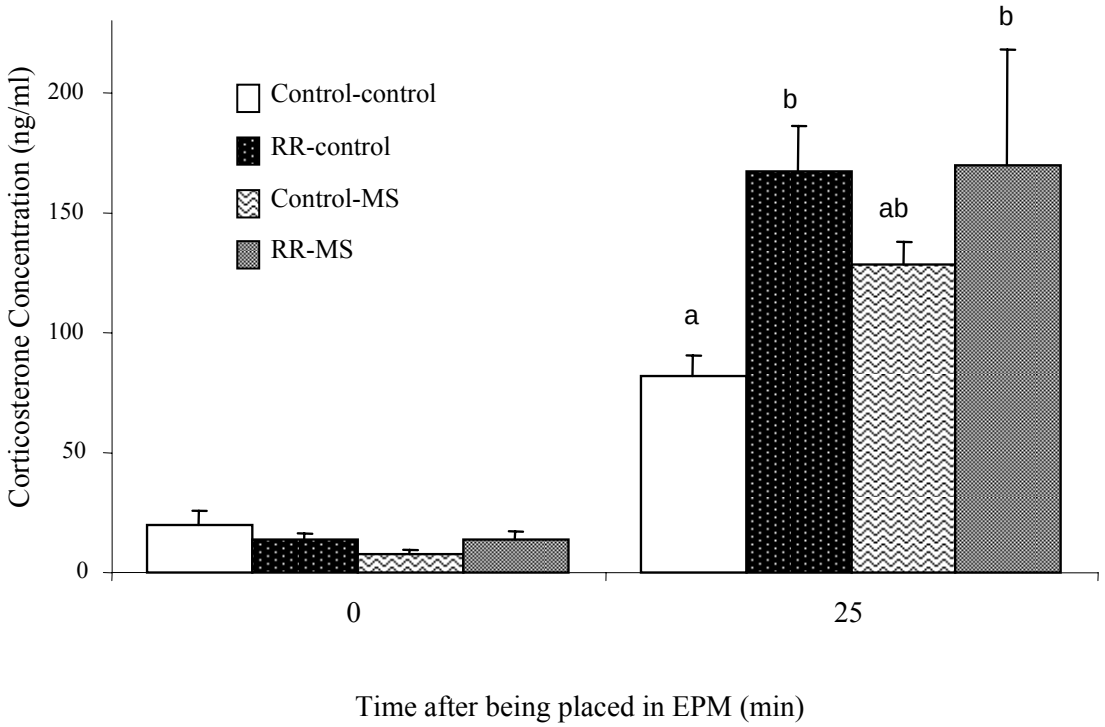

Figure 3.5: Serum corticosterone measured immediately before placement into (0) and 25 minutes after (25) the end of a 10 minute exposure to the EPM

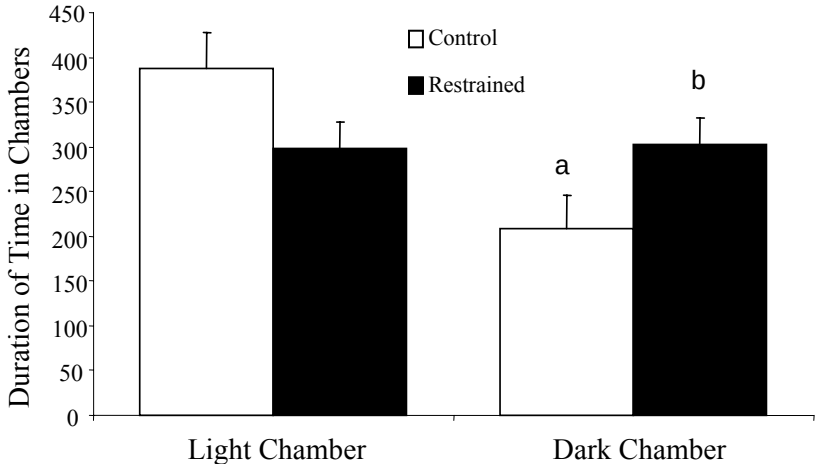

Figure 3.6: The number of entries into the dark chamber of the light-dark box during a 10 minute exposure.

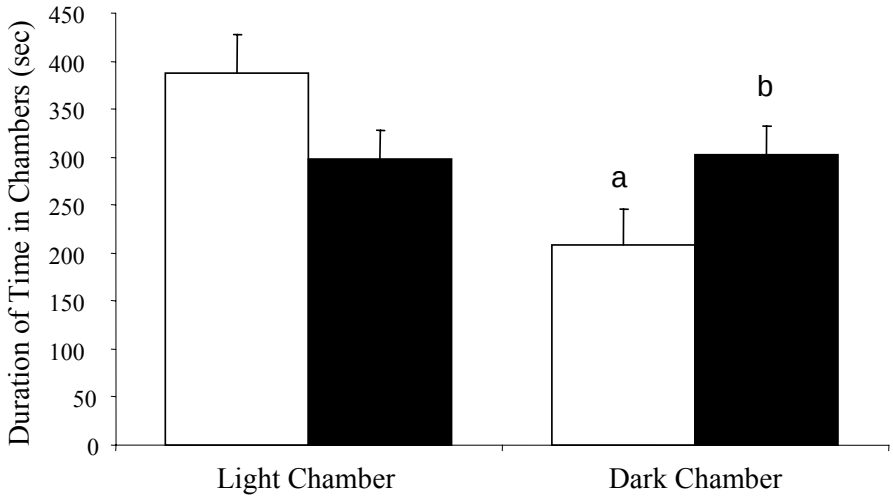

Figure 3.7: The total amount of time spent in either chamber of the light-dark box during a 10 minute exposure

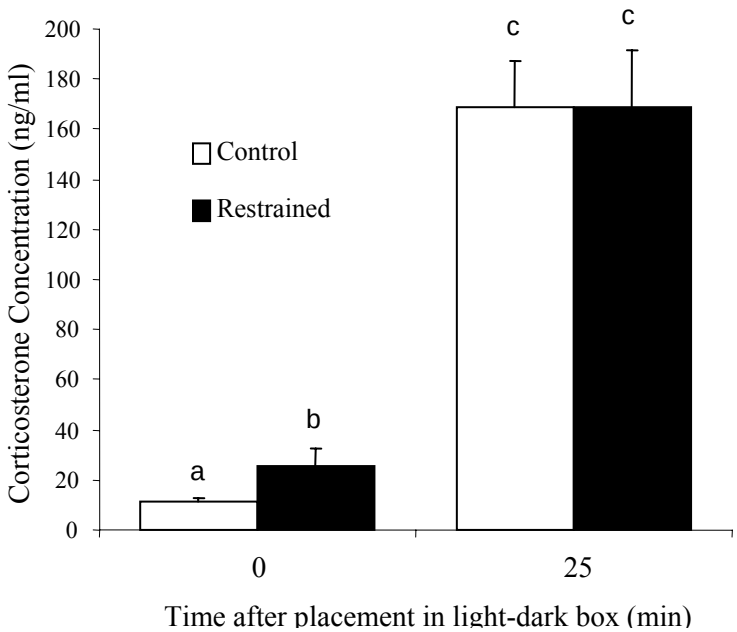

Figure 3.8: Serum corticosterone measured immediately before placement into (0) and 25 minutes after removal from (25) the light-dark box

CHAPTER 4

ANTAGONISM OF CRF RECEPTOR 1 OR 2 IN AREAS ADJACENT TO THE THIRD VENTRICLE DURING REPEATED RESTRAINT ALTERS STRESS RESPONSE IN RATS[2]

[2] Chotiwat, C. and Harris, R.B.S. *To be submitted to the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*

ABSTRACT

Exposure to 3 hours of restraint stress on each of 3 consecutive days (repeated restraint, RR) results in enduring changes in weight regulation and neuroendocrine responses to subsequent stressors. Rats lose weight in response to RR and, although they gain weight at the same rate as non-restrained controls in the post-stress period, never return to the weight of controls. Additionally, RR rats exhibit exaggerated corticosterone release in response to novel stressors in the post-stress period. The mechanisms behind these RR-induced changes are unknown. Activation of corticotropin-releasing factor receptors (CRFR) mediate many of the body's responses to stress. The response to the stressor is dependent upon the type and location of the CRFR activated. Studies described here tested the effects of specific antagonism of either CRFR1 or CRFR2 on RR-induced weight loss, hypophagia and hyperreactivity to subsequent stressors applied in the post-stress period. The results of these experiments suggest that central, but not peripheral, antagonism of CRFR1 during RR attenuates RR-induced weight loss while central CRFR2 antagonism prevents RR-induced hypophagia, but not weight loss. CRFR activation appears to be partially responsible for the hyperresponsiveness of the endocrine system in the post-RR period. Finally, results from these studies suggest that CRFR1, but not CRFR2, activation is necessary to induce the long-term changes in body weight regulation observed in RR animals and that hypophagia on the days of stress is not required for the sustained reduction in body weight of the stressed rats.

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INTRODUCTION

Exposure to a stressor results in a myriad of responses including modification of endocrine, sympathetic, metabolic, immunologic, and behavioral function. These stress-induced changes are essential, as they initiate the immediate reaction to stress as well as facilitate the return to homeostasis once the stress has ended. Corticotropin-releasing factor (CRF) is the primary initiator of many of the physiological and behavioral responses to stress (59). CRF and its homologues urocortin I (Ucn I), Ucn II, and Ucn III induce responses to stress through activation of CRF receptors (CRFR) (37, 48, 59). CRFR can be divided into two types, CRFR1 and CRFR2, which are differentially expressed in sites throughout the brain and body. Different aspects of the stress response are dependent upon the type and location of the CRFR activated. Hypothalamic-pituitary-adrenal (HPA) axis activation as well as stress-induced behaviors indicative of anxiety are associated with activation of CRFR1 (4, 47, 56-58, 60) while CRFR2 activation is associated with stress-induced changes in feeding behavior (12, 32, 44, 55).

Previous studies have shown that rats experience a significant reduction in body weight in response to 3 hours of restraint on each of 3 consecutive days (repeated restraint, RR) (26, 30, 54). Following RR, restrained rats gain weight at the same rate as non-restrained controls, however, they fail to compensate for the weight that was lost during the days of restraint and never return to the weight of controls. This sustained weight reduction of 5-15% compared with non-restrained controls has been observed as many as 80 days after the end of RR (28). Weight changes induced by environmental factors such as food restriction or overfeeding are readily reversed by removal of the factor that is disrupting energy balance (23). Thus, the sustained reduction in weight exhibited by rats exposed to RR makes this a unique model of weight loss. Although the effects of RR are enduring, RR is an acute, rather than a chronic, stressor.

Repeatedly restrained rats do not show the same increase in adrenal and decrease in thymus weight that is exhibited by chronically stressed rats (1, 22). Additionally, chronically stressed animals exhibit a chronic elevation of baseline serum corticosterone levels (2). In the case of RR, as early as the second hour of restraint, corticosterone concentration of restrained rats returns to control levels and 12 days after the end of RR, the diurnal pattern of corticosterone release is not significantly different from that of controls (31).

Although the initial stress-induced weight loss can be attributed to decreased food intake and increased energy expenditure during the 3 days of restraint, these factors do not account for the chronic reduction in weight (27). RR inhibits food intake on the days of restraint and 3 to 5 days immediately following restraint. The intake of restrained rats returns to control levels within a week of the final day of restraint (24). Energy expenditure returns to control levels within hours of the end of restraint (28). The return of energy expenditure and food intake to control levels without compensation for the decrease in body weight following RR indicates that the pathways activated by RR must induce some change in the mechanisms that normally regulate body weight.

A sustained reduction in bodyweight is not the only chronic effect of RR. Although baseline serum corticosterone levels and the daily nadir of corticosterone release remain normal, RR animals exhibit enhanced secretion of glucocorticoids when challenged by a novel mild stressor in the post-stress period (22, 31). This effect is not unique to RR, as others have reported increased sensitivity to subsequent stressors following an acute stress. For example, rats exposed to one or three daily sessions of tail or foot shock show an exaggerated corticosterone response to the same stressor applied 10 days later (9, 51). The increased sensitivity of the endocrine response to novel stressors in the post-stress period has been

associated with an impaired ability of the glucocorticoids to suppress hypothalamic-pituitary-adrenal (HPA) axis activity (8) and a lowering of the threshold for the initiation of endocrine responses to stress (22). In addition to enhanced endocrine sensitivity, studies have shown that RR animals exhibit increased anxiety-like behavior in three well-established behavior tests; the elevated plus maze (EPM), the defensive withdrawal paradigm, and the light-dark box, when behavior is measured immediately after the end of the restraint stress (25, 29, 39, 42, 52). This increase in anxiety-like behavior is long-lasting, as mice exposed to RR display increases in behavior indicative of anxiety in the EPM and the light-dark box as many as 12 and 20 days, respectively, after the end of RR (11).

We have previously shown that simultaneous antagonism of both types of CRFR adjacent to the third ventricle during RR prevents stress-induced reductions in body weight and food intake (54). The experiments described here were performed to determine whether specific antagonism of CRFR1 or CRFR2 in areas adjacent to the third ventricle would modulate the response to RR. We hypothesized that antagonism of both types of CRFR during RR would prevent the sustained reduction in bodyweight as well the HPA axis hyperreactivity to subsequent novel stressors. Additionally, we hypothesized that the response to RR would differ with the type of CRFR antagonized during RR.

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.

Experiment 1: Subcutaneous injection of NBI 27914, an antagonist of CRFR1, before repeated restraint followed by administration of a novel mild stress 12 days later

Results from a previous study indicated that αhCRF$_{(9—41)}$, a nonselective CRFR antagonist, injected into the third ventricle immediately before RR completely attenuated RR-induced decreases in body weight and food intake (54). A second study indicated that rats exposed to RR exhibited a greater release of corticosterone when exposed to a novel mild stressor in the post-stress period than non-restrained controls (22). The objective of Experiment 1 was to determine whether specific antagonism of CRFR1 immediately before exposure to RR would alter RR-induced changes in food intake, body weight, or hyperreactivity to a subsequent stressor.

Experimental Protocol: Eighty male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with body weights of approximately 320 g were individually housed in hanging wire cages with free access to tap water and standard rat chow (LabDiet 5012, PMI Nutrition International, Brentwood, MO) in a humidity (52%) and temperature-controlled (22.7°C) room on a 12:12 hour light:dark cycle. After 7 days of baseline food intake and body weight measures, the rats were divided into 4 weight-matched groups (n=20): vehicle control (V/C), vehicle restraint (V/RR), NBI 27914 control (NBI/C) and NBI 27914 restraint (NBI/RR). On each of the following three days (*days 1-3*), beginning at 9AM, rats were exposed to three hours of restraint (RR) or were non-restrained controls (C). Thirty minutes before the onset of restraint on each day of restraint rats received a subcutaneous injection of 0.2ml dimethyl sulfoxide (DMSO) (V/C and V/RR) or 11.5 nmol/kg NBI 27914 (Sigma Aldrich, St. Louis, MO) dissolved in 0.2ml DMSO (NBI/C or NBI/RR). Results of a previous study indicated that 11.5 nmol NBI 27914 delivered subcutaneously before a 20 minute period of restraint inhibited increases in

anxiety-like behavior measured immediately after restraint (53). Restrained rats were placed in Perspex restraining tubes (21.6 x 6.4 cm, Plas Labs, Lansing MI) and placed in an experimental room for 3 hours. Control rats were placed in shoebox cages in the same experimental room. All groups were denied access to food or water during the 3 hours of restraint stress. On the second day of RR, blood was collected by tailbleeding from all rats immediately prior to infusion (baseline) and 60 minutes after the onset of RR (RR rats) or placement in control cages (C rats) for measurement of serum corticosterone concentration. At the end of restraint the rats were returned to their home cages. Food intakes and body weights were monitored on the days of RR and for 12 days following the end of RR. Twelve days after the final day of restraint (*day 15*) each group of rats was subdivided into 2 weight-matched groups (n=8), with one subgroup receiving a novel mild stress (MS) and half receiving no stress (C): V/C-C, V/C-MS, V/RR-C, VRR-MS, NBI/C-C, NBI/C-MS, NBI/RR-C, and NBI/RR-MS. Beginning at 9AM on *day 15*, a small tail blood sample (Time 0) was collected for basal measurement of serum corticosterone concentration. Immediately following blood collection, MS rats were given a 1ml ip injection of sterile saline and placed in a shoebox cage in an experimental room. C rats were given no injection and were returned to their home cages. At 15, 30, 60, 90, and 120 min after administration of MS or return to home cage, additional blood samples were collected by tail bleeding for measurement of serum corticosterone concentration as described below.

Experiment 2: Third ventricle infusion of antalarmin, a CRFR1 antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

The results of Experiment 1 suggested that peripheral administration of NBI 27914 did not alter the chronic effects of RR. Additionally, blockade of CRFR1 using NBI 27914 did not change RR-induced corticosterone release. Experiment 2 was performed to determine whether central blockade of CRFR1 using a different CRFR1 antagonist, antalarmin, would affect the response to RR.

Surgery: Fifty-six male Sprague-Dawley rats were housed as described in Experiment 1. Each rat was implanted with a 26 gauge guide cannula (Plastics One, Roanoke, VA) aimed at the third ventricle (anteroposterior -2.8mm, lateral 0.0mm, ventral -8.3mm relative to bregma) using stereotaxic techniques and coordinates based on The Paxinos and Watson Rat Brain Atlas (46). The cannulae were anchored to the skull with jeweler's screws and secured with dental acrylic cement. Rats were allowed 7 days to recover from surgery.

Cannula Placement Verification: Following recovery from surgery, rats were infused icv with 20ng angiotensin II in 2µl of sterile saline. Rats that drank water within 5 minutes of infusion were included in the experiment. Rats were given approximately 2 days to recover before the experiment started.

Experimental Protocol: RR was administered in the same manner as described in Experiment 1. The rats were divided into 4 weight-matched groups (n=14): vehicle control (V/C), vehicle

restraint (V/RR), antalarmin control (Ant/C) and antalarmin restraint (Ant/RR). Thirty minutes before the start of restraint on each day of RR rats received an icv infusion of 2µl DMSO (V/C and V/RR) or 5 nmol antalarmin (Sigma Aldrich, St. Louis, MO) dissolved in 2µl DMSO (Ant/C or Ant/RR) over 2 minutes and returned to their home cages. On the second day of RR, blood was collected by tail bleeding from all rats immediately before infusion (Time 0) and 60 minutes after the start of RR (RR rats) or placement in control cages (C rats) for measurement of serum corticosterone concentration. Following RR, food intake and body weights were recorded for 12 days. Twelve days after RR (*day 15*), rats in each treatment group were divided into two subgroups; control or MS (n=7). MS rats were exposed to MS as described in Experiment 1 while control rats remained in their home cages. Blood was collected immediately before MS (Time 0) and at 30, 60 and 90 minutes after MS for measurement of serum corticosterone concentration.

Experiment 3: Third ventricle infusion of antisauvagine-30, a CRFR2-specific antagonist, before repeated restraint

Activation of CRFR2 play a role in mediation of stress-induced hypophagia (13, 32), therefore, Experiment 3 was performed to determine whether antagonism of CRFR2 immediately before RR would alter RR-induced decreases in food intake.

Experimental Protocol: Twenty-eight male Sprague-Dawley rats were housed as described in the previous experiments. Cannulae were implanted in the third ventricle, cannula placement verified, and baseline food intake and body weights were recorded for 7 days as described in

Experiment 1. RR was administered in the same manner as described in the previous experiments. The rats were divided into 4 weight-matched groups (n=7): vehicle control (V/C), vehicle restraint (V/RR), antisauvagine-30 control (ASV/C) and antisauvagine-30 restraint (ASV/RR). Thirty minutes before the start of restraint on each day of RR rats received an icv infusion of 2µl sterile saline (V/C and V/RR) or 2.5 nmol antisauvagine-30 (Sigma Aldrich, St. Louis, MO) dissolved in 2µl sterile saline (ASV/C or ASV/RR) over 2 minutes. Results of a previous study indicated that 2.5 nmol of the non-selective CRFR antagonist αhCRF injected into the third ventricle attenuated RR-induced weight loss (54), therefore 2.5 nmol was chosen as the dose for ASV-30. Blood was collected by tail bleeding 60 minutes after the beginning of restraint on *days 1* and *3* for measurement of serum corticosterone concentration. Following RR, food intake and body weights were recorded for 7 days. Rats in Experiment 3 were not exposed to MS, as HPA axis activation is primarily controlled by CRFR1 activation rather than CRFR2 activation (4).

Experiment 4: Third ventricle infusion of αhCRF$_{(9-41)}$, a non-specific CRFR antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

Experiment 2 indicated that central infusion of a CRFR1 antagonist immediately before RR prevented the sustained reduction in body weight exhibited by RR rats while Experiment 3 suggested that central antagonism of CRFR2 attenuateed RR-induced hypophagia. This experiment was performed to test whether antagonism of all CRFR in areas adjacent to the third

ventricle during RR would block the hyperreactivity of the HPA axis to subsequent stressors, RR-associated weight loss, and RR-induced hypophagia.

Experimental Protocol: Sixty-four male Sprague-Dawley rats were housed as described in the previous experiments. Experimental design was the same except that the receptor antagonist infused was αhCRF, which blocks both CRFR1 and CRFR2. Following recovery from cannula placement verification, baseline daily food intakes and body weights were recorded for 7 days. Rats were then divided into 4 weight-matched groups (n=16): vehicle control (VC), vehicle restraint (VRR), αhCRF control (αhCRF/C), and αhCRF restraint (αhCRF/RR). RR was administered in the same manner as described in the previous experiments. Thirty minutes prior to the onset of restraint on each day of restraint rats received an icv infusion of 2µl of sterile saline over 2 minutes (VC and VRR) or 2.5 nmol $\alpha hCRF_{(9—41)}$ (Sigma Aldrich, St. Louis, MO) in 2µl sterile saline over 2 minutes (αhCRF/C and αhCRF/RR) and were returned to their home cages. Following RR, food intake and body weights were recorded for 12 days. Twelve days after RR (*day 15*), rats in each treatment group were divided into two subgroups; control or MS. MS rats were exposed to MS as described in the previous experiments while control rats remained in their home cages. Blood was collected immediately before MS (Time 0) and at 15, 30, 60, 90, and 120 minutes after MS for measurement of serum corticosterone concentration.

Experiment 5: Third ventricle infusion of astressin, a non-specific CRFR antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

The results from Experiment 4 failed to duplicate the results of a previous study which indicated that third ventricle infusion of αhCRF$_{(9—41)}$ immediately before RR attenuated stress-induced weight loss and hypophagia (54). As this outcome was unexpected, this experiment was performed to determine whether the results of Experiment 4 could be replicated using a different non-specific CRFR antagonist, astressin.

Experimental Protocol: Fifty-six male Sprague-Dawley rats were housed as described in the previous experiments. Experimental design was the same as in Experiment 4 except that the receptor antagonist was astressin. RR was administered in the same manner as described in the previous experiments. The rats were divided into 4 weight-matched groups (n=14): vehicle control (VC), vehicle restraint (VRR), astressin control (Ast/C) and astressin restraint (Ast/RR). Thirty minutes prior to the onset of restraint on each day of restraint rats received an icv infusion of 2μl sterile saline (VC and VRR) or 5 nmol astressin (Sigma Aldrich, St. Louis, MO) dissolved in 2μl saline (Ast/C or Ast/RR) over 2 minutes. On the second day of RR, blood was collected by tail bleeding from all rats immediately prior to infusion (Time 0) and 60 minutes after the onset of RR (RR rats) or placement in control cages (C rats) for measurement of serum corticosterone concentration. Following RR, food intakes and body weights were recorded for 12 days. On *day 15*, 12 days after RR, the rats were exposed to MS as described in Experiment 1 with the exception that MS was administered to all rats. Blood was collected immediately before

MS (Time 0) and at 30, 60 and 90 minutes after MS for measurement of serum corticosterone concentration.

Blood Collection and Measurement of Serum Corticosterone Concentration

Rats were hand-held while a small piece (approximately 3mm) was snipped from the tail. The tail was then gently massaged to induce blood flow. Approximately 50µl of blood was collected from the tail for each time point measured. All blood samples were collected within 2 minutes of first handling the rat. Serum corticosterone concentration was then measured with a corticosterone RIA kit (MP Biomedicals, Irvine, CA).

Statistical Analysis

Food intake, change in body weight, and measurements of serum corticosterone after MS were compared using repeated measures ANOVA (Statistica, Stat Soft, Tulsa OK). Average food intake from the 3 days before RR and blood collected at Time 0 for measures of serum corticosterone concentration were used as covariates to determine changes between groups. Two-way ANOVA was used to analyze RR corticosterone data. Duncan's Multiple Range test was used for post hoc comparisons between groups. Differences were considered significant at $P<0.05$.

RESULTS

Experiment 1: Subcutaneous injection of NBI 27914, an antagonist of CRFR1, before repeated restraint followed by administration of a novel mild stress 12 days later

RR caused weight loss in all rats and RR rats continued to weigh less than controls from *day 1* to *day 13* of the experiment (Figure 4.1; RR: $P<0.001$, Day: $P<0.001$, RR X Day: $P<0.001$). Antagonism of CRFR1 with NBI 27914 had no significant effect on body weight of RR or control groups ($P<1.0$). Exposure to RR significantly suppressed food intake on the 3 days of RR as well as the average food intake on the 6 days immediately following RR but there was no significant effect of CRFR1 antagonism on food intake (Figure 4.2; Inj: $P<0.85$, RR: $P<0.001$, Inj X RR: $P<0.32$).

Serum corticosterone was increased in both Veh/RR and NBI/RR rats 60 minutes after the start of RR but CRFR1 antagonism had no significant effect on the increase in corticosterone concentration (data not shown). As expected, MS significantly increased corticosterone concentration (Figure 4.3; $P<0.001$) with a bigger response in RR-MS than C-MS rats (RR: $P<0.001$). Corticosterone concentrations were significantly higher in RR-MS than RR-C rats at 15, 30, and 60 minutes ($P<0.01$, MS: $P<0.001$, RR X MS: $P<0.02$). CRFR1 antagonism during RR had no significant effect on corticosterone concentrations in response to MS.

Experiment 2: Third ventricle infusion of antalarmin, a CRFR1 antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

Analysis of change in body weight revealed a significant decrease in body weight due to RR exposure beginning on *day 2* of the experiment and continuing through *day 10* (Figure 4.4; RR: $P<0.005$, Day: $P<0.001$, RR X Day: $P<0.001$). Ant/RR rats lost significantly more weight

than Veh/C and Ant/C rats on the days of RR (*days 1-3*) only. Beginning on *day 4* of the experiment, the body weight of Ant/RR rats did not differ from Veh/C rats although it remained significantly lower than Ant/C rats until *day 5*. Ant/RR rats weighed significantly more than Veh/RR rats from *day 9* until the end of the experiment. Veh/RR rats differed significantly from Veh/C and Ant/C rats following the first day of RR to the end of the experiment. The average food intake on days 1-3 of the experiment was decreased from pre-RR levels in all groups, presumably due to the stress of infusion. Exposure to RR further reduced the average food intake in Veh/RR and Ant/RR groups, but there was no significant effect of CRFR1 antagonism observed independent of RR (Figure 4.5; Inf: $P<0.92$, RR: $P<0.008$, Inf X RR: $P<0.74$).

When measured 60 minutes after the beginning of restraint on *day 2*, serum corticosterone concentration was significantly higher in RR than C rats (Figure 4.6: Inf: $P<0.012$, RR: $P<0.001$, Inf X RR: 0.97). Corticosterone concentration was also elevated above baseline levels in both C groups, but Veh/C rats exhibited greater increases in corticosterone release than Ant/C rats. Twelve days after the end of RR, MS increased serum corticosterone concentration in all rats and this was not changed significantly by previous exposure to RR or antalarmin infusion (data not shown). There were no differences in area under the curve of corticosterone concentration for any group in this experiment.

Experiment 3: Third ventricle infusion of antisauvagine-30, a CRFR2 antagonist, before repeated restraint

In this experiment all rats (Control and RR) lost weight on the first day of RR, but by *day 3*, the weight loss of rats exposed to RR was significantly greater than that of controls (Figure 4.7; RR: $P<0.002$, Day: $P<0.001$, RR X Day: $P<0.001$). Although ASV/RR lost less weight than

Veh/RR rats, the difference was not statistically significant. Food intake was significantly inhibited on the days of restraint compared with pre-stress levels for all rats but the inhibition was greater for Veh/RR rats than for any other group (Figure 4.8; Inf: P<0.40, RR: P<0.029, Inf X RR: P<0.69). There was no difference average food intake during the 3 days of RR of ASV/C and ASV/RR rats.

Rats exposed to RR exhibited significantly greater levels of serum corticosterone than C groups when measured 60 minutes after the beginning of restraint on *days 1* and *3* of the experiment. Infusion of antagonist had no effect on serum corticosterone concentrations (data not shown).

Experiment 4: Third ventricle infusion of αhCRF$_{(9—41)}$, a non-specific CRFR antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

While there was a significant overall effect of RR (P<0.04) on change in body weight on *days 1-10*, post hoc analysis revealed that RR groups did not experience a significantly greater weight loss than controls on any individual day of the experiment (data not shown; RR X Day: P<0.92). All rats lost weight during the period of RR, presumably because of the stress associated with infusion as well as movement to a new room during the period of RR. αhCRF/C rats lost significantly less weight than any other group on *day 1* of the experiment, however, there was no overall effect of CRFR antagonism (P<0.39) on the change in body weight. Average food intake for 3 consecutive days before, after, and during RR is shown in Figure 7A. Rats in all groups ate less during RR but there was no overall significant effect of CRFR antagonism on average food intake during the days of RR (Figure 4.9; Inf: P<0.60, RR: P<0.03, Inf X RR: P<0.37). The average food intake on the days of RR was less for αhCRF/RR rats than

αhCRF/C rats. Average food intake of all groups returned to pre-stress levels on the days immediately following RR.

Because there was no effect of RR on change in body weight, control and RR groups of rats were combined for analysis of area under the curve for serum corticosterone concentration. While there was no overall effect of antagonist infusion on corticosterone release (Figure 4.10; Inf: $P<0.11$, MS: $P<0.001$, Inf X MS: $P<0.12$), post hoc analysis shows that infusion of αhCRF on *days 1-3* significantly decreased area under curve of corticosterone concentration in rats exposed to MS ($P<0.05$).

Experiment 5: Third ventricle infusion of astressin, a non-specific CRFR antagonist, before repeated restraint followed by administration of a novel mild stress 12 days later

Rats exposed to RR lost more weight than control rats on the days of RR (*days 1-3*) and maintained a lower weight than controls in the post-stress period (*days 4-10*) (Figure 4.11; RR: $P<0.001$, Day: $P<0.001$, RR X Day: $P<0.001$). Infusion of the CRFR antagonist astressin immediately before RR had no effect on the change in body weight of RR rats, however, C rats infused with astressin weighed significantly less than Veh/C rats but significantly more than RR rats on *days 2-4* ($P<0.05$). Average food intake during the 3 days of RR dropped significantly from pre-stress levels for all groups except Veh/C rats (Figure 4.12; Inf: $P<0.001$, RR: $P<0.05$, Inf X RR: $P<0.42$). All groups infused with astressin reduced food intake equally on *days 1-3* of the experiment. Average food intake for rats in all groups returned to baseline levels in the 3 days immediately following RR.

Exposure to RR significantly increased serum corticosterone concentration when measured 60 minutes after the start of RR on *day 2*, however, CRFR antagonism had no

significant effect (data not shown; Inf: $P<0.70$, RR: $P<0.004$, Inf X RR: $P<0.85$). RR had no effect on corticosterone release in response to MS at any time point with the exception of 60 minutes after MS, when Veh/RR rats had significantly higher serum corticosterone levels than Veh/C and Ast/C rats (Figure 4.13; $P<0.027$). Area under the curve of corticosterone concentration was similar for all groups in this experiment (data not shown).

DISCUSSION

Exposure to RR, an acute stressor, results in chronic changes in rats including a sustained reduction in body weight and an enhanced sensitivity to mild stress applied in the post-RR period. Previous studies have shown that non-specific antagonism of central CRFR during RR completely blocked stress-induced weight loss and hypophagia (54). The experiments described here tested whether antagonism of CRFR1 or CRFR2, specifically, during RR would attenuate the effects of RR. The results help to elucidate the mechanism through which RR initiates its chronic effects on body weight and HPA axis hyperreactivity.

Experiments 1 and 2 tested the effects of specifically inhibiting CRFR1 during RR using two different CRFR1 antagonists, NBI 27914 and antalarmin. Neither antagonist prevented RR-induced hypophagia. This was not surprising, as stress-induced decreases in food intake are believed to be mediated primarily by CRFR2 (44, 55). In Experiment 2, infusion of antalarmin did not prevent RR rats from losing weight on *days 1* and *2* of RR, but by *day 3* of the experiment, RR rats infused with antalarmin no longer maintained a significantly reduced weight from control groups and, by *day 9*, RR rats infused with antalarmin had returned to the weight of controls. These results indicate that central antagonism of CRFR1 prevents the sustained

reduction in body weight normally induced by exposure to RR. This implies that activation of CRFR1 during RR is a necessary step in the process by which RR animals chronically down-regulate their body weight.

In contrast to the results of Experiment 2, Experiment 1 indicated that peripheral administration of the CRFR1 antagonist NBI 27914 had no effect on RR-induced inhibition of food intake or weight loss. While previous studies indicate that systemic application of NBI 27914 attenuates stress-induced elevation of ACTH and reduces restraint-induced anxiety behaviors, presumably via antagonism of CRFR1 (38, 53), our results showed that NBI 27914 had no effect on RR-induced decreases in food intake or body weight. Although given in a dose identical to that of a previous study in which subcutaneous injection of NBI 27914 inhibited restraint-induced increases in behavioral anxiety (53), it is possible that not enough of the peripherally-applied CRFR1 antagonist penetrated areas adjacent to the third ventricle (38). This would explain why central infusion of antalarmin prevented the chronic weight loss induced by RR while peripheral injection of NBI 27914 did not.

It is well established that activation of CRFR1 is primarily responsible for stress-induced activation of the HPA axis (4). Further, both antalarmin and NBI 27914 have been reported to attenuate stress-induced increases in ACTH (38, 60). We expected that CRFR1 antagonism during RR would blunt, or completely attenuate, RR-induced release of corticosterone but neither antagonist modified serum corticosterone concentration measured 60 minutes after the onset of RR. It is possible that neither peripheral CRFR1 antagonism nor antagonism of CRFR1 in areas of the brain adjacent to the third ventricle act on the CRFR1 responsible for the endocrine response to RR. Additionally, CRFR1 is not the only mechanism by which the HPA axis is activated during exposure to stress. Arginine vasopressin (AVP), is co-expressed and co-

secreted with CRF in response to stress and has been shown to act concurrently with CRF to trigger the release of ACTH, and ultimately, glucocorticoids, through activation of AVP 1b receptors in the pituitary (33, 34, 36). We have previously reported that AVP mRNA in the hypothalamus is not increased after 3 hours of restraint stress (50), however, hypothalamic expression of AVP mRNA has not been measured following RR. Others have reported that repeated exposure to the same stressor results in a substantial increase in the number of neurons co-expressing CRF and AVP and that this is associated with increased ACTH release in response to subsequent novel stress exposure (14). In addition, research suggests that during periods of chronic stress primary control of ACTH secretion shifts from CRF to AVP (5, 40, 43) and other stress-activated neurotransmitters such as serotonin, GABA, and acetylcholine also mediate endocrine responses to stress (19, 49). Therefore, levels of serum corticosterone could be increased during RR independent of CRFR1 activation.

The results of Experiment 3 indicate that central CRFR2 antagonism during RR attenuates RR-induced hypophagia. These results are consistent with reports from others who have found that concurrent lateral ventricular administration of ASV-30 with Ucn I or CRF completely reverses the decrease in food intake and body weight observed when the stress peptides alone are infused (13). The results of our experiment imply that the sustained reduction in body weight observed in response to RR exposure is independent of a reduction in food intake, as antagonist-infused RR rats in this experiment showed the same sustained reduction in body weight typically observed in RR animals that do have a reduced food intake of he days of restraint. This implies that the chronic down-regulation of body weight exhibited by RR rats in the post-RR period is due primarily to a change in energy expenditure. This conclusion is consistent with those of a previous study in which we measured energy expenditure during RR

and the 3 days following RR using indirect calorimetry (28). As expected, rats increased energy expenditure on the days of RR and did not compensate with a decrease in energy expenditure during the 3 days following RR. RR also reduced the respiratory quotient of the rats, indicating a shift from carbohydrate oxidation to protein and/or fatty acid oxidation. These results are also consistent with reports that icv infusion of CRF results in increased catecholaminergic activity (7), an increase in the availability of circulating glucose (6), and increased thermogenesis in brown adipose tissue (16, 35).

The results of Experiments 2 and 3 suggest that selective antagonism of either CRFR1 or CRFR2 during RR result in modification of the response to restraint. Curiously, in Experiment 5 non-selective CRFR antagonism before RR did not result in any of the changes seen with selective antagonism of the specific CRFR subtypes. We suggest that, while activation of CRFR1 is necessary to achieve the sustained modification in body weight induced by RR, the activity of CRFR2 is also necessary to modulate the response. While the effects of CRFR2 activation on feeding behavior have been well-documented, other contributions of CRFR2 have only begun to be explored (4, 13, 32). For example, CRFR2 KO mice exhibit heightened HPA axis activity in response to stress. Despite normal basal ACTH and corticosterone levels, CRFR2 KO mice exposed to restraint stress show a rapid, exaggerated release of ACTH compared to WT littermates (3, 12). Researchers found that an elevation in serum corticosterone could be detected 2 minutes after the onset of restraint stress in CRFR2 KO compared to detection 5 minutes after the onset of restraint in WT littermates. Additionally, corticosterone levels were still significantly elevated 90 minutes after a 5-minute restraint in CRFR2 KO compared to WT animals (12). Results from these studies suggest that CRFR2 may function to dampen, or reverse, the effects of CRFR1 activation during exposure to stressors. This may

occur through more rapid termination of ACTH secretion and/or corticosterone following the termination of stress leading to a more rapid restoration of homeostasis. Because RR-induced stimulation of the HPA axis occurs even with CRFR1 antagonism, it is possible that activation of CRFR2 is necessary to attenuate the chronic effects of RR on body weight regulation.

Contrary to the previously published report, in Experiments 4 and 5, we were unable to show that antagonism of all CRFR in areas adjacent to the third ventricle during RR attenuated stress-induced weight loss. Antagonism of CRFR in Experiment 5 (astressin) reduced food intake in control rats but had no effect in rats exposed to RR, consistent with the observation in Experiment 3 that a reduction in food intake on the days of stress is not required for the sustained, stress-induced down regulation of body weight. We also found that while RR significantly decreased body weight in Experiment 5 (astressin), RR did not significantly reduce body weight in Experiment 4 (αhCRF). This was unexpected, as exposure to RR has consistently results in a sustained decrease in body weight (22, 24, 28, 30, 54) and Smagin et al. reported that αhCRF applied before RR, attenuats RR-induced weight loss and hypophagia (54). It is possible that the absence of a significant stress effect in Experiment 4 was due to habituation of the animals to stress. Rats exposed to chronic stress of a variable nature for 10 days initially lose weight in response to stressor exposure, however, by day 5 of the intervention rats no longer lose weight in response to novel stressors (41, 45). Rats in Experiment 4 were exposed to the severe stressor of surgical intervention during the cannulation procedure, daily manipulations associated with food intakes and body weights, as well as other potentially stressful stimuli not under our control. Similar to rats exposed to chronic, variable stress, rats in Experiment 4 may have perceived RR as another stressor in a series of variable stressors. RR was therefore not

perceived as a severe stressor and this accounted for the absence of weight loss in stressed rats in Experiment 4.

We previously reported that rats subjected to RR exhibit an exaggerated glucocorticoid response to the novel MS of ip saline injection 12 days after the end of RR (21). In all of the experiments in this study, with the exception of Experiment 3, we measured corticosterone release in response to MS in rats that had been exposed to RR. CRFR1 are primarily associated with HPA axis activation during stress, therefore, corticosterone release in response to a MS applied in the post-RR period was not measured in Experiment 3, which tested the actions of CRFR2. Antagonism of all CRFR with αhCRF on *days 1-3* of the experiment blocked exaggeration of corticosterone release in response to MS in Experiment 4. This was the only experiment in which CRFR antagonism during RR had an effect on hyper-responsiveness to a subsequent stressor. The implications of this are unclear, as Experiment 4 is also the only experiment in which exposure to RR had no significant effect on weight loss. Earlier we suggested that rats in Experiment 4 did not perceive RR to be as severe a stressor as rats in other experiments. Others have shown that the perceived severity of a stressor partially determines the pattern of CRF mRNA expression and CRFR activation in response to the stressor (4, 10, 15, 17, 20). It is possible that antagonism of CRFR in areas adjacent to the third ventricle during RR in Experiment 4 resulted in a decrease in hyperreactivity to subsequent stressors only because RR was not perceived as a severe stress.

RR had no significant effect on corticosterone release in response to MS in any experiment with the exception of Experiment 1. This may be an indication that the MS used in these experiments was not an effective novel mild stress for rats. Studies have shown that even one previous exposure to a stressor can reduce plasma corticosterone levels in response to that

same stressor (18). In Experiments 2, 4, and 5 an anesthetic was administered through an ip injection during the cannulation and icv infusion procedure. This could have affected perception of the subsequent ip injection as a novel mild stress. In addition, rats in Experiments 2, 4 and 5, because of the cannulation procedure, were handled much more than rats in Experiment 1. Rats in these experiments could have become so accustomed to constant manipulation that ip injection was not perceived as a novel stressor.

In conclusion, the experiments described here demonstrate that central antagonism of CRFR1 with concurrent activation of CRFR2 during RR attenuate the sustained reduction in body weight exhibited by RR rats. This chronic effect of RR on body weight appears to be independent of food intake during the days of restraint. Peripheral antagonism of CRFR1 does not affect hypersensitivity of the HPA axis during the post-stress period. Further studies are needed to determine which nuclei adjacent to the third ventricle are responsible for the sustained effects of RR.

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FIGURES

Figures 4.1-4.3: Change from baseline body weight from *day 1* to *day 13* (4.1), average food intake for 3 consecutive days before, after, and during RR (4.2), and change in serum corticosterone concentration after exposure to MS (4.3) in Experiment 1. Body weight and food intake values are means ± S.E.M. for 20 rats. Corticosterone values are means ± S.E.M. for 10 rats. Astericks indicate significant differences between control and restrained animals ($P<0.001$), # indicates a significant effect of RR on corticosterone concentration in response to MS ($P<0.01$), and food intake values in a specific group of days that do not share a common superscript are significantly different at $P<0.001$.

Figures 4.4-4.6: Change from baseline body weight from *day 1* to *day 13* (4.4), average food intake for 3 consecutive days before, after, and during RR (4.5), and serum corticosterone concentration measured 60 minutes after the start of RR on *day 2* (4.6) in Experiment 2. Body weight, food intake, and serum corticosterone concentration values are means ± S.E.M. for 14 rats. Astericks indicate a significant differences between Ant/RR and control animals ($P<0.05$), # indicates a significant difference between Ant/RR and Veh/RR animals ($P<0.05$), and food intake and corticosterone values in a specific group of days (food intake) or time points (corticosterone) that do not share a common superscript are significantly different at $P<0.001$.

Figures 4.7 and 4.8: Change from baseline body weight from *day 1* to *day 9* (4.7) and average food intake for 3 consecutive days before, after, and during RR (4.8) in Experiment 3. Body weight and food intake are means ± S.E.M. for 7 rats. Astericks indicate a significant differences between RR and control animals ($P<0.005$) and food intake and values of serum corticosterone

concentration in a specific group that do not share a common superscript are significantly different at $P<0.05$.

Figures 4.9 and 4.10: Average food intake for 3 consecutive days before, after, and during RR (4.9) and area under the curve of serum corticosterone concentration in response to MS (4.10) in Experiment 4. Food intake values and corticosterone values are means ± S.E.M. for 16 rats. Intake values in a specific group of days that do not share a common superscript are significantly different at $P<0.05$. Corticosterone values that do not share a common superscript are significantly different at $P<0.5$.

Figures 4.11-4.13: Change from baseline body weight from *day 1* to *day 13* (4.11), average food intake for 3 consecutive days before, after, and during RR (4.12), and change in serum corticosterone concentration after exposure to MS (4.13) in Experiment 5. Body weight and food intake values are means ± S.E.M. for 14 rats. Corticosterone values are means ± S.E.M. for 7 rats. Astericks indicate significant differences between control and restrained animals ($P<0.001$), δ indicates a significant difference ($P<0.05$) between Ast/C and all other groups, # indicates a significant effect of RR on corticosterone concentration in response to MS ($P<0.027$), and food intake values in a specific group of days that do not share a common superscript are significantly different at $P<0.001$.

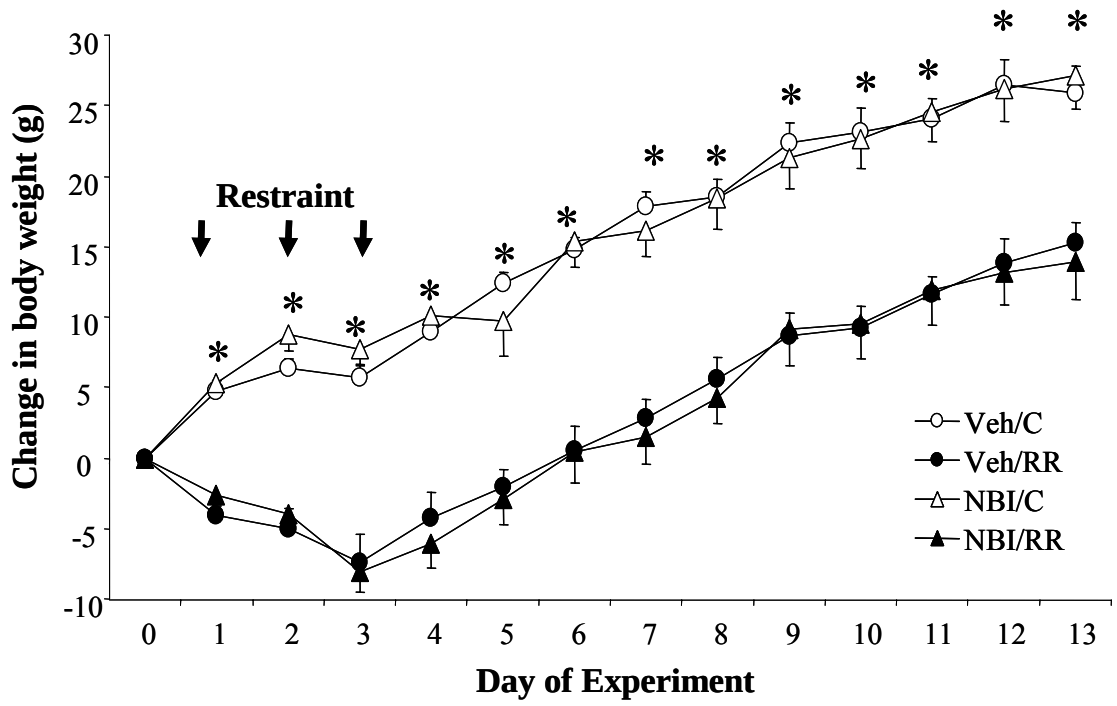

Figure 4.1: Change in body weight from the day before RR to *day 13* in Experiment 1

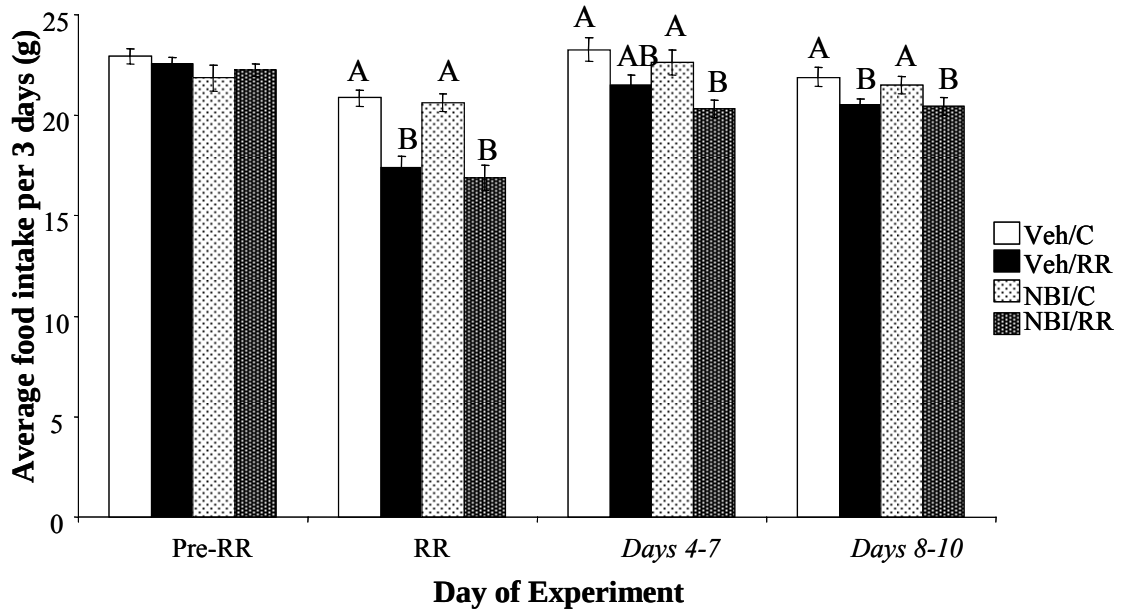

Figure 4.2: Average food intake for 3 consecutive days before, after, and during RR in Experiment 1

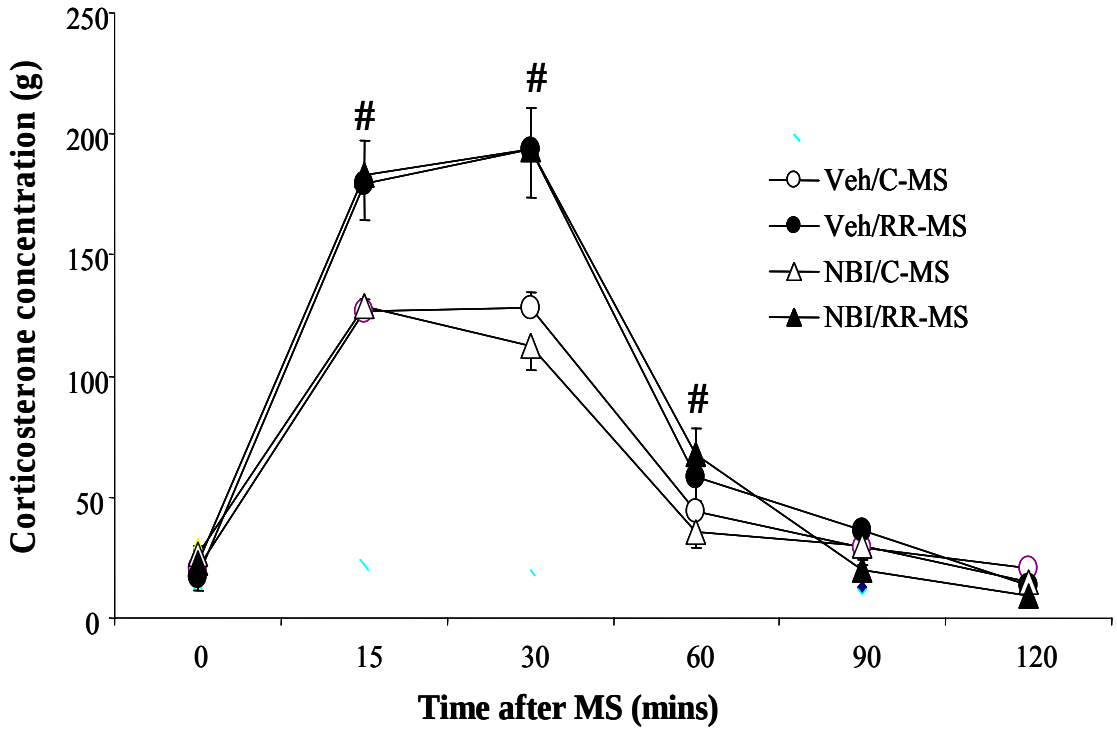

Figure 4.3: Serum corticosterone concentration after MS in Experiment 1

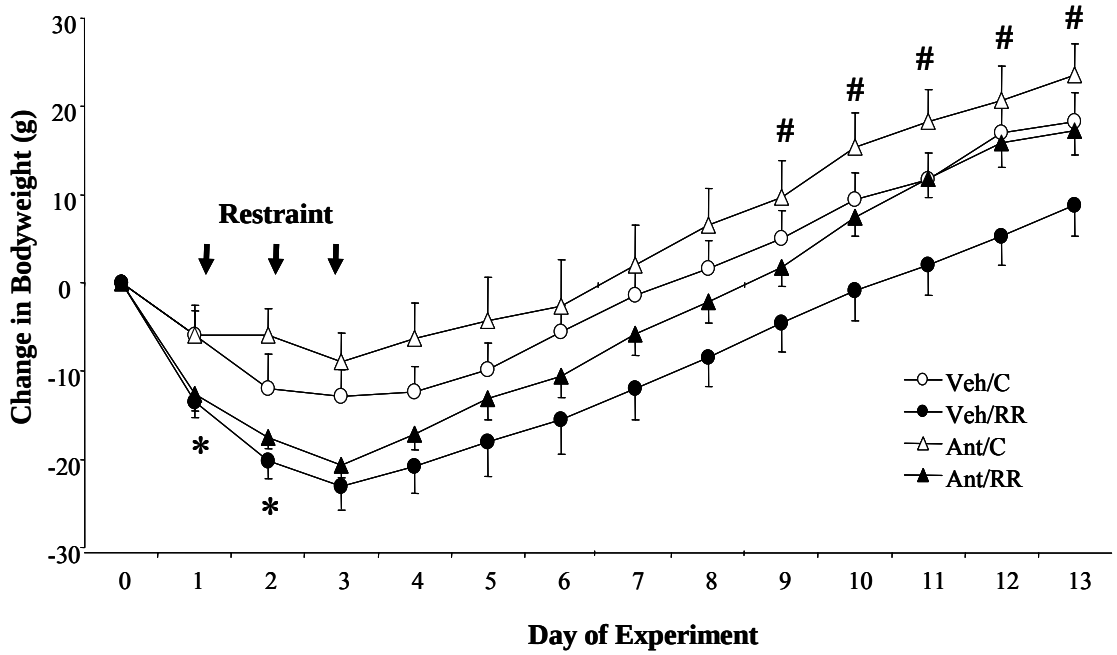

Figure 4.4: Change in body weight from the day before RR to *day 13* in Experiment 2

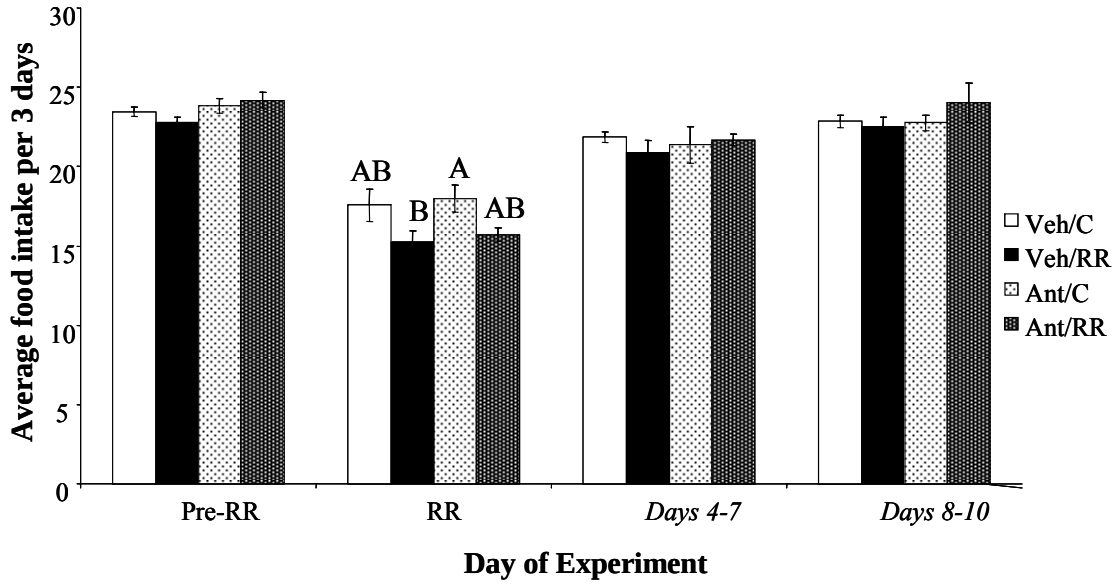

Figure 4.5: Average food intake for 3 consecutive days before, after, and during RR in Experiment 2

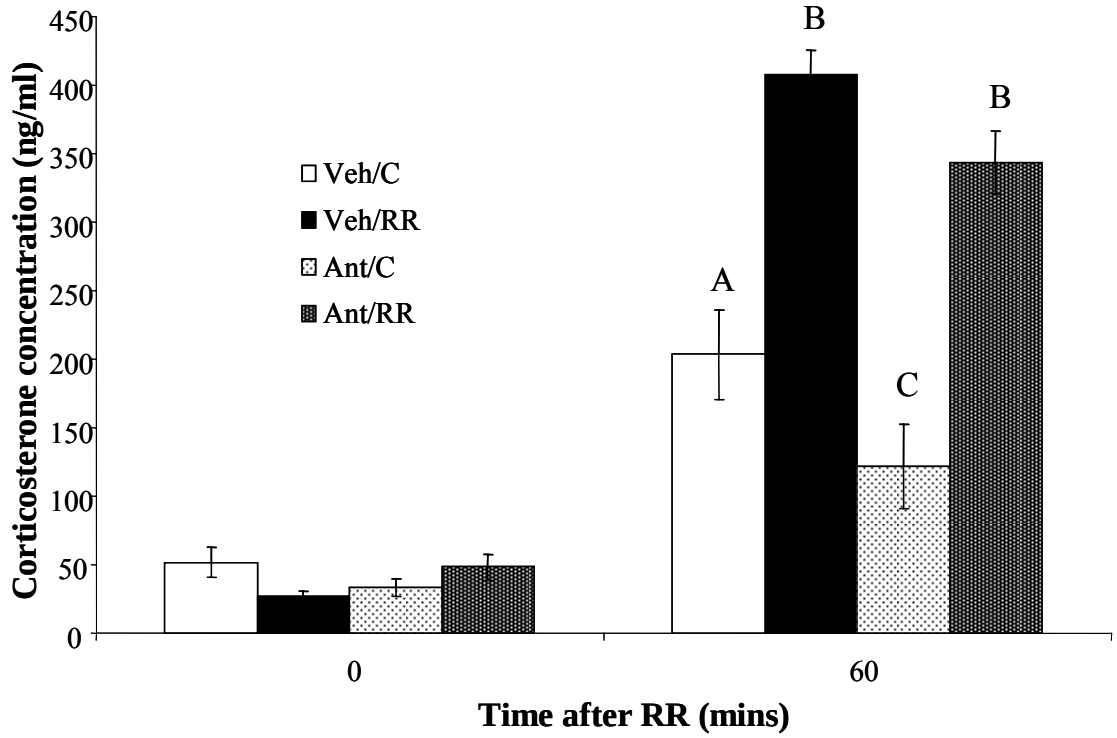

Figure 4.6: Serum corticosterone concentration after RR in Experiment 2

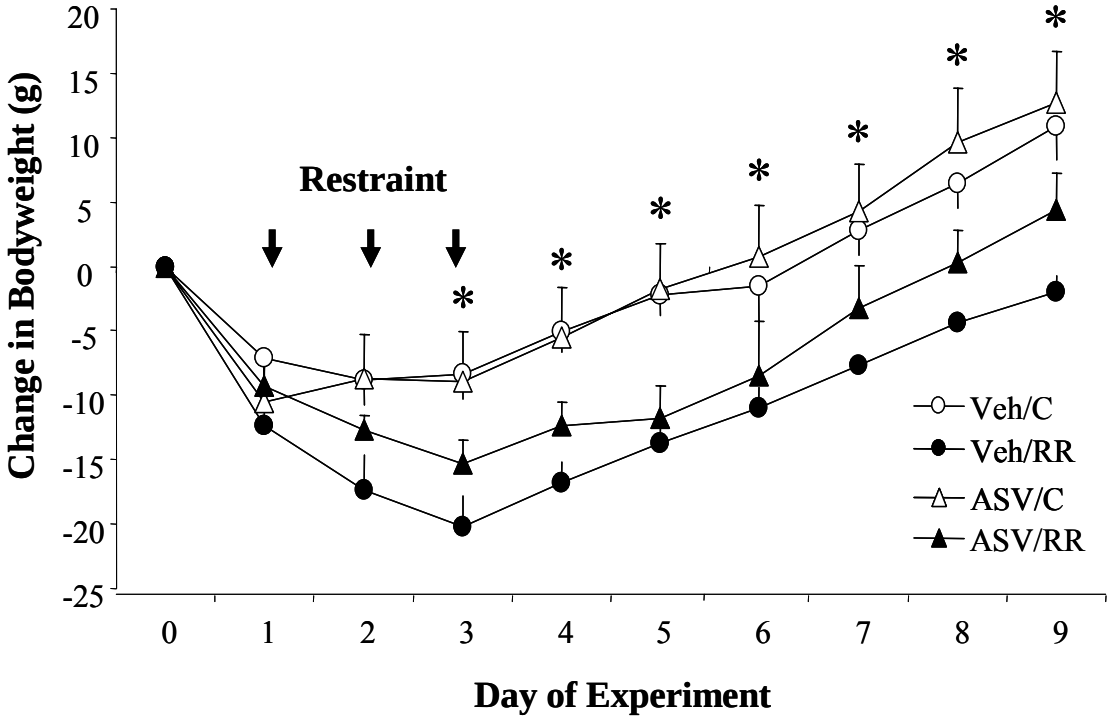

Figure 4.7: Change in body weight from the day before RR to *day 9* in Experiment 3

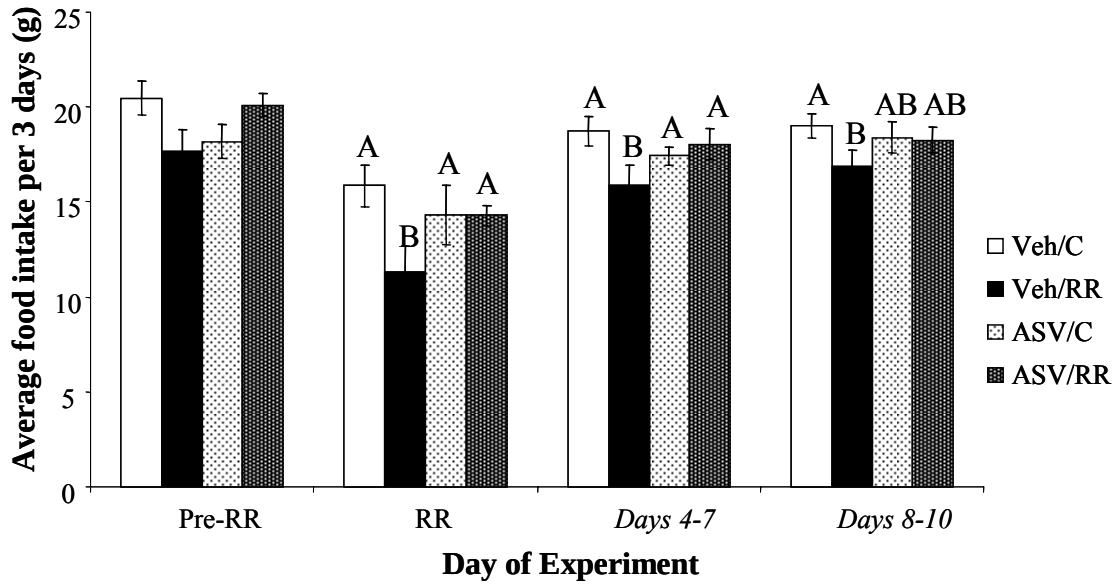

Figure 4.8: Average food intake for 3 consecutive days before, after, and during RR in Experiment 3

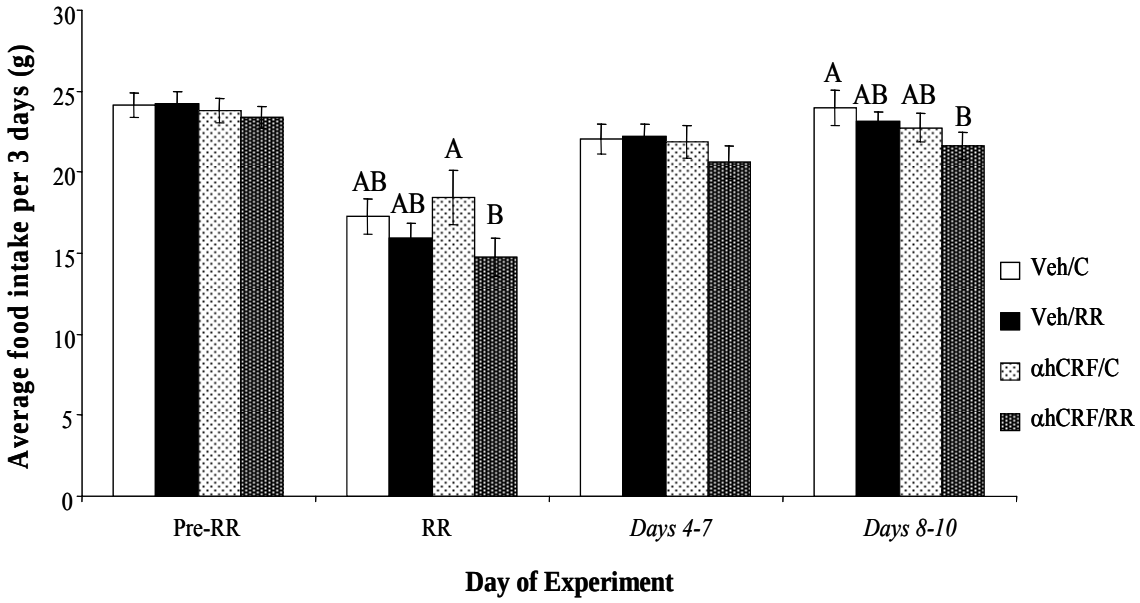

Figure 4.9: Average food intake for 3 consecutive days before, after, and during RR in Experiment 4

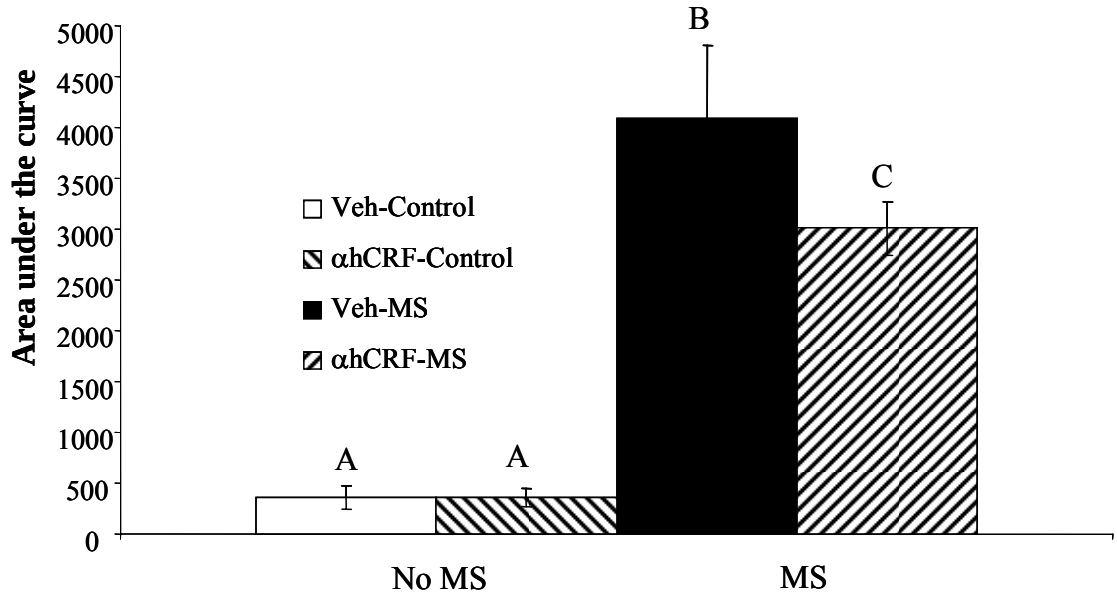

Figure 4.10: Area under the curve of corticosterone release for 120 minutes after MS in Experiment 4

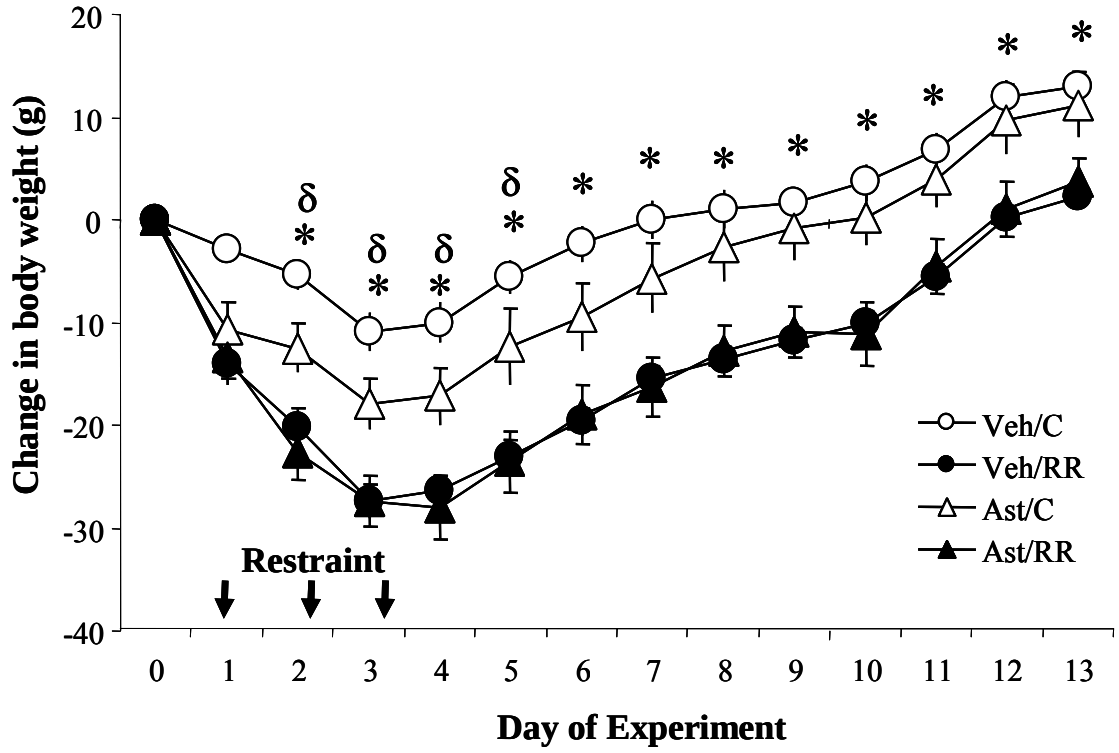

Figure 4.11: Change in body weight from the day before RR to *day 13* in Experiment 5

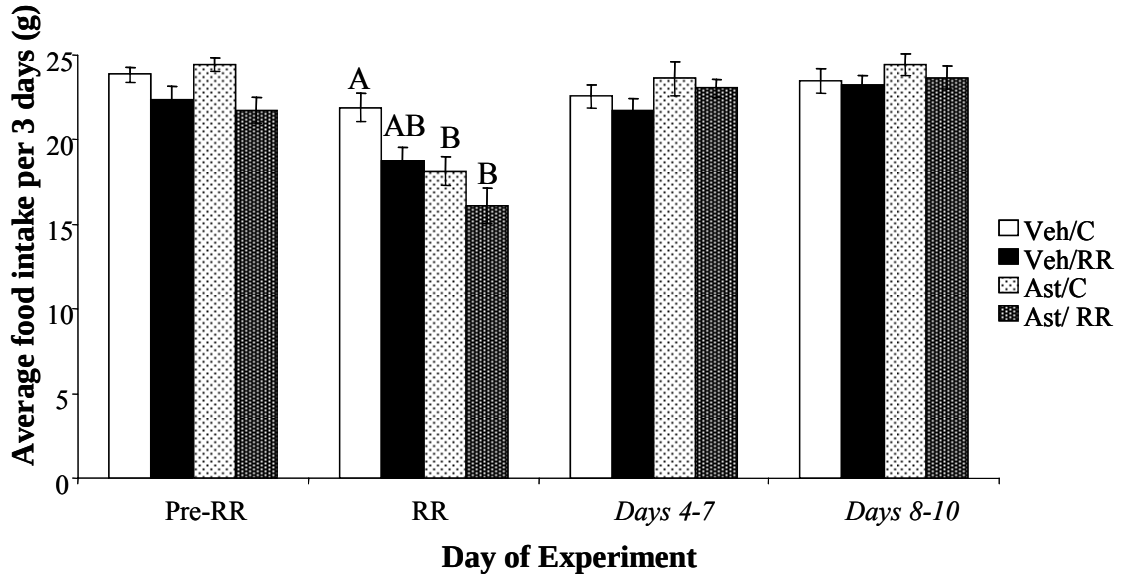

Figure 4.12: Average food intake for 3 consecutive days before, after, and during RR in Experiment 5

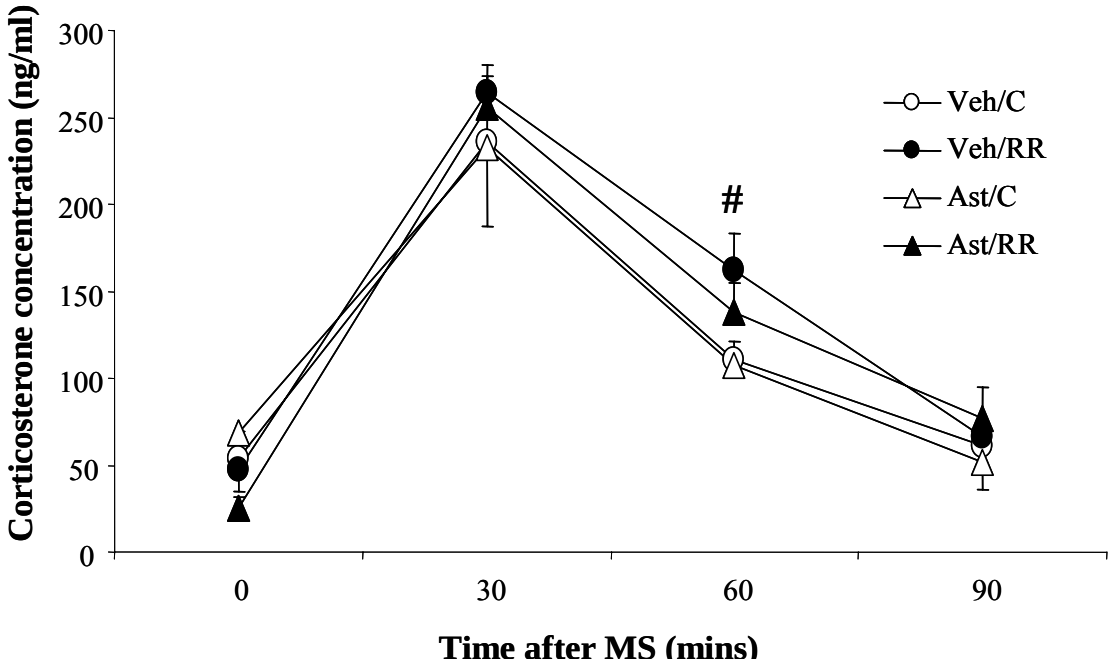

Figure 4.13: Serum corticosterone concentration after MS in Experiment 5

CHAPTER 5

SUMMARY AND CONCLUSION

Although regulated by a number of neurotransmitters, hormones and receptors, the primary initiator of the stress response appears to be corticotropin-releasing factor (CRF) which mediates responses to stress through activation of the two types of CRF receptors (CRFR) (1, 15). CRFR1 activation is implicated in stimulation of the hypothalamic-pituitary-adrenal (HPA) axis response and increases in behaviors indicative of anxiety (2, 13, 14), while CRFR2 are believed to mediate stress-induced hypophagia and increases in energy expenditure (3, 4). Response to stress is dependent upon the type and location of the CRFR activated as well as the length of exposure and the perceived severity of the stressor (10, 11).

Rats exposed to RR (3 hours of restraint on each of 3 consecutive days) experience an inhibition of food intake and a loss of body weight during the days of restraint (8). In the post-stress period, RR rats gain weight at the same rate as non-stressed controls but never return to the weight of controls (6). Additionally, RR animals exhibit enhanced secretion of glucocorticoids when challenged by a novel mild stressor in the post-stress period (5, 9). Mice exposed to 2 hours of restraint stress on each of 3 consecutive days exhibit similar chronic changes in body weight and endocrine hyperreactivity (7).

The objective of the first study described here was to determine whether RR induces long-lasting changes in other aspects of the stress response, particularly those that can be determined through behavioral measures of anxiety. We found that mice exposed to RR exhibited increases in anxiety-like behavior as many as 20 days after RR. These results indicate

that exposure to acute stress can induce long-lasting changes in the responsiveness of a variety of neuroendocrine systems. Future studies will utilize CRFR knockout (KO) mice, which either do not express CRFR1 or do not express CRFR2, to explore the role of the different CRFR subtypes in promoting hyperresponsiveness to novel stressors in the post-RR period. KO mice, deficient in either CRFR1 or CRFR2, are an ideal model for this purpose, as the brain areas responsible for these RR-induced changes have not been determined. Results of these studies would reveal the role that each type of CRFR plays in post-RR hyperreactivity.

The objective of the second set of studies was to test the effects of antagonism of either CRFR1 or CRFR2 during RR on RR-induced changes in body weight, food intake, and subsequent endocrine hyperreactivity to mild stressors. Because results from a previous study indicated that injection of a non-selective CRFR antagonist, αhCRF, in areas adjacent to the third ventricle before RR prevented RR-induced weight loss, we infused specific CRFR1 and CRFR2 antagonists into third ventricle before RR (12). From these experiments we concluded that central, but not peripheral, antagonism of CRFR1 during RR attenuates the sustained decrease in body weight following exposure to RR. The chronic down-regulation of body weight appears to be due primarily to an increase in energy expenditure, because antagonism of CRFR2 during RR prevented RR-induced hypophagia, but not weight loss. CRFR1 antagonism during RR did not attenuate the increase in corticosterone release either during RR or in response to a subsequent stressor. This suggests that CRFR1 in areas adjacent to the third ventricle are not responsible for RR-induced increases in serum corticosterone.

Interestingly, we could not replicate the results of the study by Smagin et al. in which non-specific CRFR antagonism completely blocked RR-induced weight loss (12). Furthermore, antagonism of both CRFR1 and CRFR2 with a different non-specfic CRFR antagonist during RR

did not block RR-induced weight loss or hypophagia. This was unexpected, as antagonism of either CRFR1 or CRFR2 individually blocked different aspects of the response to RR. Two explanations exist for the absence of an effect of non-specific CRFR antagonism on RR-induced changes in body weight and hypophagia. The first is that astressin, the non-specific antagonist infused in Experiment 5, is not an effective antagonist of CRFR in the third ventricle. To test this theory, we propose to co-infuse astressin with CRF into the third ventricle and measure food intake of hungry rats. If astressin is an effective antagonist of CRFR in the third ventricle, co-infusion of astressin with CRF will prevent CRF-induced hypophagia.

Alternatively, we suggest that both CRFR2 activation and CRFR1 antagonism, during RR are necessary to prevent the RR-induced sustained weight loss (See Figure 9). We propose that a nucleus of the brain distant from the third ventricle is directly responsible for RR-induced weight loss. Hypothalamic CRFR1 activation during RR feeds forward to this second nucleus, exaggerating or perpetuating the weight loss. Hypothalamic activation of CRFR2 may also act indirectly on this nucleus to inhibit weight loss, however, its effects may be overshadowed by the effects of CRFR1 activation. Antagonism of CRFR1, then, would allow the effects of inhibition of weight loss by CRFR2 activation to be expressed. Antagonism of both hypothalamic CRFR1 and CRFR2 would prevent the inhibiting effects of CRFR2 on the unknown nucleus, resulting in RR-induced weight loss. This would explain why specific antagonism of CRFR1 during RR prevents RR-induced weight loss, but non-selective antagonism of CRFR does not.

Based on the results of the studies described here, we propose that future studies further investigate the role of CRFR1 in individual brain nuclei adjacent to the third ventricle during RR. Measurement of c-Fos activation in brains of rats exposed to RR would indicate the areas adjacent to the third ventricle that are activated by RR. Infusion of CRFR1 antagonist into the

third ventricle before RR and subsequent measurement of C-Fos immunoreactivity would help identify areas affected by CRFR1 antagonism during RR. The effects of site-specific CRFR1 antagonism in these specific areas would then be tested. Completion of these studies would yield a better understanding of the mechanisms by which acute stressors act to induce long-term changes in body weight regulation.

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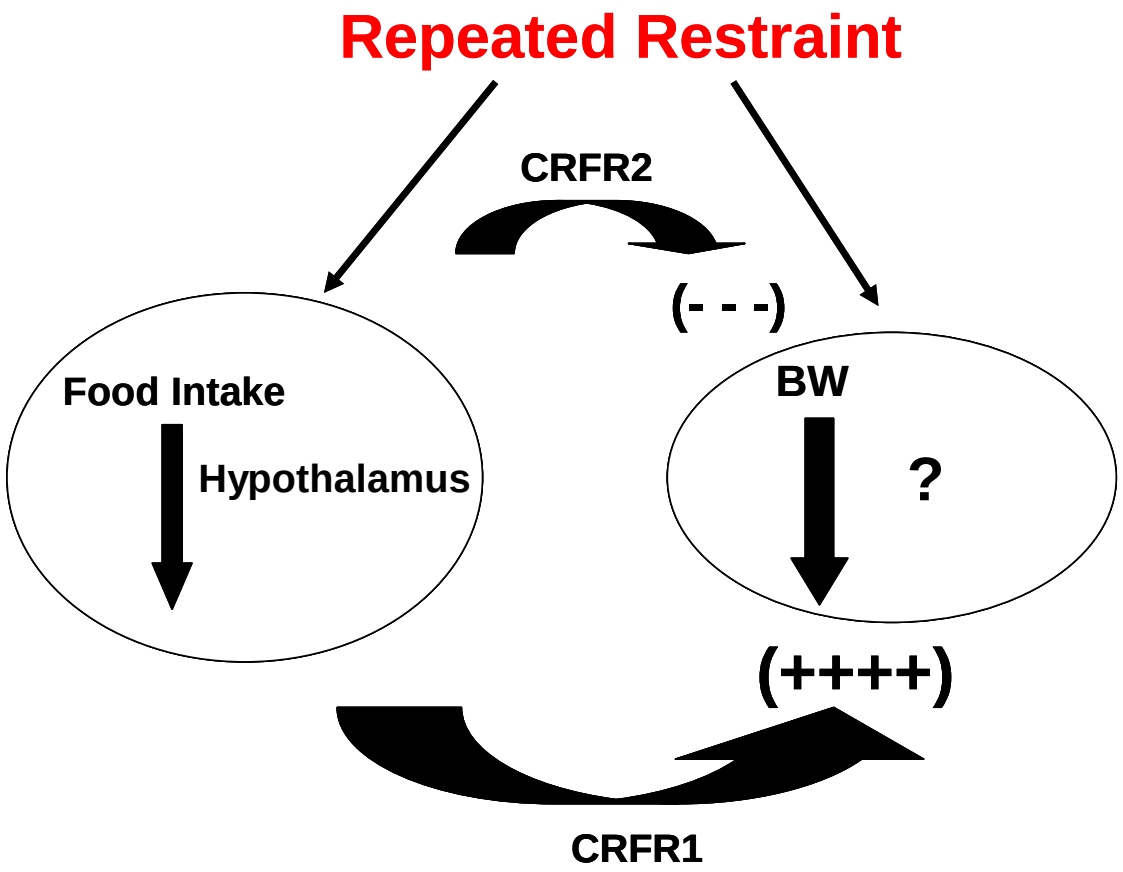

Figure 5.1: The proposed interaction between hypothalamic CRFR1 and CRFR2 activation during RR