THE EFFECT OF CORTICOSTERONE ON STRESS-INDUCED WEIGHT LOSS AND STRESS RELATED NEUROPEPTIDE mRNA EXPRESSION IN THE LIMBIC SYSTEM

by

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

ABSTRACT

Rats exposed to repeated restraint (RR) lose weight and do not return to the weight of non-stressed controls (CTRL). This study tested the effect of corticosterone (cort) on sustained weight loss by subjecting intact, adrenalectomized (ADX) and ADX with cort replacement (ADX CORT) rats to RR. RR ADX CORT rats had the same initial and sustained weight loss as RR intact rats. CTRL ADX CORT and RR ADX rats lost half as much weight and maintained the weight loss for less time than RR intact. Thus both stress-induced cort and an undefined aspect of RR are required for sustained weight loss. In a second set of rats, PVN CRF mRNA expression was increased in ADX and RR rats compared with CTRL intact rats. Cort did not inhibit expression in ADX rats. This suggests that cort contributes to initial and sustained weight loss in RR rats independent of PVN CRF mRNA.

INDEX WORDS: rats, stress, repeated restraint, in situ hybridization, paraventricular nucleus of the hypothalamus, corticotropin-releasing factor, arginine vasopressin

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CHAPTER 1

INTRODUCTION

Statistics from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) estimate that 66% of adults and 17% of children and adolescents are overweight or obese (1). Obesity and overweight have been linked to increased risk for chronic disease such as diabetes, cardiovascular disease, stroke and hypertension (71). Individuals who manage to lose weight often are unsuccessful in maintaining that weight loss (3).

Several animal models exist that allow researchers to investigate the mechanisms behind weight loss. Rodent models used in body weight and energy balance research include calorie restriction (28), sleep deprivation (62) and cold exposure (9). Rats exposed to these interventions regain weight quickly after the treatment ends. However, rats exposed to repeated restraint (RR) lose weight on the days of restraint and do not return to the weight of their controls after stress ends (29). RR is a unique model that allows researchers to investigate the mechanisms responsible for sustained weight loss.

RR animals lose weight by reducing food intake and increasing energy expenditure on the days of stress. After stress, RR rats return to normal eating patterns and gain weight at the same rate as control rats, but maintain a chronic reduction in body weight that has been documented for up to 3 months (29). Despite chronic effects of RR, restraint is classified as an acute stress. Chronic stress results in elevated corticosterone (cort) levels, an exaggerated endocrine response to a novel stress, an enlarged adrenal gland, a reduced thymus gland, and decreased immune function and impaired memory (2, 51). Acute stress such as RR results in

normal baseline levels of cort, normal thymus and adrenal sizes, and enhanced immune function and memory (27, 30, 51).

Stress initiates neurological responses that include an activation of noradrenergic neurons in the locus coerulus (LC) in the brainstem to release norepinephrine and CRF neurons in the paraventricular nucleus of the hypothalamus (PVN) and the central amygdalae (CeA) (26). The CeA integrate information from the LC and hippocampus and send a signal along the bed nucleus of the stria terminalis (BNST) to the PVN (35). Stimulation of the PVN results in the secretion of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from neurosecretory cells into the hypophyseal portal system (64). This allows the peptide hormones to reach the pituitary gland and stimulate the secretion of corticotrophin or adrenocorticotropic hormone (ACTH) (60). Circulating ACTH stimulates the adrenal cortex to synthesize and release glucocorticoids (GCs) such as cortisol in humans and corticosterone (cort) in rats. This major pathway of stress endocrine response is called the hypothalamic-pituitary-adrenal (HPA) axis (26). Cort peaks between 30 and 60 min after HPA axis activation and causes various physiological responses in the body including increased blood pressure, increased blood glucose, suppressed immune function and negative feedback to the hypothalamus and pituitary to down-regulate the response to stress (21, 56).

Adrenalectomized (ADX) rats given basal cort replacement and subjected to RR have been shown to lose weight on the days of stress (24). Preliminary studies from our lab indicate that although ADX rats also lose weight on the days of stress, they fail to show the sustained weight loss of intact RR rats. Further exploration of cort's role in sustained weight loss and HPA axis feedback after acute stress is required.

CRF has been identified as the primary initiator of the stress response, including activation of the HPA axis. Under stress conditions AVP works with CRF to release ACTH from the pituitary (45). Some investigators suggest that AVP does not play a major role in the response to acute or restraint stress (45), while others propose that a greater change in AVP mRNA in the PVN after stress, independent of cort release, indicates that feedback control over the HPA axis shifts from CRF to AVP control (49). Previous studies have shown that RR increases CRF mRNA in the PVN (29, 36, 38, 47, 49) but has no effect on AVP mRNA in the PVN (44). In contrast, peripheral injections of cort or centrally infused cort decrease CRF mRNA in the PVN (22, 41, 42, 46, 48). This supports the notion that cort released during stress acts as negative feedback to the PVN inhibiting the HPA axis response (37). Cort binds with highest affinity to mineralocorticoid receptors in the hippocampus, hypothalamus and pituitary to downregulate the secretion of CRF and ACTH (57). ADX rats have elevated levels of ACTH and increased expression of CRF mRNA in the PVN (50), which suggests that ADX results in generalized activation of the HPA axis. When ADX rats are stressed, they show a greater neurological response than intact rats since ADX rats lack the normal glucocorticoid negative feedback mechanisms (49, 50).

The experiments described here tested whether stress-induced levels of cort are necessary for the initial and sustained weight loss in RR rats. Arguments can be made that disruption of the adrenal hormone negative feedback may result in changes in pituitary hormones that also could affect weight loss after stress. Therefore, we also tested whether body weight changes in RR rats are dependent on the presence of a functional pituitary. In addition, the studies described here also tested the effects of cort on mRNA expression of CRF and AVP in the PVN. We

hypothesized that sustained weight loss in rats exposed to RR results from activation of the limbic system by stress-induced elevations in corticosterone concentration.

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CHAPTER 2

LITERATURE REVIEW

Overweight and Obesity

The prevalence of overweight and obesity is rising; in the United States, 66% of adults and 17% of children and adolescents ages 2-19 years are overweight or obese (1). Obese individuals are at increased risk for complications such as diabetes, cardiovascular disease, stroke and hypertension, some of the leading causes of death (71). One of the most effective ways of reducing these risks is to lose weight, but it is difficult to lose weight and only 15% of people who do lose weight are successful in maintaining weight loss after 1 year (3).

An animal model that serves as a unique tool for researching sustained weight loss is repeated restraint stress (RR). In the RR model, rats or mice are placed in restraining tubes and stressed for 3 hours on 3 consecutive days. RR animals lose weight during restraint. After stress ends, RR animals gain weight at the same rate as controls but maintain a reduced body weight compared with their controls. In other animal models of weight loss, such as calorie restriction, animals regain weight as soon as the treatment ends (28). RR allows us to investigate the mechanisms behind sustained weight loss. A better understanding of body weight regulation may enable us to aid individuals struggling with obesity.

Stress

Stress is defined as a disruption of normal homeostasis through physical or psychological stimuli (56). When stressed, the body reacts with various neural, endocrine, metabolic and behavioral changes. The primary neural response occurs in the limbic system, a set of brain

structures including the hippocampus, amygdalae, pituitary and hypothalamus that control emotions, behavior and memory (26). The limbic system is involved in initiating the "fight-or-flight" response and acts in part by influencing the endocrine system (26). The endocrine system influences growth and metabolism of an organism by hormone signaling (26). One pathway that includes the limbic system and endocrine system that has been implicated as a major player in stress response is the hypothalamic-pituitary-adrenal (HPA) axis (26).

Hypothalamic-pituitary-adrenal (HPA) Axis

The HPA axis is a well-characterized neuroendocrine system that uses a classic feedback mechanism. The physiological response to stress occurs in the hippocampus, amygdalae and brainstem areas such as the locus coerulus (LC), which are activated by stress and release norepinephrine (NE) as the first step to alerting the body to danger (26). NE and signals from the amygdalae stimulate the hypothalamus to secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), primarily from the parvocellular cells of the paraventricular nucleus of the hypothalamus (PVN). CRF and AVP are released into the hypophyseal portal system which signals the anterior pituitary gland to secrete corticotrophin or adrenocorticotropic hormone (ACTH). ACTH is synthesized from pre-proopiomelanocortin (pre-POMC) released into the blood stream and triggers the cortex of the adrenal gland to synthesize and release glucocorticoids (GCs). GCs, including cortisol in humans and corticosterone (cort) in rats, peak approximately 30 to 60 min after activation of the HPA axis and act primarily to increase glucose concentrations in the blood. GCs mobilize energy stores by stimulating enzymes involved in gluconeogenesis, the conversion of products such as amino acids, glycerol and lactate into glucose. GCs are needed for normal insulin response and energy storage (16), as well as to provide a negative feedback mechanism for the HPA axis.

Cort is considered the principal GC resulting from the HPA axis stress response in rodents. Cort causes various physiological responses in the body such as increasing blood pressure, increasing blood glucose, suppressing the immune system and providing negative feedback to the hypothalamus and pituitary (56). ACTH stimulates a circadian rhythm of GC release with the highest GC levels at the end of the resting period which is at the end of the light cycle for rats, but the end of the dark period for humans (23).

One of the key roles of GCs is to act as a negative feedback system. Circulating GCs bind to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in the hippocampus, hypothalamus and pituitary to inhibit the release of CRF and ACTH (4, 57). MR have a high affinity for corticosteroids and are occupied 70-90% of the time in resting conditions (11). GR have a lower affinity for corticosteroids and are only occupied during times of high circulating levels of GCs (11). Therefore, it is thought that GR are primarily responsible for terminating the GC response (61, 68). Rats who received MR and GR antagonists prior to restraint have elevated ACTH and plasma cort responses, indicating that both types of corticosteroid receptors are necessary for post-stress HPA axis downregulation (11). GCs are thought to enhance HPA axis sensitivity to CRF responses in the limbic system and influence responses to subsequent stressors (13, 54). Prolonged elevations of GCs as are suspected to cause HPA axis dysregulation which can lead to health-damaging behaviors and stress-related diseases such as abnormal metabolism, impaired immune function, behavioral disturbances and cardiovascular problems (18, 19, 52).

Stress and Energy Balance

In humans, stress can cause weight gain or weight loss (55). Animals such as hamsters and nonhuman primates often gain weight in response to stress (59, 67). When rats and mice are subjected to stress, they tend to lose weight (69). Weight change is dependent on the balance

between food intake and energy expenditure (5). Areas of the brain that play a role in food intake and energy expenditure include the brainstem and the arcuate nucleus (ARC) within the hypothalamus (6). Neuropeptide regulators of food intake within the ARC include neuropeptide Y (NPY), agouti-related peptide (AgRP), glucagon-like peptide-1 (GLP-1) and proopiomelanocortin (POMC), the precursor for α-melanocyte-stimulating hormone (α-MSH), and cocaine- and amphetamine-regulated transcript (CART) (5, 6). The ARC is located near the third ventricle and the hypophyseal portal system allowing wide distribution of neuropeptides into the brainstem and areas of the hypothalamus including the PVN (6, 12).

RR has been shown to decrease food intake and increase energy expenditure during the stress period resulting in weight loss on the days of stress (29). This temporary shift of energy balance may be regulated by hypothalamic neuropeptides. The third ventricle of the brain is located near the hypothalamus and is often used experimentally as a delivery site for neuropeptides or neuropeptide antagonists. CRF infused into the third ventricle causes a decrease in food intake while NPY and AgRP infusions cause an increase in food intake (32, 63). When the third ventricle is infused with CRF antagonist and then infused with NPY, food intake increases further (32). This suggests that CRF has control over NPY induced food intake (32). Studies have shown that CART, GLP-1 and CRF act independent of AgRP (20). These two neuropeptide systems may act individually to regulate energy balance, in combination with gut peptide signals and hormone signals.

Corticotropin-Releasing Factor (CRF)

CRF is considered the primary initiator of responses to stress, including activation of the HPA axis. Catecholamines, such as NE, are released in response to stress and trigger the secretion of CRF from peripheral tissues and sites throughout the brain, including the

parvocellular neurosecretory neurons in the PVN (60). CRF then activates a chain of reactions which result in the secretion of ACTH and β-endorphin from the anterior pituitary (60).

CRF has 3 homologues, urocortin I (UCN I), UCN II and UCN III. CRF and UCN mediate different aspects of the stress response via G protein-coupled CRF receptors. There are two types of CRF receptors (CRFR), CRFR 1 and CRFR 2. Each receptor is differentially distributed throughout the brain (70). CRFR1 are located at greater concentrations in the brain stem, pituitary and cortex while CRFR2 are located in the peripheral tissues and subcortical areas (70). CRF and UCN have different affinities for each receptor. CRF binds primarily to CRFR1 and has a lower affinity for CRFR2 (4, 8). UCN I binds to both receptors with equal affinity (4, 8). UCN II and III bind only to CRFR2 (4).

As CRFR1 and CRFR2 are located differentially throughout the brain and body, they are believed to play different roles in stress response based on their selective distribution and varying affinities for CRF and UCN. Research using CRFR antagonists and genetically engineered knockout mice which lack CRFR1, CRFR2 or both receptors has clarified the distinct roles CRFR1 and CRFR2 play in response to stress. CRFR1 activation has been found to activate the sympathetic nervous system, specifically trigger the activation of the HPA axis, and increase anxiety and depression (4). CRFR2 is responsible for changes in food intake and metabolism (66). CRFR2 activation results in decreased food intake, decreased anxiety, and decreased depression (4). Since UCN has a higher affinity for CRFR2, it may also play a role in energy balance. UCN receptors are located in brain areas related to energy balance (70).

Arginine Vasopressin (AVP)

Arginine vasopressin (AVP) is synthesized in the hypothalamus and acts to maintain water balance in the body. Under stress conditions AVP works with CRF to release ACTH from

the pituitary (45). Immunocytochemistry provides evidence that approximately half of CRF axons in the median eminence, which connects the hypothalamus to the pituitary, contain AVP peptides (72). Previous studies show that when AVP antagonists are administered, CRF is unable to increase secretion of ACTH (39). In chronic stress the link between AVP and CRF has clearly been shown in studies where repeated daily stress adapts CRF neurons in the hypothalamus which results in increased AVP stores (17). However, studies with animals exposed to repeated restraint stress indicates that acute stress has no effect on AVP mRNA in the PVN (44). In contrast, ADX has been shown to increase AVP mRNA in the PVN (43, 45, 50, 58). Some investigators suggest that AVP does not play a major role in response to acute or restraint stress (45), while others propose that a greater sensitivity to AVP mRNA in the PVN after stress independent of cort release indicates that feedback control over the HPA axis shifts from CRF to AVP control (49).

Central Control of the HPA Axis

The literature identifies several areas of the brain that are involved in activation of the HPA axis. Stress is sensed the hippocampus, brainstem and central amygdala (CeA) which have connections with the bed nucleus of the stria terminalis (BNST) (26). The hippocampus and BNST provide pathways for signals to stimulate the PVN to secrete CRF (26). CRF initiates the endocrine response via ACTH secretion in the pituitary which ultimately results in GC secretion from the adrenal glands.

Paraventricular nucleus of the hypothalamus (PVN)

One of the primary brain areas that shows stress-induced mRNA changes is the PVN. The PVN contains magnocellular neurosecretory cells which are responsible for the secretion of the hormones oxytocin and vasopressin (31). The parvocellular region of the PVN has been

identified as the primary source of CRF secretion (34, 42, 46, 49, 60). The parvocellular neurosecretory cells of the PVN project into the median eminence to release CRF, AVP and thyrotropin-releasing hormone (TRH) into the hypophyseal portal system (64). This portal system allows the peptide hormones to reach the pituitary and signal the production and release of ACTH. Several studies have shown that RR increases CRF mRNA in the PVN (29, 36, 38, 47, 49). This supports the theory that stress initiates a neurological response resulting in the release of CRF in the PVN to activate the HPA axis.

Decreases of CRF mRNA in the PVN have been seen when cort is injected or infused (22, 41, 42, 46, 48). Cort released during stress has been shown to act on the PVN to inhibit the HPA axis response (37). Cort binds with highest affinity to MR in the hippocampus, hypothalamus and pituitary to downregulate the secretion of CRF and ACTH (57). ADX rats have increased expression of CRF mRNA in the PVN (50), which suggests that ADX results in generalized activation of the HPA axis. When ADX rats are stressed, they show a greater neurological response since they lack the normal glucocorticoid negative feedback mechanisms (49, 50).

Central amygdala (CeA)

Additional brain structures that are implicated in stress response are the amygdalae, which are thought to activate the HPA axis (34). The amygdalae are known to process memory and emotional reactions. The amygdalae can be described as 3 nuclei with different functional traits: the cortical nucleus, the basolateral complex, and the centromedial complex. The cortical nucleus receives information from olfactory neurons and is responsible for the sense of smell and processing of pheromones. The basolateral complex of the amygdalae mediate memory response to adrenal stress hormones and neurotransmitters (53). Glucocorticoids bind to GR in the

basolateral complex to enhance memory via β-adrenoreceptors which are activated by catecholatmines (53). The centromedial complex can be separated into the central amygdala nucleus and the medial amygdala nucleus which are both believed to be the primary activators of the HPA axis (35). Both areas express MR and GR, with the medial amygdala (MeA) having noticeably fewer MR (35). The MeA seems to be activated by psychological stressors such as predator exposure and social interaction (15, 34, 35, 73). The central amygdala (CeA) appears to be activated by physiological stressors such as hypoxia or hemorrhage (35). Studies have shown that peripheral and intracerebral delivery of cort increases CRF mRNA in the CeA (25, 38, 46, 47, 65). RR stress has also been shown to stimulate CRF mRNA expression in the CeA (15) and in the MeA (35). Since studies show increased activity in the CeA and MeA during HPA axis activation, it is hypothesized that the amygdalae play a positive feed forward role in stress response (35).

The roles of the PVN and CeA in HPA axis in response to stress have been documented in frogs (73) and sheep (14) suggesting that cort regulated control of CRF is an ancient phylogenetic trait.

Bed nucleus of the stria terminalis (BNST)

The location and relationship of the bed nucleus of stria terminalis (BNST) to the amygdala suggests that the BNST plays an important role in stress response. More recent studies of CRF mRNA expression in the brain have shown that dorsolateral BNST CRF mRNA increases with stress, while the ventrolateral BNST shows decreased activity during stress (10, 33, 47, 73).

During stress, the CeA receive information from the LC, the BNST and the hippocampus (26). The CeA integrate this information and send it to the BNST (35). Due to the dense

population of GABA expressing neurons, this information is assumed to be processed via GABAergic neurons from the CeA and other overlapping projections from the limbic system to the PVN (26, 35). The BNST provides a pathway for the signal to travel to the PVN of the hypothalamus (26) while the hippocampus provides negative feedback to the PVN (26). Stimulation of the PVN produces CRF which results in the activation of the HPA axis (26).

Edinger-Westphal nucleus (EW)

The Edinger-Westphal nucleus (EW) is part of the parasympathetic nervous system which supplies signals for constriction of the muscles of the iris. A homolog of CRF, UCN I, is most abundantly expressed in the EW, but is also located in brain areas high in CRFR 2 expression (8, 40). UCN I has been shown to increase in the EW during RR (29). UCN I activity in the EW remains elevated for up to 18 hours after acute stress (40). In addition, rats exposed to chronic stress have increased expression of UCN III in the rostal perifornical area and the MeA when compared to rats exposed to acute stress (29). In mice with over abundant expression of CRF mRNA, UCN I mRNA in the EW is greatly decreased and the reverse is seen in mice lacking CRF (40). These studies suggest that UCN I plays a role in the stress feedback mechanism (40). UCN I neurons in the EW appear to respond specifically to stress, while UCN I in both the EW and CRF in the PVN act to end central responses to stress (40).

Differences in CRF and UCN mRNA expression between the PVN, CeA, BNST and EW may provide insight into the mechanisms behind sustained weight loss.

Repeated Restraint (RR) Animal Model

RR animals lose weight on the days of stress by reducing their caloric intake and expending more energy (29). When stress ends, the rats do not compensate for the weight loss and return to normal eating patterns (29). After stress, RR rats gain weight at the same rate as

CTRL rats (29). Therefore, the sustained reduction in body weight of RR rats can not be attributed to differences in food intake or energy expenditure in the post-stress period. Weight loss during RR is primarily from lean body mass (30). In the post-stress period, body composition of RR rats shifts to that of lean body mass and fat mass percentages seen in CTRL rats (74). The difference in body weight between RR and control rats has been documented for up to 3 months (29).

Serum ACTH and cort levels peak approximately 30 to 60 min after the onset of stress (21) but levels return to normal as early as the second hour of the 3 hour restraint (30). RR does not affect the diurnal pattern of cort or ACTH release in the post-stress period (27), however rats subjected to a novel stress some time after RR exhibit changes in behavior and an exaggerated corticosterone response (7). The resulting hypersensitivity of the HPA axis in RR rats is thought to result from a decreased ability for glucocorticoids to suppress HPA activity (27). The neurophysiological functions of the limbic system support the notion that it plays a key role in integrating feedback which results in HPA axis adaptation (26). Although RR has been well documented, the mechanisms behind this model of sustained weight loss are still unclear.

Despite the chronic effects of RR, restraint is a form of acute stress. Chronic stress results in an exaggerated endocrine response to a novel stress, elevated levels of baseline cort, an enlarged adrenal gland, and a reduced thymus gland (2). Chronic stress also suppresses the immune system and results in adaptive plasticity in brain and memory function (51). Acute stress enhances immune function and enhances memory (51). RR rats do not have elevated levels of baseline cort (27) and have normal thymus and adrenal gland sizes (30).

Stress in Adrenalectomized (ADX) Rats

Unpublished observations indicate that adrenalectomized (ADX) rats lose weight on the days of

restraint and regain the weight after stress ends. ADX rats return to the weight of their controls and do not show the sustained weight loss of RR rats. ADX rats do not have adrenal glands and therefore are unable to produce endogenous cort, the primary glucocorticoid secreted by rodent adrenal glands. Cort plays a major role in regulation of stress response as well as metabolism. The importance of cort in relation to sustained weight loss is unclear and requires further investigation. Several studies have replaced basal levels of cort in ADX rats (24, 41), but no daily body weight measures have been recorded for ADX rats that received stress levels of cort during restraint.

In a study done by Makino, et. al., the effect of stress on CRF mRNA expression in the brain was determined by implanting sham operated or ADX rats (n = 7-11 per group) with subcutaneous cort pellets (39mg, 21 day release) to maintain basal levels of cort (about 50 ng/mL) (49). Sham and ADX rats were then subjected to one of three treatments: 14 days of handling (nonstress, NS), 1 day of immobilization for 2 hours (on day 1), or 14 days of immobilization for 2 hours every morning (14 days) (49). Rats were killed at the end of treatment (on day 14), brains were collected and sections were hybridized for measurement of CRF (identified as corticosterone releasing hormone (CRH) in this data) mRNA and AVP mRNA in the PVN (49). Rats exposed to immobilization had higher levels of CRF mRNA but not AVP mRNA in the PVN than NS rats. ADX+CORT rats had higher CRF mRNA and AVP mRNA expression than sham rats after 1 day of immobilization suggesting that ADX rats with basal cort replacement are more responsive to stress than sham rats, especially with regards to AVP expression (49). This research alludes to the importance of the adrenal gland in feedback control of the HPA axis. In this experiment ADX rats were given basal levels of cort, but they

lacked the cort peak that occurs during stress. This cort peak may be crucial in activating CRF mRNA in the brain.

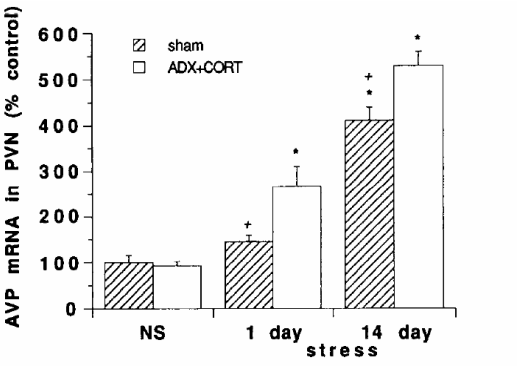

FIG. 4. AVP mRNA hybridization levels in the hypothalamic PVN after acute (1-day) and repeated (14-day) immobilization stress in sham and ADX + CORT groups. Values show percent changes compared to the NS sham group and are the mean ± SEM. *, $P < 0.01$ *vs.* NS group. +, $P < 0.01$ *vs.* ADX + CORT group.

(49)

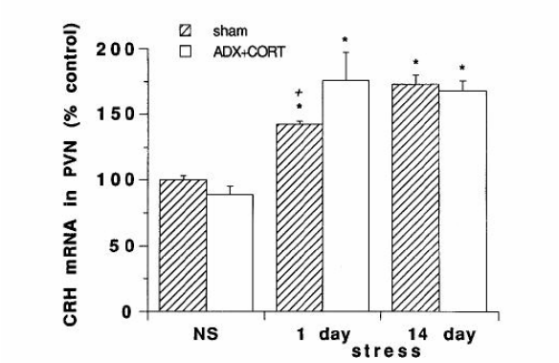

FIG. 2. CRH mRNA hybridization levels in the hypothalamic PVN after acute (1-day) and repeated (14-day) immobilization stress in sham and ADX + CORT groups. Values show percent changes compared to the NS sham group and are the mean ± SEM. *, $P < 0.01$ *vs.* NS group. +, $P < 0.05$ *vs.* ADX + CORT group.

(49)

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CHAPTER 3

THE EFFECT OF CORTICOSTERONE ON STRESS-INDUCED WEIGHT LOSS AND STRESS RELATED NEUROPEPTIDE mRNA EXPRESSION IN THE LIMBIC SYSTEM[1]

[1] Scherer, I., Holmes, P.V. and Harris, R.B.S. *To be submitted to the American Journal of Physiology.*

ABSTRACT

Rats subjected to 3 hours of restraint on 3 consecutive days (repeated restraint, RR) lose weight and do not return to the weight of non-stressed controls (CTRL). This study tested the importance of corticosterone (cort) in mediating sustained weight loss by subjecting intact, adrenalectomized (ADX) and ADX with cort replacement (ADX CORT) rats to RR. Both CTRL ADX CORT and RR ADX CORT groups were injected with 2.0 mg/kg cort before each stress. All other rats were injected with 2.0 mL/kg saline. Cort measurements on day 2 of RR confirmed the effect of ADX and replication of stress-induced cort in the cort injected rats. RR ADX CORT rats had the same initial and sustained weight loss as RR Intact rats. CTRL ADX CORT and RR ADX rats lost half as much weight as RR Intact rats on the days of stress and maintained the weight loss for a shorter period of time than RR Intact or RR ADX CORT rats. Thus both stress-induced cort and a second, undefined aspect of RR are required for the pattern of weight loss found in RR intact rats. In a second set of rats, PVN CRF mRNA expression was increased in all ADX rats and in RR Intact rats compared with CTRL Intact rats at the end of 3 hours of restraint on the second day of restraint. Cort injections did not inhibit expression in ADX rats. PVN AVP mRNA expression was increased in ADX rats but there was no significant difference in AVP mRNA expression in cort or RR rats. This suggests that cort contributes to initial and sustained weight loss in RR rats through a mechanism independent of PVN CRF mRNA.

Key words: rats, stress, repeated restraint, in situ hybridization, paraventricular nucleus of the hypothalamus

INTRODUCTION

Rats exposed to repeated restraint (RR) for 3 hours on 3 consecutive days lose weight on the days of stress and do not return to the weight of their non-stressed controls (9). Initial weight loss can be attributed to decreased food intake and increased energy expenditure on the days of stress (9). However, after stress ends RR rats do not compensate for weight loss and regain weight at the same rate as their controls (9). This suggests that the normal pathway allowing for recovery of body weight may be disrupted. The mechanism behind this chronic reduction in body weight is still unclear.

Studies indicate that RR activates the hypothalamic-pituitary-adrenal (HPA) axis resulting in release of corticotropin-releasing factor (CRF) and arginine vasopression (AVP) from neurosecretory cells of the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal portal system (29). CRF and AVP trigger the pituitary to secrete corticotropin or adrenocorticotropic hormone (ACTH) (25). Circulatory ACTH stimulates the synthesis and release of glucocorticoids (GCs) from the adrenal glands. The primary GC in rodents, corticosterone (cort), peaks between 30 and 60 min after HPA axis activation (5). This HPA axis response is under feedback control from GCs. Circulating GCs bind to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in the hippocampus, hypothalamus and pituitary to inhibit the release of CRF and ACTH (1, 27). The post-stress role of GC feedback is still unclear, but it may contribute to the sustained weight loss in RR rats.

Adrenalectomized (ADX) rats given basal cort replacement subjected to RR have been shown to lose weight on the days of stress (7). Preliminary studies from our lab indicate that although ADX rats lose weight on the days of stress, they fail to show the sustained weight loss of intact RR rats. Further exploration of cort's role in sustained weight loss and HPA axis

feedback after acute stress is required.

CRF is considered the primary central initiator of the stress response, including activation of the HPA axis. Under stress conditions AVP works with CRF to release ACTH from the pituitary (21). Some investigators suggest that AVP does not play a major role in response to acute or restraint stress (21), while others propose that a greater response of AVP mRNA in the PVN after stress, independent of cort release, indicates that feedback control over the HPA axis shifts from CRF to AVP (25). Previous studies have shown that RR increases CRF mRNA in the PVN (9, 11, 16, 23, 25) but has no effect on AVP mRNA in the PVN (20). In contrast, peripheral injections of cort or centrally infused cort decrease CRF mRNA in the PVN (6, 17, 19, 22, 24). This supports the notion that cort released during stress acts as negative feedback to the PVN inhibiting the HPA axis response (14). Cort binds with highest affinity to mineralocorticoid receptors in the hippocampus, hypothalamus and pituitary to downregulate the secretion of CRF and ACTH (27). ADX rats have elevated levels of ACTH and increased expression of CRF mRNA in the PVN (26), which suggests that ADX results in generalized activation of the HPA axis. When ADX rats are stressed, they show a greater neurological response than intact rats since ADX rats lack the normal glucocorticoid negative feedback mechanisms (25, 26).

The first experiment described here tested whether stress-associated levels of cort were necessary for initial and sustained weight loss of both intact and adrenalectomized rats. Arguments can be made that disruption of the adrenal hormones may result in changes in pituitary hormones that also could affect weight loss after stress. Hypophysectomized (hypox) rats were used to determine the whether body weight changes in RR rats were dependent on the presence of a functional pituitary. In addition, we measured CRF and AVP mRNA expression in the PVN to determine central differences in response to stress.

METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Pilot study: Accurate replication of RR cort peak by cort injection

The amount of cort to be injected to accurately replicate cort levels seen in RR rats was based on that reported by Fleschner et. al. (4) who produced an acute increase in cort in rats by subcutaneous injection of 1.0mg/kg cort dissolved in propylene glycol (4). We tested this amount to verify that the maximum cort peak would be similar to 400 ng/mL seen in restrained rats (1). The cort peak only reached 200 ng/mL. When the dose was doubled, the maximum cort value reached 400 ng/mL and accurately replicated the natural cort peak of RR rats.

Experiment 1: The effect of stress-associated levels of cort injections in adrenalectomized rats

We previously found that ADX rats subjected to RR did not lose weight (unpublished observations, therefore the objective of this study was to test whether stress associated levels of corticosterone restored sustained weight loss in ADX rats subjected to RR. Seventy-two male rats weighing approximately 310 g (Harlan Sprague Dawley, Indianapolis, IN) were housed in individual hanging wire mesh cages in a room at 21°C with the lights on for 12 hours starting at 7am. Rats were given free access to chow (LabDiet Rat Chow 5012, Richmond, IN) and water. Daily body weights and food intakes were measured daily at the start of the light period.

After one week of adaptation to housing and handling, two-thirds of rats were anesthetized with isoflurane/oxygen and bilaterally adrenalectomized via a dorsal approach. After removal of the adrenal glands, the incisions were sutured. A subcutaneous injection of analgesic (2 mg/kg ketofen) was given immediately before surgery and on the day following surgery. ADX rats had free access to 25µg cort/mL in 0.1% solution of NaCl until the end of the

experimental period.

After eight days of baseline measurement of body weights and food intakes, all rats were weight-matched into seven groups. Intact rats were separated into three groups: control with saline injections (CTRL SAL), control with cort injections (CTRL CORT) and repeated restraint with saline injections (RR SAL). There was no intact RR rat group that received cort injections. ADX rats were split into 4 groups: CTRL ADX SAL, CTRL ADX CORT, RR ADX SAL and RR ADX CORT. All groups were moved to an experimental room during times of stress. Stressed rats were placed in Perspex restraining tubes (21.6 cm long, 6.4 cm diameter; Plas Labs, Lansing, MI) for 3 hours. Non-stressed control rats were placed in shoebox cages in the same experimental room without food or water for the same duration. Before placing rats in restraining tubes, cort rats were subcutaneously injected with 2.0mg/kg corticosterone in propylene glycol. Rats not in cort groups, were given subcutaneous injections of 2.0 mL/kg sterile saline to control for the stress effect of injection. After 3 hours, rats were returned to their home cages and were given access to food and water. The same procedure was repeated on the two subsequent days. Body weights and food intakes were measured for an additional 2 weeks after the end of stress or injection.

On the second day of RR, blood samples (50 µL) were collected by tail bleeding immediately prior to placement in the restraining tubes (time point 0) and at 15, 30, 60, 90, 120 and 180 minutes after the onset of stress. Blood was collected within 2 minutes of picking up the rat and was used to measure serum corticosterone (Corticosterone Double Antibody – 125I RIA Kit Rats & Mice, MP Biomedicals, Solon, OH).

Experiment 2: The effect of stress-associated levels of cort injections in intact rats

The previous study indicated that corticosterone partially restored the sustained weight

loss in ADX repeated restrained rats, therefore the objective of this study was to determine whether corticosterone would cause sustained weight loss in non-stressed intact rats. Thirty male rats (Harlan Sprague Dawley, Indianapolis, IN) were allowed free access to chow, housed in wire mesh hanging cages and adapted to housing and handling for one week. Body weights and food intakes were measured daily. After 7 days of baseline measurements, rats were split into three weight-matched groups: intact control (CTRL), intact RR (RR) and intact rats given cort injections (CORT). RR were stressed for 3 hours on 3 consecutive days, while CTRL and CORT rats were placed in shoebox cages in the same room for the same duration. RR and CTRL rats were given saline injections and CORT rats were given cort injections prior to RR. Blood was collected on the second day of RR by tail bleeding to measure cort levels. Food intakes and body weights were measured for 2 weeks after the end of stress.

Experiment 3: Measurement of CRF mRNA and AVP mRNA

The objective of this study was to determine whether stress-induced levels of corticosterone altered CRF or AVP mRNA expression in the PVN.

Fifty-six rats were adapted to housing and handling for 7 days. Thirty-two rats were anesthetized with isoflurane/oxygen and bilaterally adrenalectomized via a dorsal approach, as described above. ADX rats had free access to 25µg cort/mL in 0.1% solution of NaCl.

Rats were divided into 7 weight-matched groups of 8 rats each. Intact rats were split into 3 groups: RR, CTRL SAL and CTRL CORT. ADX rats were split into 4 groups: CTRL ADX SAL, CTRL ADX CORT, RR ADX SAL, and RR ADX CORT. Rats were restrained for 3 hours on 2 consecutive days. Tail blood was collected at time 0 min and at time 30 min on day 2 of restraint to determine serum cort levels. Rats were decapitated at the end of 3 hours of restraint or housing in a shoe box cage on the second day of restraint. Blood was collected at the kill to

verify cort concentrations. Brains were collected, frozen in dry ice and stored at -80°C. Using the coordinates given in the rat brain atlas, the paraventricular nucleus of the hypothalamus (PVN) (AP -2.12mm) (28) was collected and analyzed for mRNA expression of CRF or AVP. All coordinates are given in relation to bregma [anteroposterior (AP)]. Fifteen micrometer sections were collected and mounted on gelatin and chromium potassium sulfate coated slides. Every tenth section was stained with 0.1% thionin to verify neuroanatomical location.

Slides were treated with 4% formaldehyde in 0.12 M sodium phosphate-buffered saline (PBS) for 5 min, rinsed with PBS three times, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine in 0.9% saline for 10 min. Slides were then treated with 70%, 80%, 95% and 100% ethanol washes, soaked in chloroform for 5 min, rinsed in 100% ethanol and rinsed in 95% ethanol before air drying.

Terminal deoxynucleotidyl transferase (Tdt, Roche, Indianapolis, IN), 35S-dATP (1250 Ci, Perkin Elmer) and tailing buffer were used to label oligodeoxynucleotide probes at the 3' end. Labeled probes were filtered through a chromatographic column (Stratagene Nuctrap Column). The CRF and AVP oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Oligonucleotide	Sequence
CRF bases 496-543 (15)	5'-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3'
AVP last 48 bases (13)	5'-GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG-3'

Slides were treated with radiolabeled probes in a buffer of 50% formamide, 600 mM NaCl, 80 mM Tris-HCl, 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 0.2 mg/mL heparin sulfate and 10% dextran sulfate for 24 hours. Slides were rinsed three times with 1xSSC and washed three times for 20 min each in 2xSSC/50% formamide at 40°C, followed by two washes in 2xSSC/50% formamide at 22°C. After one 30 min wash in 1xSSC, a brief dip in

deionized water and 70% ethanol, the slides were left to dry. Slides were exposed to autoradiographic film (GE Healthcare, Amersham Hyperfilm MP) in a dark room for 2-4 weeks. Film was developed and density of the PVN mRNA expression was measured by tracing the areas and quantifying the density using the NIH computer image analysis program ImageJ. Mean measurements were corrected for background density.

Experiment 4: The effect of cort or RR on body weight of hypophysectomized rats

A critical component of the HPA axis is the pituitary gland. There is increased release of ACTH in ADX rats due to the lack of cort negative feedback. The objective of this study was to test the role of the pituitary in stress-associated weight changes by using hypophysectomized (hypox) rats.

Thirty eight adult male rats, half of which were hypox rats (Harlan Sprague Dawley, Indianapolis, IN), were housed in hanging wire mesh cages in a 21°C room with a 12 hr light/dark cycle. All rats had free access to chow and water. Hypox rats also had continuous access to a 5% sucrose solution. Rats were adapted to housing and handling for one week before beginning daily measurements of body weights, food intakes and sucrose consumption. After one week of baseline measurements, rats were divided into five weight-matched groups: intact CTRL, intact RR, hypox CTRL, hypox RR and hypox CORT. RR groups were subjected to RR for 3 hours on 3 consecutive days. CTRL and CORT groups were placed in the same experimental room as RR for the same duration of time. Prior to placement in the experimental room, RR and CTRL rats all received an injection of saline while CORT rats received a cort injection. Tail blood was collected on day 2 of RR just prior to injections (time 0 min) and at 60 min after injections (time 60 min) to verify cort levels. Body weights and food intakes were measured for two additional weeks after the end of stress or cort injections.

Statistical Analysis

All body weight data was analyzed by two-way ANOVA, adjusted for repeated measures (Statistica). Significant differences between specific groups on particular days were determined by post hoc Duncan's multiple range test. $P \leq 0.05$ was considered significant.

All ISH data was analyzed by one-way ANOVA. Significant differences between groups were determined by post hoc Duncan's multiple range test. $P \leq 0.05$ was considered significant.

RESULTS

Experiment 1

In this study, all RR and all CORT rats lost weight on the days of stress (Figures 1A and 1B). RR ADX SAL rats lost about half of much weight as RR SAL and RR ADX CORT rats . The weight loss of RR ADX SAL rats was significantly different from that of RR ADX CORT rats or RR SAL rats only on the 3 days of RR (Figure 1B). All RR rats maintained a lower body weight than their controls for two weeks after the end of RR (Figure 1B). CTRL ADX CORT rats lost as much weight as RR ADX Sal rats (Figure 1C) and sustained a lower body weight than the other control rats until 8 days after the end of RR (Figure 1A). Neither CTRL SAL nor CTRL ADX SAL rats lost weight during the days of RR but CTRL ADX SAL rats gained weight more slowly than CTRL SAL rats (Figure 1A).

On day 2 of RR basal serum corticosterone levels ranged from 44 to 84 ng/mL at time 0 min (Figure 2). Saline injected ADX CTRL SAL and ADX RR SAL rats maintained low levels of serum cort throughout the test period (Figure 2). Serum cort of CTRL SAL rats increased to a maximum of of 251 ng/mL at time 30 min (Figure 2), but cort levels returned to basal levels at time 60 min (Figure 2). Serum cort of RR SAL, CTRL ADX CORT and RR ADX CORT rats

peaked between 450 and 550 ng/mL at time 60 min (Figure 2).

There were no differences in the food intake between groups in the 3 days prior to stress or the 3 days immediately following stress (Figure 3). During the 3 days of RR, CTRL SAL rats ate more than any other group. Food intake was reduced in both CTRL ADX SAL and CTRL ADX CORT rats compared to CTRL SAL rats and was inhibited even further in all three groups of RR rats (Figure 3).

Experiment 2

In this study, as expected, RR rats lost weight on the 3 days of RR and maintained a reduced body weight compared to controls until the end of the experimental period (Figure 4). By contrast, CORT rats did not lose weight on the days they were injected with corticosterone and both CTRL and CORT rats gained weight steadily until the end of the experiment (Figure 4).

On the second day of RR, serum corticosterone levels of saline injected CTRL rats peaked at time 30 min and were reduced to basal levels by time 60 min (Figure 5). Serum corticosterone of RR and CORT rats peaked at time 60 (Figure 5).

There were no differences in total food intake of the three groups during the 3 days prior to stress or the 3 days immediately following stress (Figure 6). Total food intake was reduced on the 3 days of stress in both RR and CORT groups when compared to CTRL rats (RR: $P<0.002$, CORT: $P<0.05$) (Figure 6).

Experiment 3

Intact rats killed on the second day of stress after the end of RR were heavier than ADX rats (Table 1). Serum cort values on day 2 of RR at time 0 min averaged 102 ng/mL. CTRL ADX SAL and RR ADX SAL rats maintained low serum cort values throughout the entire stress

procedure (Table 1). RR SAL rats had serum cort peaks at time 30 min (Table 1). Rats given cort injections also had serum cort peaks at time 30 min (Table 1). Food intakes were not measured.

The results of the in situ hybridization for CRF mRNA in the PVN are quantified in Figure 7A and typical examples of images from rats from different treatment groups are shown in Figure 7B. Both ADX and RR had increased CRF mRNA expression in the PVN when compared to CTRL SAL (Figure 7A). By contrast, cort injections had no significant effect on mRNA expression in either CTRL CORT or ADX rats.

The results for in situ hybridization for AVP mRNA in the PVN are shown in Figure 8. Neither ADX nor RR had a significant effect on mRNA expression and the only significant difference was between intact RR SAL and RR ADX SAL rats (Figure 8).

Experiment 4

None of the Hypox rats gained any weight during the study, but both Intact RR and Hypox RR rats lost weight on the days of stress and maintained a reduced body weight compared with their respective control groups until the end of the experimental period (Figure 9). Both Hypox CTRL and Hypox RR had low levels of serum cort at time 0 min and time 60 min on day 2 of RR (Figure 10). Intact CTRL and Hypox CTRL rats had a low peak of serum cort at time 30 min (Figure 10). Intact RR and Hypox CORT rats had similar serum cort peaks at time 60 min (Figure 10).

Intact RR rats had decreased total food intake on days 2 and 3 of RR when compared with Intact CTRL food intake (Figure 11). Hypox CORT rats had decreased food intake on the days 0, 1, 3 and 4 (Figure 11).

Hypox CTRL and Hypox SAL rats lost weight on the days of injections (Figure 12).

Hypox CORT rats continued to lose weight until day 7 (Figure 12). Hypox CORT rats had significant differences in weight from Hypox CTRL rats on days 5, 6 and 7 (Figure 12).

DISCUSSION

It is well known that the hypothalamic-pituitary-adrenal (HPA) axis, including cort, plays a major role in the regulation of stress response (8). The experiments described here tested the effect of stress associated levels of corticosterone on sustained weight loss in ADX rats. In addition, we measured mRNA expression of CRF and AVP in the PVN to determine activation or inhibition by corticosterone of stress-related neuropeptides.

In experiment 1, intact RR SAL rats had the same initial and sustained weight loss as RR ADX CORT rats. This indicates that cort replacement during RR acted similarly to natural cort in intact rats with regards to body weight regulation. Rats who were exposed to RR and elevated cort levels during RR exhibited both initial and sustained weight loss. In contrast, RR ADX SAL rats and CTRL ADX CORT rats lost half as much weight as RR SAL or RR ADX CORT rats. Exposure to only RR or only elevated cort levels, resulted in only part of the complete weight response. RR ADX SAL and CTRL ADX CORT rats also maintained a reduced body weight for a shorter length of time following RR. This implies that both RR and cort are each required for both initial and sustained weight loss.

Since CTRL ADX CORT rats lost weight and maintained a reduced body weight for a period of time during experiment 1, it appears that cort, independent of RR, plays part of the role in body weight regulation. Experiment 2 tested whether intact rats given cort injections would have a similar weight response without RR. Surprisingly, intact rats given cort injections did not show any changes in body weight despite having serum cort values similar to the CTRL ADX

CORT rats. Since ADX rats given cort injections lost weight but intact rats given cort injections did not, this suggests that the adrenal gland is playing a key role in feedback to prevent weight change, but that this feedback is modified during stress. Again, this confirms that both high levels of cort and some other, unidentified aspect of the stress response are required for weight loss. Cort binds to mineralocorticoids and glucocorticoids in the brain (1, 27). These signals are integrated by the central amygdala bed nucleus of the stria terminalis which then initiate action to downregulate the stress response (10). Intact CTRL CORT rats only receive the stress signals from cort, therefore the cumulative signal does not trigger physiological actions that would decrease body weight. However, in ADX rats CRF mRNA levels in the PVN and possibly in other stress-associated areas of the brain are already increased (26). When cort is injected, the brain receives sufficient signaling to trigger the physiological response to decrease body weight. ADX removes inhibition of stress so the basal state is closer to the threshold for a stress response. While cort is important in the stress weight response, it is not the sole determinant of weight response.

In experiment 4, Hypox RR rats lost weight and maintained weight loss. This confirms that weight loss caused by RR is not dependent on pituitary hormones and that body weight is regulated after acute stress in the absence of a functional pituitary gland. Hypox Cort rats had decreased food intake on the days of stress which indicates that stress effects are mediated by the adrenal gland and are independent of the pituitary. Hypox Ctrl and Hypox Cort rats lost weight on the days of stress but Hypox Cort rats continued to lose weight until 4 days after the end of stress. It appears that cort extended the amount of time needed to terminate the stress response. In intact rats, cort binds to mineralocorticoid receptor in the hippocampus, hypothalamus and pituitary to inhibit the release of CRF and ACTH (1, 27). The pituitary is known to have high

levels of CRFR1 expression, the receptor linked with HPA axis regulation (30). Hypox rats lack the feedback signal normally provided by cort binding to receptors in the pituitary and therefore have exaggerated weight loss in response to acute stress. These observations suggest that while cort acts through the adrenal gland to initiate the stress response, the pituitary plays a role in relaying negative feedback to decrease the response to stress.

As expected, food intakes of RR groups were reduced during the days of RR when compared to their controls (9). However, interestingly, food intakes were also reduced in CTRL ADX SAL and CTRL ADX CORT rats. Food intake reductions were not as substantial as those seen in RR groups but they were still significantly reduced in comparison with CTRL SAL intake during RR. While basal levels of cort normalize food intake in ADX rats (2), other investigators have shown that high levels of cort in ADX rats reduce food intake (3). Since the reduction was the same in both the cort injected and saline injected CTRL ADX rats, this suggests that the mild stress of injection or the relocation to a foreign environment on the days of RR was enough to impact food intake. We have previously shown no changes in food intake on RR days in CTRL animals (9), therefore we suggest that the stress of experimental manipulations, independent of a rise in cort, was responsible for the decreased food intake in ADX CTRL rats. This is consistent with the theory that ADX rats are more sensitive to stressors than intact rats (26). Reductions in food intake may have been potentiated by the fact that food intake and fluid intake usually occur together (31). If ADX rats drank less water, they may also have been reducing their basal level of serum cort causing further reductions in food intake.

In experiment 2, the decrease in food intake of RR rats is consistent with our previous findings (9). Contrary to findings from others (3), food intake of intact rats injected with cort was also decreased. Since food intake was decreased in RR and intact CORT rats, but weight loss

was only seen in RR rats, this indicates that cort either was not significant enough to cause weight loss or that some counter mechanism prevented weight loss. This indicates a dissociation between food intake and weight change and implies a role for energy expenditure. It may be that cort alone is unable to affect body weight and some aspect of RR other than HPA activation is required for weight change. This is consistent with the theory that cort is necessary but not sufficient to stimulate the physiological responses resulting in weight loss mediated by the CeA and BNST (10). These findings are consistent with our previous observations of a dissociation between weight loss and cort in RR rats.

In agreement with previous studies by other researchers (9, 11, 16, 23, 25), the results of experiment 3 indicated an increase of CRF mRNA in the PVN due to RR. This increase results from stress stimulating parvocellular neurosecretory cells of the PVN to release CRF (29). This implies that RR is responsible for the observed increase in CRF mRNA. We also found increased CRF mRNA expression in the PVN of ADX rats, which is consistent with previous findings (26). ADX appears to cause generalized HPA axis activation due to a lack of negative feedback by cort resulting in elevated CRF mRNA levels in the PVN. Although CTRL ADX SAL rats had increased levels of CRF mRNA in the PVN, CTRL ADX CORT, RR ADX SAL and RR ADX CORT rats did not have CRF mRNA PVN levels significantly different from those of intact CTRL SAL. This indicates that cort did not acutely downregulate CRF mRNA expression. Surprisingly, cort had no effect on PVN CRF mRNA in intact CTRL, ADX CTRL, or ADX RR rats. Cort appears to have caused an insignificant decrease of CRF mRNA in the PVN of intact CTRL rats and an insignificant increase of CRF mRNA in the PVN of RR ADX rats. This indicates that cort's actions may depend on whether the brain is receiving any additional stress signals.

By contrast, neither RR nor ADX had any effect on AVP mRNA in the PVN. The non-significant increase of AVP PVN mRNA in CTRL ADX SAL rats when compared to intact CTRL SAL rats is consistent with findings by other researchers who have shown that basal cort replacement in ADX rats normalizes AVP mRNA expression in the PVN (12). This was expected as all ADX rats had free access to cort in their drinking water. The lack of effect of RR on AVP mRNA is consistent with reports from others (20). RR ADX SAL showed decreased AVP mRNA expression when compared to intact RR SAL rats. This suggests that without glucocorticoid feedback, RR decreases AVP expression in the PVN. However, both CTRL ADX CORT and RR ADX CORT rats had the same level of AVP mRNA expression as intact CTRL SAL rats. This implies that while RR decreases AVP expression in ADX rats, AVP mRNA expression is normalized in ADX rats when cort is injected, indicating that cort plays a major role in negative feedback on AVP mRNA.

Serum cort levels of cort injected rats accurately matched intact RR SAL levels but did not fall as quickly as RR SAL cort levels. This may be due to delivery method. The natural release of cort in RR SAL rats indicates a quick rise in cort which peaks around 60 min, followed by a steady decrease. This is most likely due to the degradation of cort by the liver as the half-life of cort is 10 min (18). CTRL ADX CORT and CTRL ADX RR rats were injected with cort dissolved in propylene glycol for slow release and accurate replication of the levels and time points seen in natural cort responses to stress. The injected cort may have still been diffusing from the propylene glycol at time points after 60 min when normal cort secretion from the adrenal gland would decrease and degradation of cort by the liver would be increased. This may account for the slower rate of serum cort level reduction in cort injected rats.

In conclusion, the results of these experiments demonstrate that RR and cort from the adrenal glands independently contribute to the initial and sustained weight loss observed in RR rats. Elevated levels of CRF mRNA in the PVN, whether from ADX or RR, are required for cort's action on weight regulation following acute stress. The pituitary gland is not required for activation of this stress-associated weight response, but may play a negative feedback role in terminating the stress response. Further research is needed to determine cort's specific site of action.

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FIGURES

Figure 3.1A: Body weight change of control rats groups (n=9-12) in Experiment 1. Days of stress are denoted with arrows. Significant differences (P<0.05) between CTRL ADX CORT and CTRL ADX SAL are indicated by asterisks.

Figure 3.1B: Body weight change of RR rat groups (n=10-13) in Experiment 1. Days of stress are denoted with arrows. Significant differences (P<0.05) between RR ADX CORT and RR ADX SAL are indicated by asterisks.

Figure 3.1C: Total weight loss during stress in Experiment 1. Superscript letters indicate a significant difference between groups (P<0.05).

Figure 3.2: Serum corticosterone levels measured on day two of RR in Experiment 1. Data are means for 9 or 10 rats.

Figure 3.3: Total average food intake per rat as measured three days pre-stress, during stress and post-stress. A superscript letter indicates a significant difference (P<0.05) between groups.

Figure 3.4: Body weight change of rat groups (n=10) in Experiment 2. Days of stress are denoted with arrows. Significant differences (P<0.05) between RR and CTRL or CORT are indicated by asterisks.

Figure 3.5: Serum corticosterone levels measured on day two of RR in Experiment 2. Data are means for 9 or 10 rats.

Figure 3.6: Total food intake as measured three days pre-stress, during stress and post-stress. Superscript letters indicate a significant difference (P<0.05) from the CTRL group.

Figure 3.7A: CRF mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) in Experiment 3. Superscript letters indicate significant differences between groups (P<0.05).

Figure 3.7B: Autoradiographic images from different treatment groups in Experiment 3.

Figure 3.8: AVP mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) in Experiment 3. Asterisks indicate a significant difference from the Intact RR SAL group ($P<0.05$).

Figure 3.9: Body weight change in rat groups (n=9) subjected to repeated restraint in Experiment 4. Arrows indicate days of RR. Asterisks indicate a significant difference between Hypox RR and Hypox CTRL groups ($P<0.05$). Pounds (octothorpes) indicate a significant different between Intact Control and Intact RRS ($P<0.05$).

Figure 3.10: Serum corticosterone levels measured on day two of RR in Experiment 4. Data are means for 9 or 10 rats.

Figure 3.11: Food intake in rat groups (n=9) subjected to repeated restraint or corticosterone injections in Experiment 4. Arrows indicate days of RR or cort injection. Asterisks indicate significant differences between Intact CTRL and Intact RR ($P<0.05$) Plus signs indicate significant differences between Hypox CTRL and Hypox CORT ($P<0.05$).

Figure 3.12: Body weight change in rat groups (n=9) subjected to corticosterone injections in Experiment 4. Arrows indicate days of injection. Asterisks indicate significant differences in Hypox CORT compared to Hypox CTRL ($P<0.05$).

TABLES

Table 3.1: Body weights and serum cort values of rat groups in Experiment 3

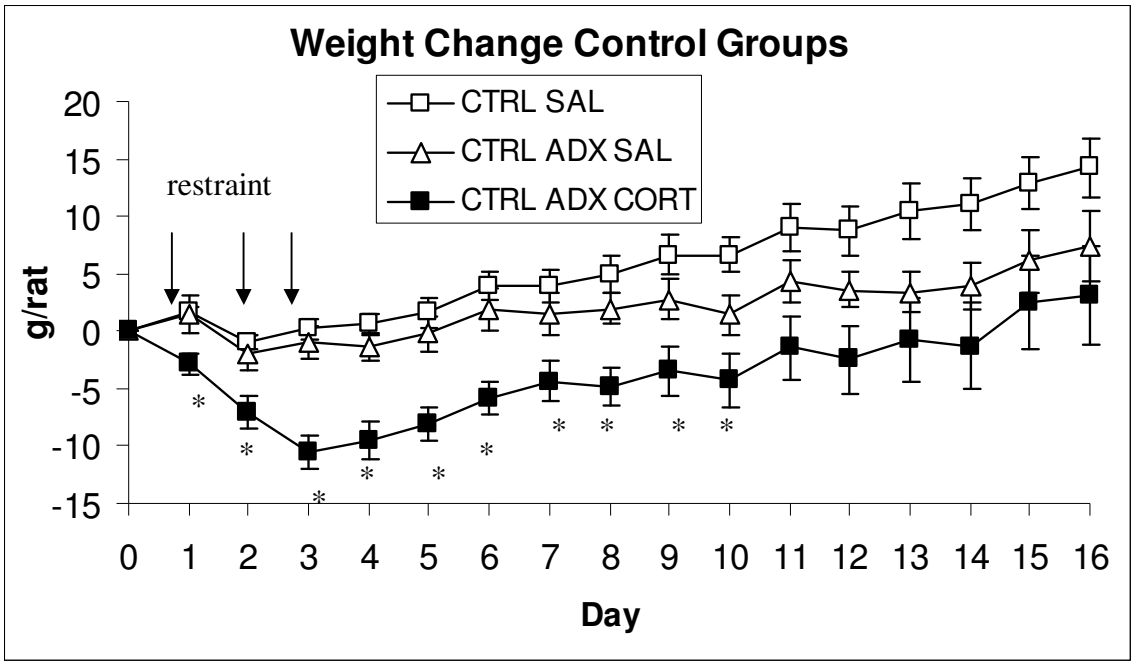

Figure 3.1A: The effect of RR on body weight change in control groups

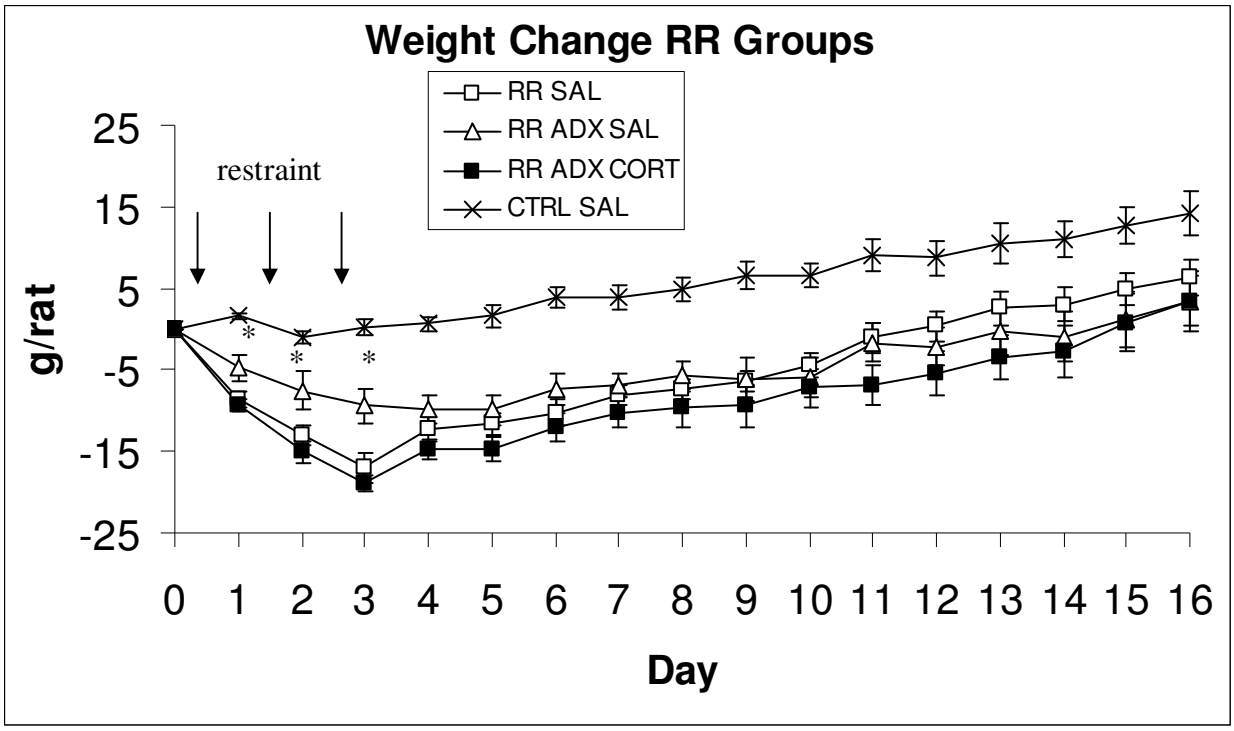

Figure 3.1B: The effect of RR on body weight change in RR groups

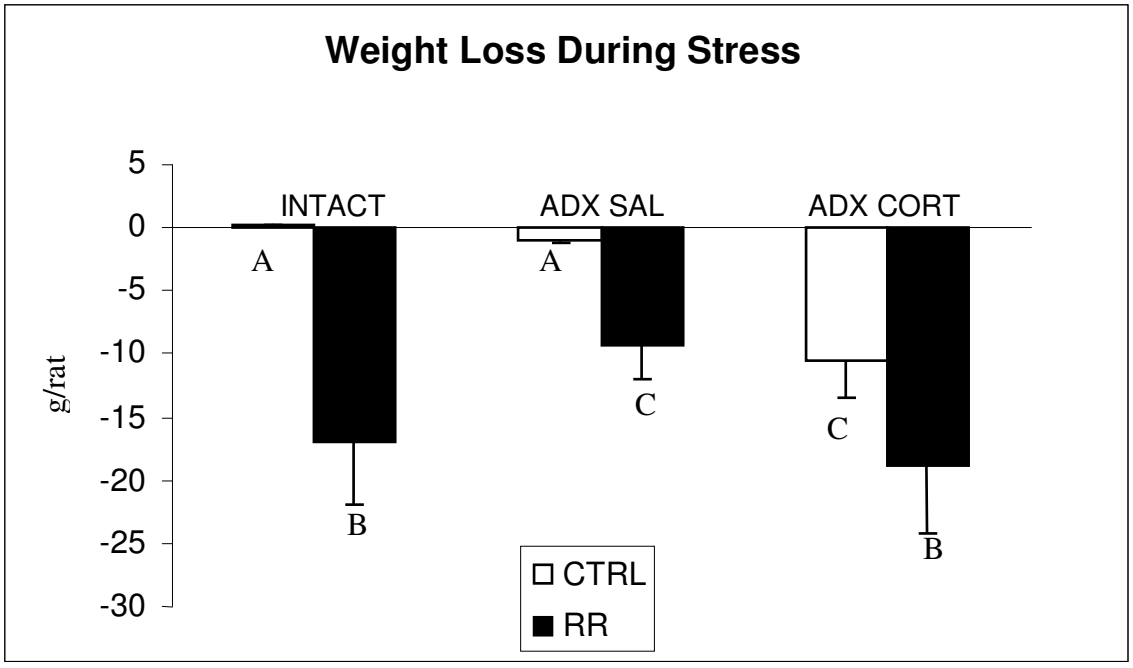

Figure 3.1C: Total weight loss during stress

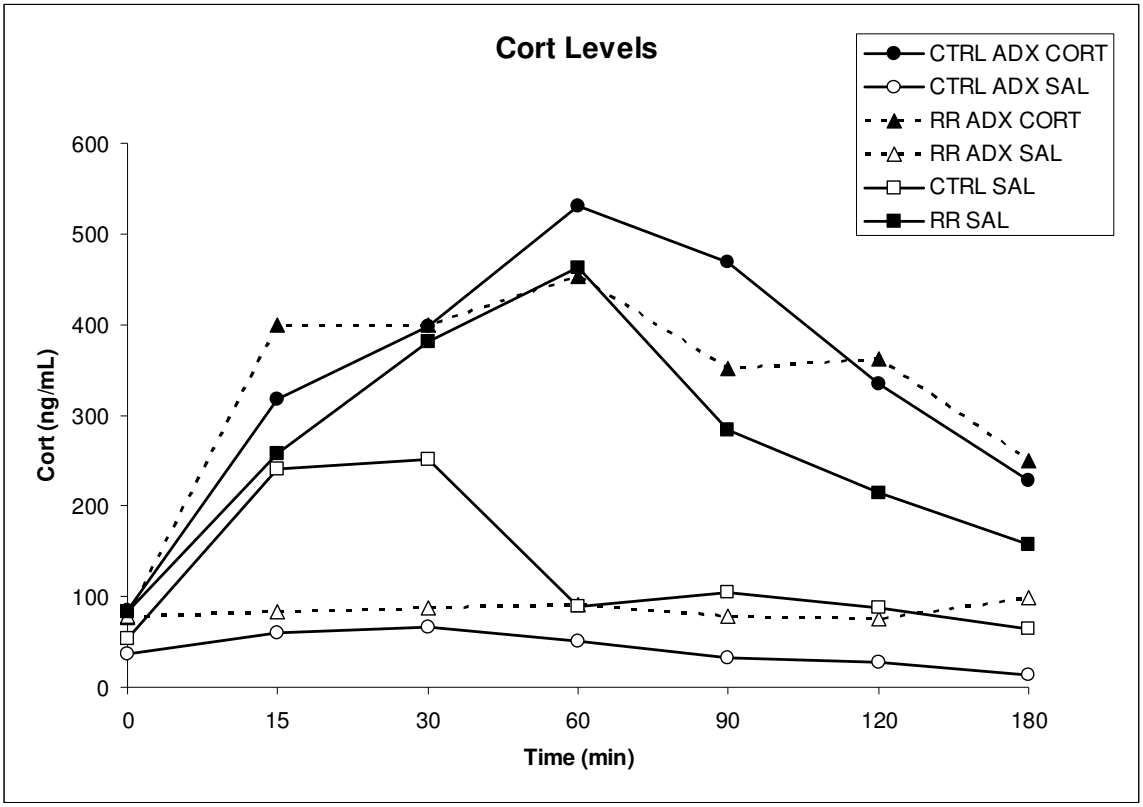

Figure 3.2: Corticosterone levels on day 2 of RR

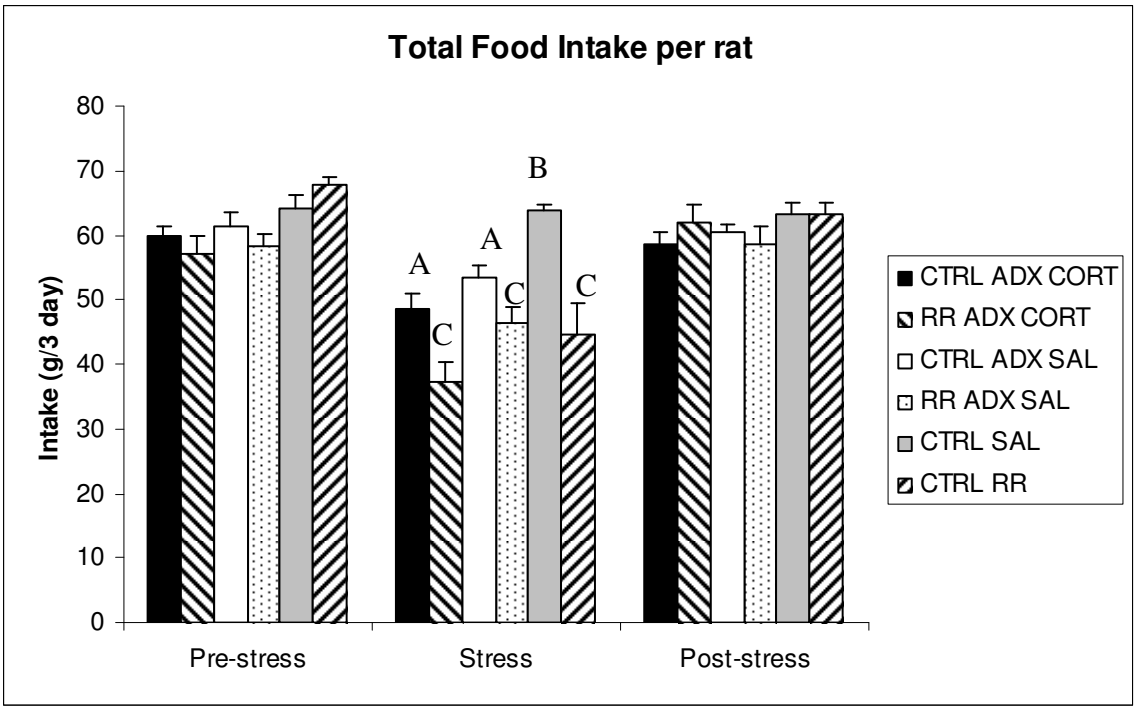

Figure 3.3: Food intake on the three days prior to stress, the three days of stress and the three days following the end of stress

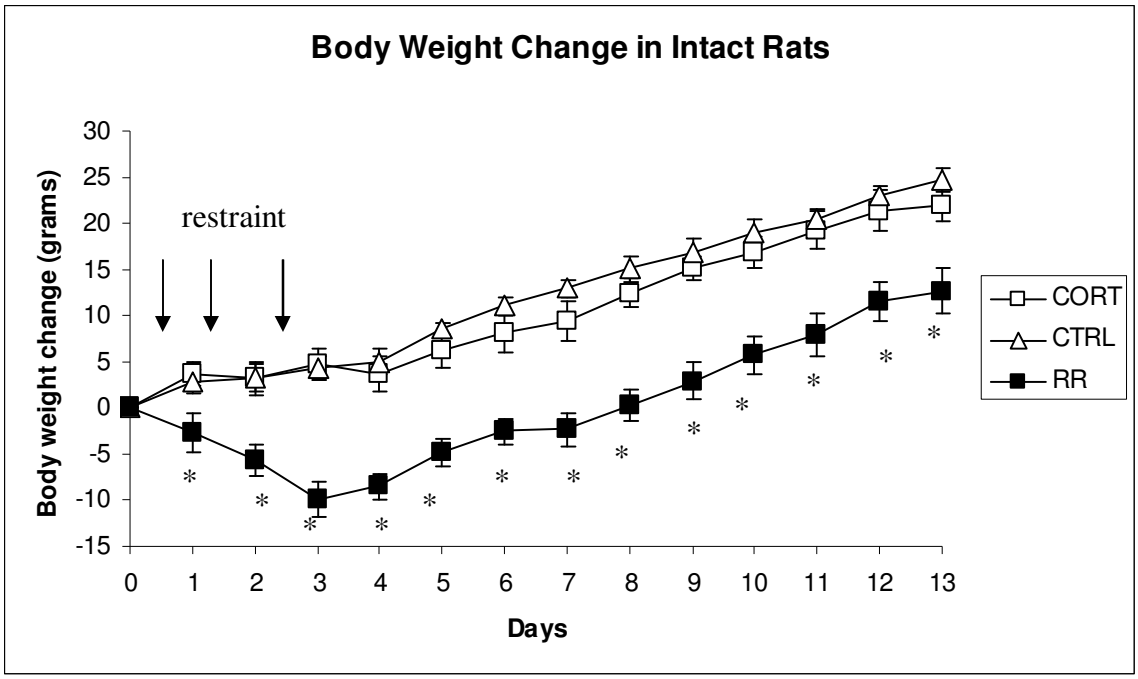

Figure 3.4: The effect of RR or corticosterone injection in intact rats

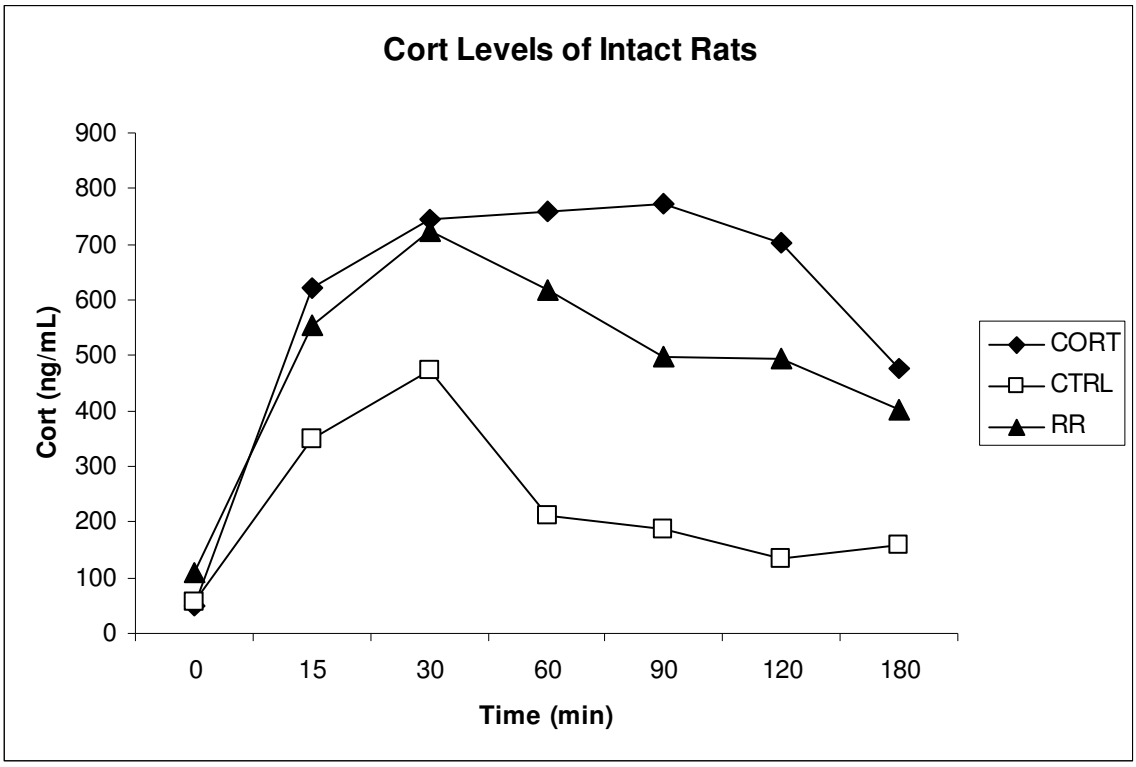

Figure 3.5: Corticosterone levels on day 2 of RR

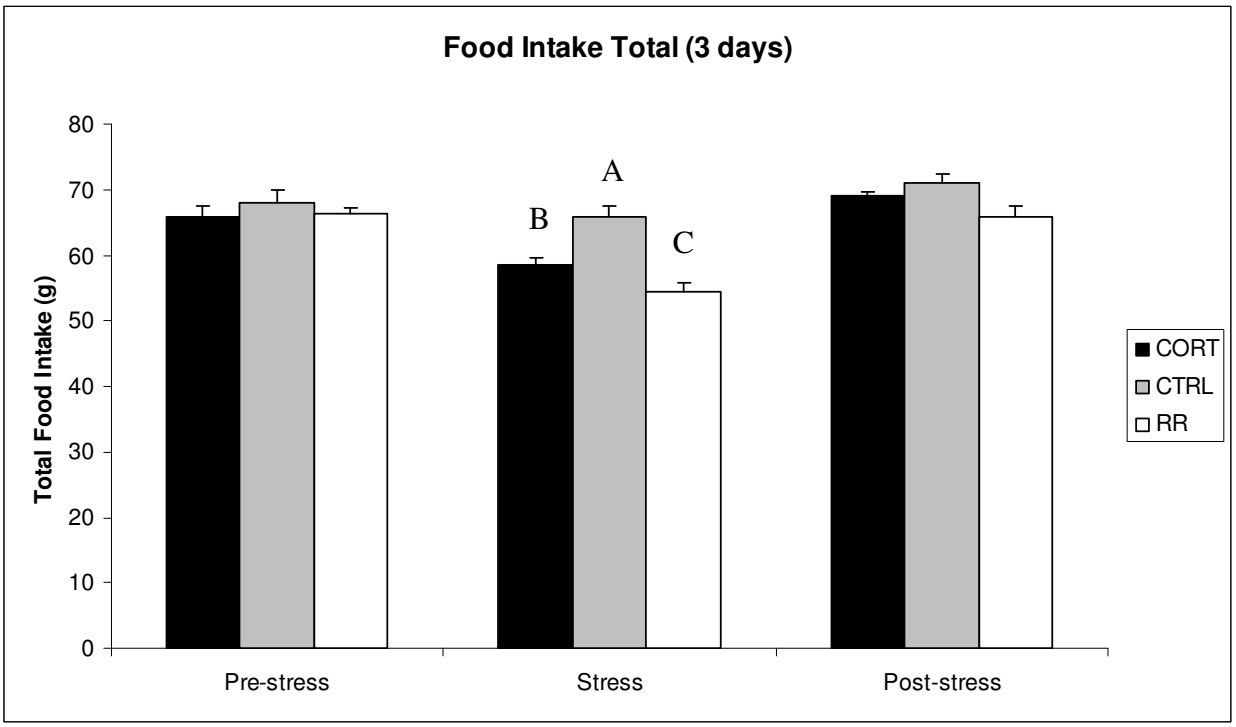

Figure 3.6: Food intake of intact rats on the three days prior to stress, the three days of stress and the three days following the end of stress

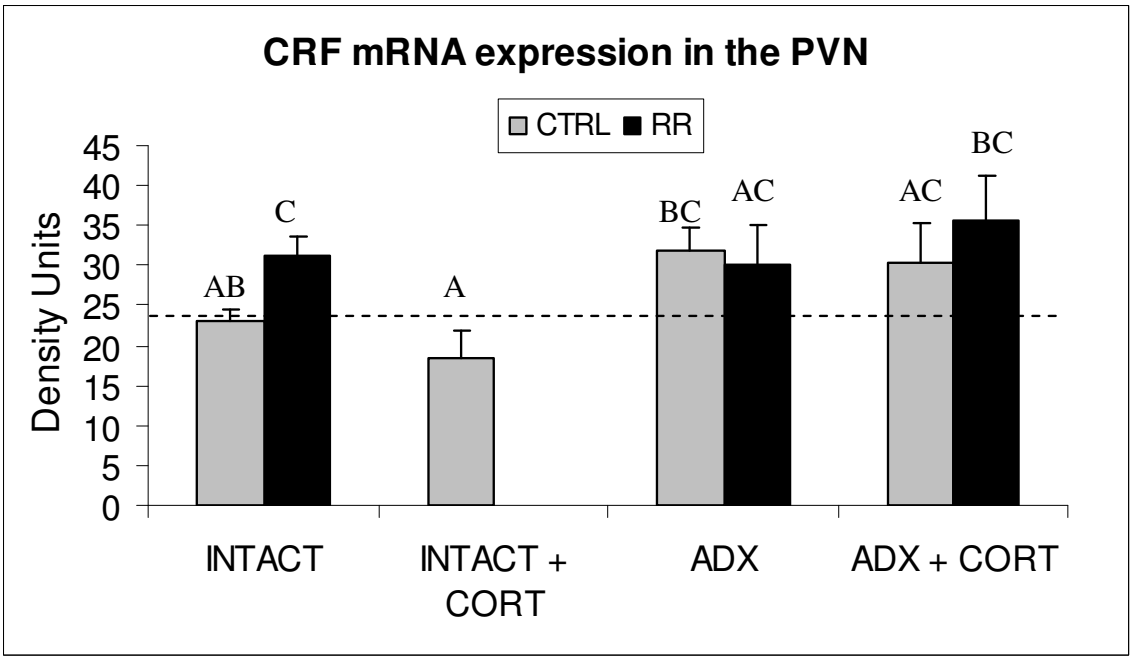

Figure 3.7A: CRF mRNA expression in the PVN

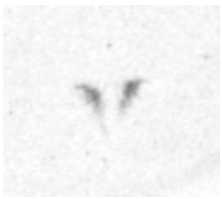

Figure 3.7B: CRF mRNA expression images from the PVN

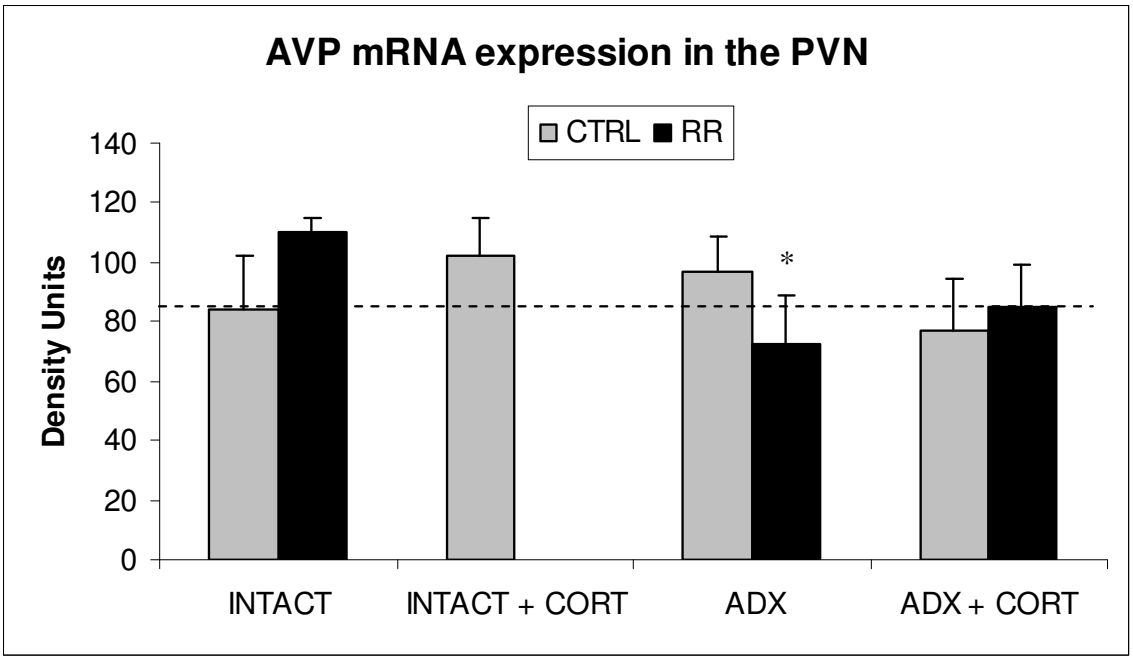

Figure 3.8: AVP mRNA expression in the PVN

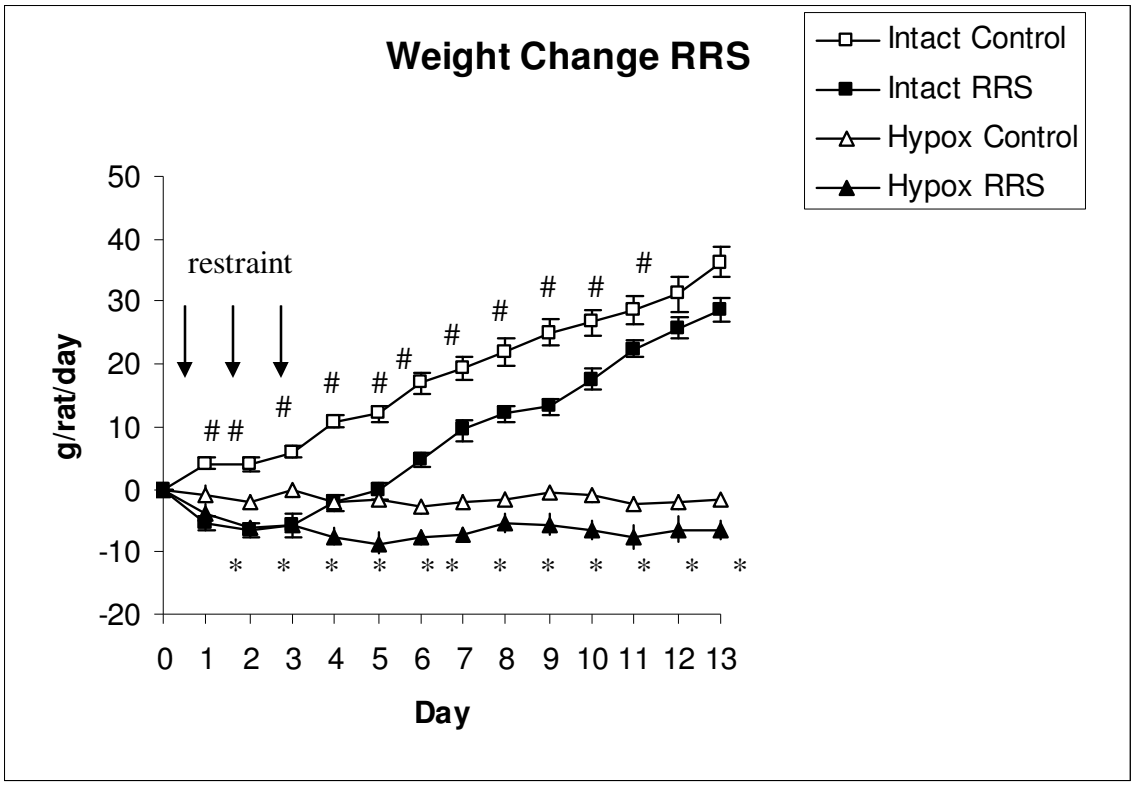

Figure 3.9: The effect of RR on body weight in intact and hypox rats

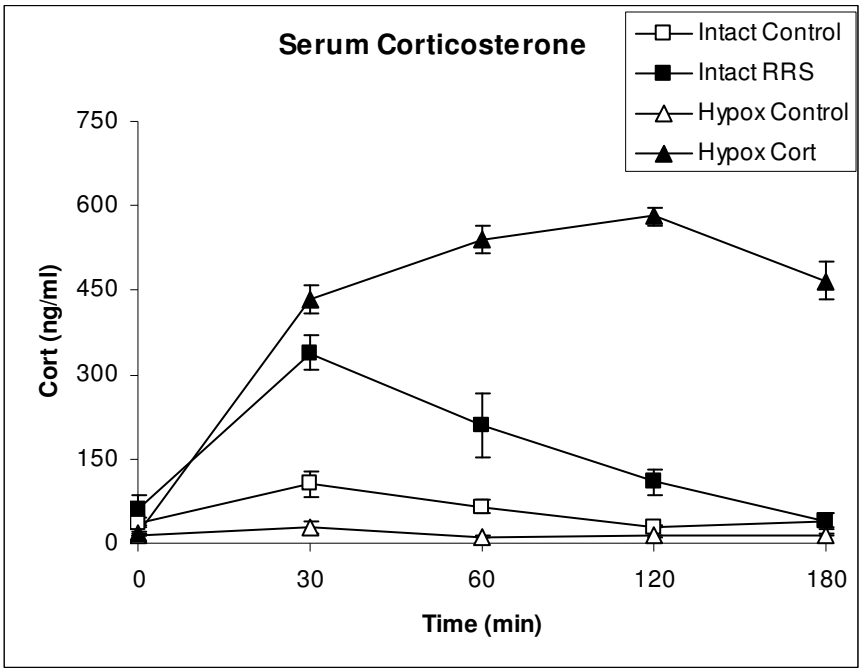

Figure 3.10: Corticosterone levels on day 2 of RR

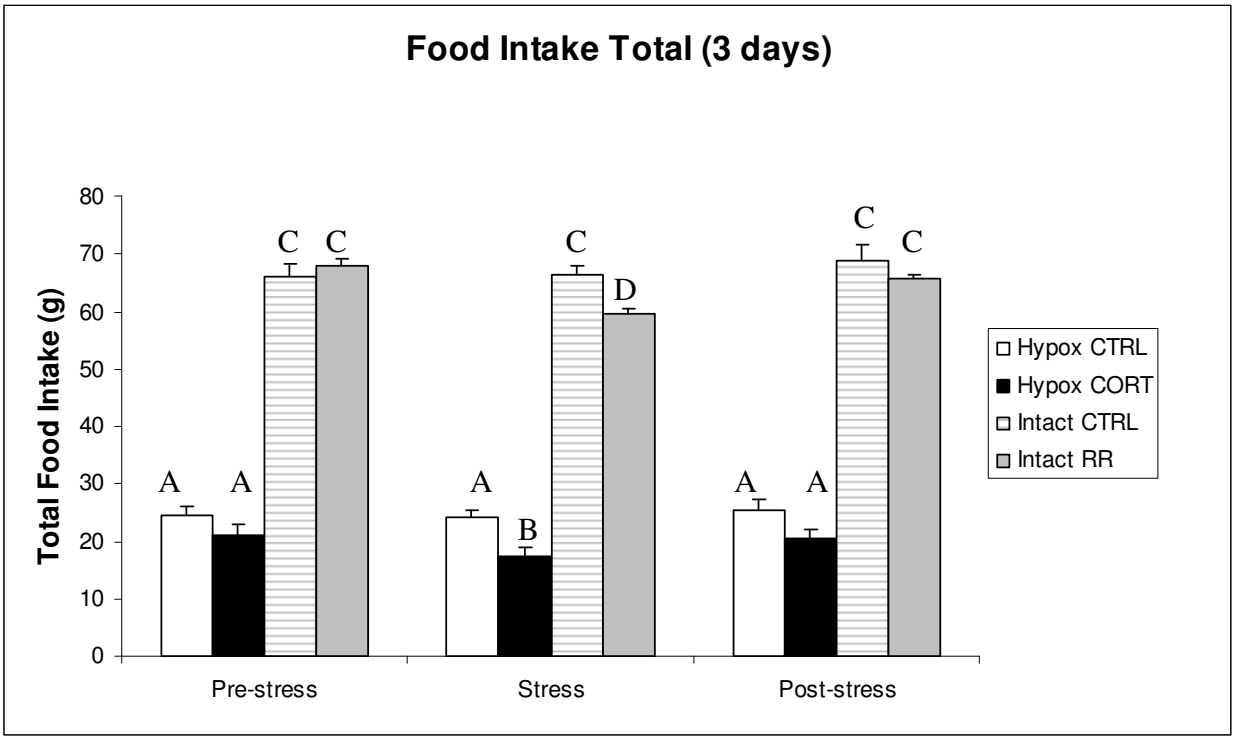

Figure 3.11: Food intake of hypox and intact rats

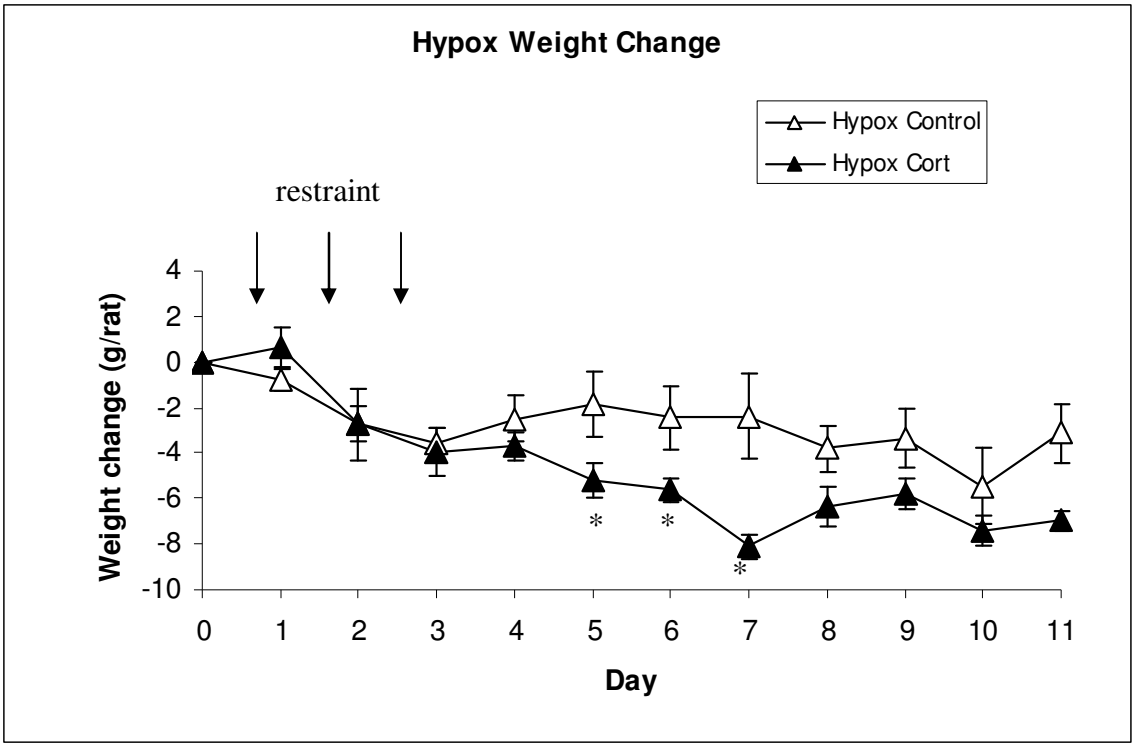

Figure 3.12: The effect of corticosterone injections on body weight in hypox rats

Surgery	Intact			Adrenalectomized			
Body weight (g)	373			296			
Stress	CTRL		RR	CTRL		RR	
Injection	SAL	CORT	SAL	SAL	CORT	SAL	CORT
Serum cort (ng/mL)							
0 min	68	69	90	136	63	140	151
30 min	431	679	646	55	563	117	575

Table 3.1: Body weights and serum cort values of rat groups in Experiment 3

CHAPTER 4

SUMMARY AND CONCLUSION

The hypothalamic-pituitary-adrenal (HPA) axis is a well-characterized neuroendocrine system that uses a classic feedback mechanism. Stress and other threats to homeostasis stimulate the limbic system in the brain to trigger activation of the HPA axis. Activation induces corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) secretion from stress responsive areas in the brain which ultimately results in a secretion of glucocorticoids from the adrenal glands (2). Glucocorticoids, such as corticosterone (cort) in rodents, bind to mineralocorticoid and glucocorticoid receptors throughout the body and brain and act to downregulate the stress response (1). Stress may make permanent changes to physiological responses resulting in chronic effects of stress.

A unique animal model for investigating the mechanism of stress response is the repeated restraint (RR) rat model. Rats exposed to 3 hours of restraint on 3 consecutive days lose weight on the days of restraint and do not return to the weight of their controls following the end of stress (3). RR allows researchers to investigate the mechanism behind this interesting paradigm of chronic body weight reduction.

The first objective of the studies described here was to determine the effect of cort on initial and sustained weight loss in RR rats. We found that cort fully restored initial and sustained weight loss in restrained adrenalectomized (ADX) rats. Rats that were only exposed to RR or only received cort peaks, had only part of the post-stress weight response. Rats with exposure to both RR and elevated cort levels exhibit both initial and sustained weight loss. These results

indicate that cort and RR each play a role in the post-stress weight response. While ADX rats given cort lost weight, intact rats given cort did not. This indicates that the adrenal glands play a critical role in feedback of weight response to stress. Cort injections in hypox rats produced an extended period of weight loss, suggesting the importance of the pituitary in terminating the response to stress. Future studies will determine the specific sites of cort action, focusing on mineralocorticoid and glucocorticoid receptors. Results of these studies will help elucidate the mechanism behind sustained weight loss.

The second objective of the studies was to determine cort's effect on CRF and AVP mRNA expression in the PVN. Our results agree with findings from previous studies that indicated elevated levels of CRF mRNA in the PVN of RR (3-7) and ADX rats (8). From these experiments we concluded that under stress conditions, AVP may be sensitized by cort and feedback control may shift from CRF to AVP control (7). Other researchers have measured mRNA expression in the limbic system of ADX rats with basal concentration of cort (7). Therefore, our data offer new information to the central mechanisms of HPA axis control following stress-induced cort levels. Future studies will explore CRF receptor expression in response to elevated cort levels.

We propose that while cort plays an important role in the post-stress weight response of RR rats, there is an independent factor of restraint that is required for the complete initial and sustained weight loss. This factor is separate from the adrenal gland, because RR ADX SAL rats experienced weight loss following stress.

Based on the results of the studies described here, we propose that future studies further investigate the role of mineralocorticoid and glucocorticoid receptors in mediating changes in body weight following stress. The effects of cort on these receptors may provide valuable insight

into HPA axis regulation. Completion of these studies would yield a better understanding of the mechanisms behind the sustained weight loss of RR rats and provide insight into the cause of weight regain in other models of weight loss.

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