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Neurochemical Mechanisms Involved in the Anticipation, Ingestion and Termination of a Meal : Focus on Corticotropin-Releasing Hormone and Bombesin-like Peptides

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Neurochemical Mechanisms Involved in the Anticipation, Ingestion and Termination of a Meal: Focus on Corticotropin-Releasing Hormone and Bombesin-Like Peptides

A Doctoral Dissertation

By

Judith F. McIntosh

Submitted as partial fulfillment of the requirements for the degree of

Doctor of Philosophy to the School of Graduate Studies,

University of Ottawa

Dedicated to

My wonderful Dad who had the gift of honesty and wisdom

Henry Kennedy McIntosh
1911 – 1971

My loving Mom who dedicated her life to her family

Martha Dreghorn (Orr) McIntosh
1910 – 2001

My precious Brother who was my hero

Andrew Kennedy McIntosh
1944 - 1999

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Abbreviations

ACTH	Adrenocorticotropin hormone
α-MSH	Alpha-melanocyte-stimulating hormone
AH	Anterior hypothalamus
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BN	Bombesin
BN-LPs	Bombesin-like peptides
BNST	Bed nucleus of the stria terminalis
CART	Cocaine- and amphetamine-regulated transcript
CeA	Central nucleus of the amygdala
CCK	Cholecystokinin
CRH	Corticotropin-releasing hormone
CRH-BP	CRH binding protein
DA	Dopamine
DM	Dorsomedial hypothalamus
DOPAC	Dihydroxyphenylacetic acid
5-HT	Serotonin
5-HIAA	5-hydroxyindoleacetic acid
GABA	Gamma-aminobutyric acid
GRP	Gastrin releasing peptide
H	Neonatal handling (brief 15-min exposure)
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
ICV	Intracerebroventricular
Ir-	Immunoreactive
KRB	Kreb's Ringer Phosphate
LH	Lateral hypothalamus
mPFC	Medial prefrontal cortex
mPOA	Medial preoptic area
MS	Maternal separation (protracted 3-hour separation)
NE	Norepinephrine
NH	Not handled (neonatal)
NMB	Neuromedin B
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
RIA(s)	Radioimmunoassay(s)
Ucn	Urocortin
VMH	Ventromedial hypothalamus

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Abstract

The apparently simple decision to initiate or terminate a meal is extremely complex. However, the need to determine the physiological and psychological mechanisms regulating these processes has taken on an air of urgency due to escalating levels of eating disorders in our society. Ingestive patterns are influenced by a number of factors, including signaling circuits utilizing peptides, such as corticotropin-releasing hormone (CRH) and bombesin-like peptides (BN-LPs). As some of the peptidergic systems under study have also been implicated in the mediation of the stress response, some peptidergic linkages between 'stress' and 'satiety' responses are also explored. The overall objective of this thesis was to examine the pattern of utilization or release of BN-LPs and CRH at specific brain loci during 1) various phases of food ingestion, 2) the presentation of anticipatory cues paired with an appetitive event, and 3) exposure to stressors. Using *in vivo* microdialysis, we demonstrated that both CRH and BN-LPs are released from the central nucleus of the amygdala in response to both restraint stressor exposure and spontaneous food ingestion, an effect that was mirrored at the anterior pituitary. Furthermore, we demonstrated that CRH and BN-LPs were released at the medial prefrontal cortex in response to the "anticipation" of a palatable snack. These results raised the intriguing possibility that some aspects of both the feeding behavior and the organism's response to stressor exposure are being subserved by a common neurochemical mechanism(s). As such, it would be expected that variables that affect an organism's stress response would also have an impact on feeding behavior and related neurochemical processes. In fact we found that early life experience, gender and genetics variables (that appear to impact the stress response) also impact on the animal's

neurochemical response(s) to ingestion as well as their propensity to consume a palatable snack. These experiments extend our current knowledge of the mechanism(s) underlying the control of food intake and support our contention that these systems may also serve to draw attention to events or cues of biological salience. Finally, these results also provide new insights into the potential mechanism(s) underlying various stress and eating dysfunctions, including anorexia nervosa, anorexia associated with AIDS, cancer, sickness or stress, bulimia nervosa, obesity and depression.

Summary

The regulation of complex behaviors such as feeding potentially depends on multiple and overlapping neuronal (including distinct neuropeptidergic) networks. In that regard, corticotropin-releasing hormone (CRH), noted for its ability to stimulate the hypothalamic-pituitary-adrenal (HPA) axis in response to stressful stimuli, has also been shown to suppress food intake. Moreover, endogenous levels of CRH, particularly at certain hypothalamic and amygdaloid sites, fluctuate in close temporal association with the feeding status. In addition to CRH, bombesin (BN), a tetradecapeptide of amphibian (*Bombina bombina*) origin, and its mammalian counterparts (gastrin releasing peptide and neuromedin B) are well recognized for their ability to reduce food intake while inducing a repertoire of satiety-like behaviors. Furthermore, changes in endogenous levels of BN-like peptides (BN-LPs) at various feeding-relevant brain nuclei accompany spontaneous food ingestion and satiety. It is of interest to note that, like CRH, central administration of BN has been shown to stimulate the HPA axis. These findings suggest that both BN-LPs and CRH might be involved in the regulation of two seemingly diverse behaviors - response to stressor exposure and feeding. Thus, the overall focus of this thesis is to present evidence supporting the contention that 1) CRH and BN-LPs are released in response to both aversive and appetitive events and b) factors that influence an organism's response to stressor exposure also impact feeding behavior. Summarized below are the six specific objectives addressed and their key outcomes:

1. Prior research in our laboratory had demonstrated that post mortem tissue levels of BN-LPs and CRH fluctuated at various feeding-relevant brain sites, including

hypothalamic nuclei and the central nucleus of the amygdala, in close temporal association with the animals' feeding status. In order to determine whether these changes translated to dynamic changes in peptidergic release, we used *in vivo* microdialysis sampling (followed by *ex vivo* radioimmunoassay), to monitor the interstitial (rather than tissue) levels of CRH and BN-LPs during appetitive (food intake) and stressful (restraint) events. Our findings indicated that the *in vivo* release of CRH and BN-LP(s) at the central nucleus was markedly increased by both stressor exposure and food ingestion. Paralleling these findings, circulating adrenocorticotropin hormone and corticosterone levels were also increased in response to both food intake and restraint. These results indicate that either food ingestion is interpreted as a "stressful" event by certain neural circuits involving the central nucleus of the amygdala, or that the CRH and/or BN-related peptidergic system(s) may serve a much broader role than previously envisioned.

2. It is known that the central nucleus of the amygdala and the HPA axis are intimately and reciprocally connected. Thus, in the second experiment, we set out to explore whether the increased release of CRH and BN-LPs at the central nucleus in response to food intake translated into increased availability of these peptides at the anterior pituitary, one of the primary targets of CRH action. In addition to the rapid responses, stressor exposure can also induce protracted changes (including phenotypic changes) in peptidergic expression. In the next experiment, we set out to test the effects of acute (followed by the passage of time) and chronic exposure to restraint stress on basal as well as feeding-induced changes in the release of CRH,

BN-LPs (gastrin releasing peptide and neuromedin B), arginine vasopressin and corticosterone at the anterior pituitary. Using *in vivo* push-pull perfusion we found that, in response to the presentation and consumption of a palatable snack, there was increased availability in the interstitial levels of CRH, BN-LPs neuromedin B and gastrin releasing peptide, at the anterior pituitary in 'non-stressed' control rats. Furthermore, exposure to either an acute or chronic stressor elevated basal levels of CRH, gastrin releasing peptide, neuromedin B, and arginine vasopressin within the anterior pituitary. Finally, animals that had increased basal levels of CRH, neuromedin B and gastrin releasing peptide displayed a relatively blunted peptidergic response to the consumption of a palatable snack. Taken together, these findings provide further evidence for an interrelationship between the systems governing feeding behavior and the response to stressor exposure. They raise an intriguing possibility that stressor exposure may have protracted impact on neurochemical, particularly peptidergic, responses to appetitive events.

3. The above-mentioned experiments showed that the release of CRH and BN-LP (gastrin releasing peptide) at the central nucleus of the amygdala and the anterior pituitary was provoked by an appetitive event (feeding). However, it is not clear whether this is purely a physiological response or could it be subserving a psychological response(s) as well. In the third study, using auditory, olfactory and visual cues, we trained one group of rats to expect or "anticipate" the daily presentation of a palatable snack (Cue Relevant group). Two control groups were similarly trained, but, for one group, the snack was discontinued at least two weeks

prior to testing (Extinction group), and for the other group, the snack was never paired with the cues (Cue Irrelevant group). We used *in vivo* microdialysis sampling followed by radioimmunoassays and high performance liquid chromatography to monitor the release of CRH, BN-LPs, 5-hydroxyindoleacetic acid (5-HIAA), dopamine and dihydroxyphenylacetic acid (DOPAC) at the right and left medial prefrontal cortex in response to the "anticipatory" cues and subsequent consumption of the snack. We found that the release of CRH at the medial prefrontal cortex was increased in response to the anticipatory cues and that this release displayed a right-sided bias. 5-HIAA release was similarly increased in response to snack consumption. The release of BN-LP(s) (gastrin releasing peptide), which was most prominent in the left medial prefrontal cortex, was of a similar magnitude in both the Cue Relevant and Extinction group of animals, but only reached statistical significance in the Extinction group. Behaviorally, Cue Relevant animals were more vigilant during the "anticipatory" sampling period. These results support the view that these systems may serve to allocate incentive salience and/or reward value to biologically significant stimuli.

4. If the organism's response to both ingestion (an appetitive event) and stressor exposure (an aversive event) is being subserved by common neurochemical mechanisms, one would expect that stimuli that affect the stress response would impact on ingestive behavior. An individual's response to a stressor depends on a multitude of variables including experiential factors such as early life experiences. Neonatal handling and maternal separation are known to induce permanent alterations

in the HPA axis and behavioral responses to stressors. However, little is known about the impact of early life experiences on appetitive responses. Experiment 4 examined the effect of brief handling/separation or protracted separation from the dams on the animals' physical development, anxiety level, ingestion of a palatable snack, and response to known satiety peptides. At weaning, the Maternal Separation rats weighed significantly less than both the Handled and Non-Handled animals. When tested on the elevated plus-maze, Handled male and female rats and Maternal Separation females displayed less anxiety than Non-Handled gender-matched controls. The Handled animals of both genders, and the females of the Maternal Separation group, consumed more of the palatable "snack" than their Non-Handled counterparts. The feeding suppressant response to the various satiety peptides (BN, cholecystokinin, and amylin) was not affected by the early life experience, with exception of cholecystokinin and amylin (females only). These results suggest that early life events may contribute to anxiety and/or ingestive disorders and support our hypothesis that variables that affect the stress response also impact on feeding behavior.

5. In addition to experiential variables, organismic factors (gender, genetics, and age) can also affect a mammal's responses to its environment. Research over the past few decades has highlighted the fact that males and females are different in a variety of ways including their neurochemical responses to stressor exposure. In Experiment 5, we monitored the consummatory behavior of male and female rats. Non food-deprived rats were given a daily one-hour concomitant access to a palatable high

carbohydrate "snack" (Graham Wafers) and rat chow pellets. At the end of one month, the snack access was discontinued and 24-hour chow intake was measured for an additional week. As a proportion to their body weight, females consumed almost twice as much of the Graham Wafers during the snack period than the males. Furthermore, the females' 24-hour intake exceeded that of the males whilst the snack period was part of their diet, an effect that disappeared after the snack was discontinued. Finally, the females' Graham Wafer intake decreased during the estrus phase of their cycle. Taken together, the results of this study suggest that the females display a greater propensity to consume a palatable snack, which may be significant when considering disordered feeding patterns such as obesity, anorexia nervosa and bulimia nervosa, that are more prevalent in the females.

6. Genetic makeup is another organismic factor that impacts on the organism's response to stressor exposure. Our laboratory has characterized the stress responses of two lines of rats selectively bred for differences in amygdala kindling (Fast and Slow), and found that these rat lines differ in their endocrine responses to stressor exposure. Experiment 6 was designed to investigate potential differences between Fast and Slow animals in their feeding behavior as well as in their feeding-related neurochemical responses. When Fast and Slow rats were compared for their propensity to consume a palatable snack, the Slow rats consumed more Graham Wafers and sucrose than their Fast counterparts. Slow rats were more sensitive to diazepam, requiring relatively low doses to enhance sucrose intake. The Slow rats were also more sensitive to the hypophagic effect of a novel environment, which was

reversed with diazepam. At the end of the experiment, the animals were sacrificed and their brains were extracted. A micropunch survey of feeding-relevant brain nuclei revealed that, in response to the ingestion of a palatable snack, Slow animals (compared to the Fast animals) exhibited higher tissue levels of CRH and BN-LP (gastrin releasing peptide) levels in the ventromedial nucleus of the hypothalamus. Also, in the Slow rats, feeding-induced levels of CRH were significantly higher in the medial amygdala. While basal levels of gastrin releasing peptide were similar in these two lines of rats, basal levels of CRH were elevated in the hypothalamic paraventricular nucleus of the Slow animals. Further, basal levels of ACTH and corticosterone were similar, but the increase in plasma ACTH levels following feeding was significantly greater in the Slow rats compared to their Fast counterparts, although this did not translate into greater (than Fast animals) plasma levels of corticosterone.

In summary, the research contained in this thesis challenges the traditionally held views of CRH (as being primarily a stress peptide) and BN-LPs (as being purely satiety peptides) and provides a new body of evidence suggesting that both of these peptide families are significantly involved in both the stress/anxiety and feeding behaviors. In this regard, this thesis should provide new insights into the mechanism(s) underlying various dysfunctions related to stress and anxiety as well as of ingestive behavior, such as anorexia nervosa, and anorexia associated with AIDS, cancer, sickness or stress.

Introduction

The apparently simple decision to initiate or terminate a meal is extremely complex.[310,336] Eating is controlled not only by our internal condition and need to maintain energy balance, but by many external factors such as time of day, social setting, stress, boredom, palatability/reward and, of course, the availability of food.[266] Due to escalating levels of obesity, the need to determine the physiological and psychological mechanisms regulating food intake has taken on an air of urgency. Indeed, the World Health Organization and the former U.S. Surgeon General C. Everett Koop have declared that obesity has become epidemic, as approximately 50% of the population in the U.S. is considered to be overweight and nearly 25-30% can be categorized as being clinically obese.[371,493] In addition, eating disorders such as anorexia nervosa and bulimia nervosa, although rare in comparison to obesity, involve approximately 3% of the population, predominantly young women.[488]

It is clear that ingestive patterns are influenced by a number of factors, including those that are environmental, social/cultural, psychological, genetic and, important for this thesis, biological.[73,124,194] Multiple neurochemical systems have been implicated in the etiology of eating disorders, including dopamine (DA), norepinephrine (NE), neuropeptide Y (NPY), serotonin (5-HT) and endogenous opioids.[13,124,196,205] The newly discovered hormone, leptin, has also been implicated, particularly in obesity, possibly exerting its influence through interactions with NPY, orexin, cocaine- and amphetamine-regulated transcript (CART), alpha-melanocyte-stimulating hormone (α-MSH), and agouti-related protein.[261] Eating disorders are further complicated by high rates of

comorbidity with other disorders such as anxiety.[70] In fact, antecedent anxiety is a potential risk factor for eating disorders.[70] In this respect, the functioning of stress systems, in particular the hypothalamic-pituitary-adrenal (HPA) axis may be relevant, as depression, anxiety/stress, panic disorder and eating disorders such as obesity, anorexia nervosa and normal weight bulimia have all been associated with disturbances in the HPA axis.[32,97,100,206,226,243,318,364,436,447,500]

It has been known for a long time that food intake and HPA activity are intimately associated.[369] Glucocorticoids (corticosterone in rats and cortisol in humans), the end products of HPA stimulation, are increased in response to food intake as well as fasting.[369] Underweight anorectics display hypercortisolemia and low plasma adrenocorticotropic hormone (ACTH) levels, as well as a blunted ACTH and cortisol response to intravenous administration of corticotropin-releasing hormone (CRH), the principal activator of the HPA axis.[226,318] Moreover, increased HPA axis activity resulting from chronic stressor exposure has been shown to change body energy stores away from muscle and toward fat, and may be meaningful in the development of abdominal obesity.[97] Similarly, monkeys exposed to chronic insubordination stress displayed (among other things) high levels of visceral fat, hyperglycemia and insulin resistance, adrenal gland hypertrophy and enhanced adrenal responsiveness to ACTH.[364]

It is this association between the HPA axis, stress/anxiety and feeding behavior that has inspired the experiments contained in this thesis. This introduction, therefore, is devoted to not only discussing the physiology of feeding and the neurochemical

regulators involved in this(these) process(es), but also to describing the HPA axis and its role in both the stress response and the regulation of feeding behavior. Research in our lab has focused on CRH, one of the key regulators of the HPA axis, and bombesin-like peptides (BN-LPs), regulators of feeding behavior, and particularly the satiety response. These neuropeptides, along with NPY will be discussed in detail in terms of their roles in both feeding and the stress response. Globally, this thesis investigates neurochemical signals that trigger meal initiation, meal termination and the ensuing intermeal interval. In particular it will examine whether similar neurochemical mechanisms govern both appetitive and aversive events and whether factors known to influence HPA reactivity affect patterns of food intake.

The Physiology of Feeding:

Prior to embarking on a discussion about the physiology of feeding behavior, it is important to understand some of the basic terminology of feeding. First of all, eating can be regarded as a discontinuous process in which periods of eating (meals) are interspersed with periods of non-eating (inter-meal intervals).[45] The initiation of a meal is generally preceded by hunger, which can be defined as the motivation to seek and consume food.[177] Digestion refers to the gastrointestinal process of breaking food down and absorbing its constituents into the body.[371] The physiological process that terminates eating (or brings a meal to an end) is called satiation.[45,439] The term "satiety" refers to the inhibition of hunger and eating (i.e. the state of noneating) and begins at the end of one meal and lasts until the onset of the next meal.[45,439] In rodents, satiety is generally accompanied by a repertoire of behaviors including a brief period of exploration and

grooming followed by sleep. Satiation determines meal size and satiety determines the length of the post meal interval.[45,177] These terms all refer to normal physiological processes. On the other hand, anorexia refers to a lack or loss of appetite[401] and is usually thought of as a pathological state.

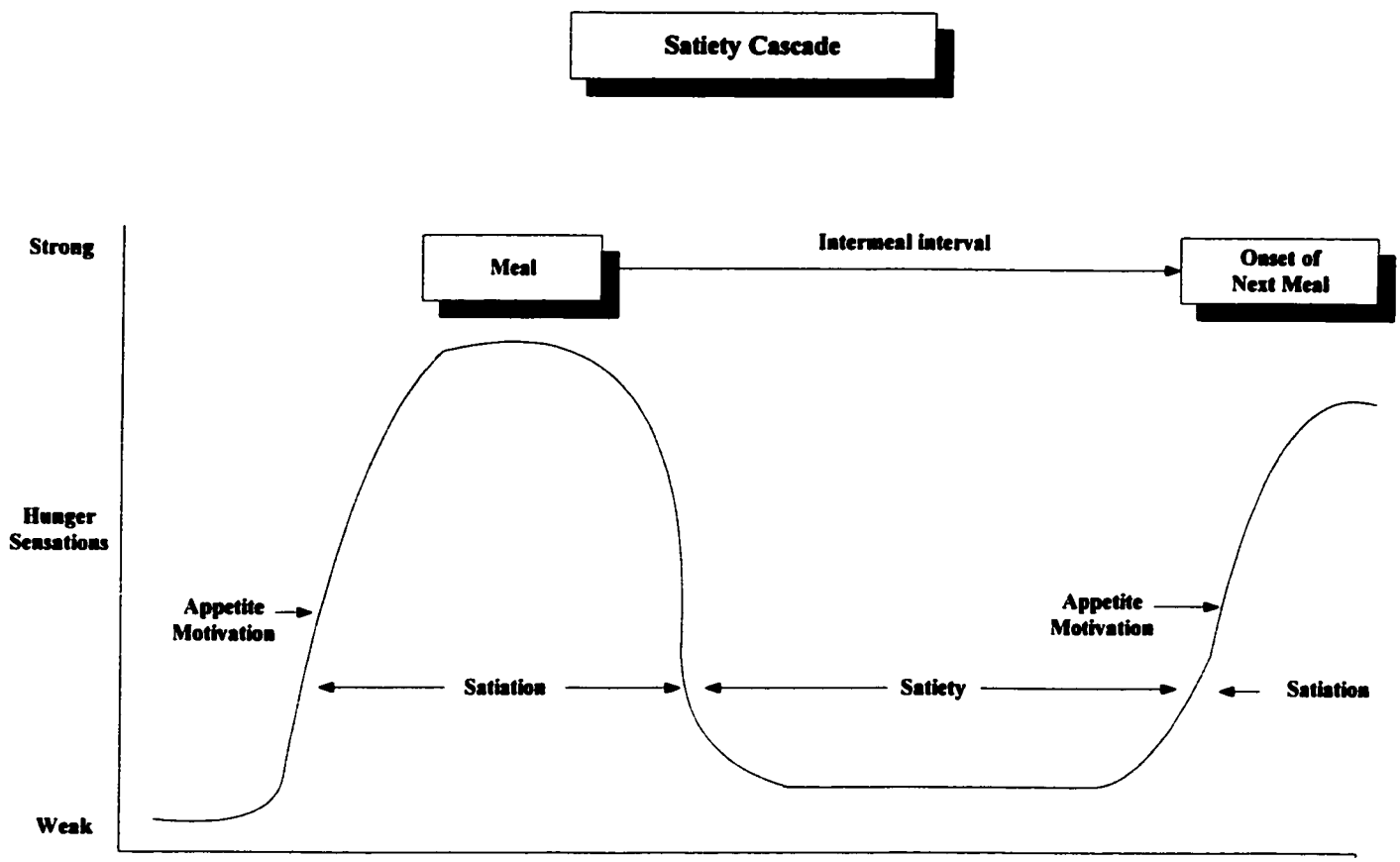


Figure 1: The operational domains of feeding behavior[45]

One way to look at feeding is in terms of energy balance. In that regard, eating becomes a means for the organism to supply its energy requirements.[371] Food provides three forms of energy: fats, amino acids (breakdown products of protein), and glucose (breakdown products of carbohydrates). The body also stores energy in three forms: fat (adipose tissue is the most common storage compartment), glycogen (liver and muscle) and proteins (muscle). In addition, there are three phases of energy metabolism. The *cephalic* or *preparatory* phase often begins with the sight, smell or thought of food and ends when digested food starts to be absorbed into the blood stream. The *absorptive* phase is the period during which energy absorbed into the blood stream from the meal begins to meet the body's immediate energy needs. The *fasting* phase is the period during which all of the unstored energy acquired from the previous meal has been used and the body draws on its energy stores to meet its immediate energy requirements.[371] Two pancreatic hormones, insulin and glucagon are critically involved in the control of energy flow. During the cephalic and absorptive phases, insulin levels are high and glucagon levels are low. Under this condition, these hormones promote the utilization of blood glucose as an energy source, convert excess blood glucose to glycogen and fat, convert amino acids to proteins, and store glycogen in the liver and muscle, fat in adipose tissue, and protein in muscle. During the fasting phase, the reverse is true: insulin levels fall and glucagon levels rise. Fats are converted to free fatty acids and ketones, and glycogen and protein are converted to glucose.[371]

Several theories have emerged in an attempt to reconcile the processes regulating hunger, eating and body weight.[371] Ingrained in our thinking since the 1940s and 1950s

has been the *set point theories* of energy balance. Briefly, these theories state that eating is regulated by systems designed to maintain energy stores at a set point. For example, the glucostatic set point theory (thought to account for meal initiation and termination) states that when the blood glucose level falls below a set point, we start to ingest food and when the blood glucose level returns to its set point, we feel satiated and stop eating. Likewise, the lipostatic set point theory (thought to account for long-term regulation of food intake) suggests that each individual has a set point for body fat and if there is a deviation from that point, the person adjusts his/her level of eating. However, there are some fundamental flaws with these concepts.[371] For example, these theories do not fit well with feeding-related evolutionary pressures. In order to survive famine and drought, our ancestors often found themselves in a situation where it was necessary to consume large quantities of food when available, in order to store energy for the times when food was in short supply. It was not enough to have a regulatory system that responded only to energy deficits. Moreover, blood glucose levels are tightly regulated within a specific range even during the fasting phase, and the reductions in blood glucose required to induce feeding in experimental conditions rarely occur naturally. Likewise, a significant portion of the population has an excess of fat deposits, and yet they still feel hunger and consume food. Finally, set point theories fail to recognize the contribution of other factors including taste, learning and social influences. This inability for set point theories to explain or account for our eating patterns has led to the *positive-incentive* theory, which states that organisms are driven to eat by the anticipated pleasure of eating.[371] Many factors can influence the incentive salience of the food, including taste, the amount

of time since the last meal, the type and quality of the food, post ingestive consequences of consuming the food, blood glucose levels, and social factors.[371]

Despite the failures of the set point theories of ingestive behavior, over the long run, we are normally able to maintain energy balance and body weight within tight limits (energy homeostasis).[97,418] Small animals such as rodents appear to be particularly efficient in this regard.[316] Thus, it would appear that, under normal circumstances, there exists a highly efficient regulatory system that controls what we eat, how much we eat, and when we should cease eating. Yet, even today, despite years of extensive research, we do not have all the answers.

The idea that feeding behavior may be regulated by central mechanism(s) began to appear around the mid 1800's when several cases of extreme obesity were associated with hypothalamic-hypophysial tumors as well as tumors not involving the pituitary.[19] The first experimental evidence that the hypothalamus may be the regulatory center for feeding behavior came in 1921 when Bailey and Bremer reported a case of obesity in a dog with a surgically induced hypothalamic lesion.[19] The hypothalamus is now fairly well recognized as the principal site in the brain that maintains endocrine and behavioral (vegetative) homeostasis.[415] Experiments during the 1950's implicated two regions within the hypothalamus that may be specifically related to the control of energy balance and food intake, the ventromedial (VMH) and lateral (LH) hypothalamic nuclei.[19,371] VMH lesioned rats became extremely obese, while LH lesioned rats stopped eating and died unless given proper care. Moreover, electrical stimulation of VMH neurons

suppressed food intake, while the same stimulus applied to the LH enhanced food intake.[371] Thus, the VMH and the LH became known respectively as the 'satiety' and 'feeding' centers of the brain. That notion subsequently came under heavy criticism and, although these nuclei are still recognized as being involved in energy balance and eating behavior, they are no longer regarded as the sole regulatory systems for ingestion.[162,371,387] It has become clear that an activity such as feeding behavior, which is so complex and critical to the survival of the organism, must involve a coordinated regulatory system that includes discrete neuronal pathways.[420]

The HPA Axis: Stress/Anxiety and Feeding

As previously mentioned, the HPA axis and antecedent anxiety are very relevant to the issue of disordered eating. Exposure to stressors has been shown to influence feeding behavior in a variety of ways and, thus, will be discussed in some detail.

Stress is a difficult concept to define. Classically, stress is defined as "a nonspecific response to any demand (usually noxious) upon the body and is accompanied by various physiological [and psychological] changes including activation of the [limbic] hypothalamic-pituitary-adrenal-axis"[188,206,278] (i.e. a "state of *threatened homeostasis*").[449] Stressors, defined as "any perturbation that disrupts homeostasis" can be physical (anoxia, hypoglycemia, extremes of heat or cold, and physical strain or injury), or psychological (stimuli that induce fear, anxiety or frustration)[207] in nature. In people, adaptation to stressor exposure involves the activation of a repertoire of behavioral and physiological responses.[207,449] Individual responses to stressors are

dependent on a multitude of factors including the severity (e.g. weak or strong), duration (e.g. acute or chronic), or quality (e.g. physical or psychological) of the stressor, as well as on experiential (e.g. early life experiences or previous adult stressor exposure) and organismic variables (e.g. gender, age, or species).[309] It is a complex interplay of these variables that will influence the overall success of the organism's response to stressor exposure. For example, severe, prolonged and uncontrollable exposure to stressors are thought to lead to pathophysiological conditions such as depression, anorexia nervosa, malnutrition, panic disorder, obsessive-compulsive disorder, chronic excessive exercising, and chronic active alcoholism.[364] Many of these conditions are associated with excessive HPA axis activation resulting from a failure to shut down (discontinue the stress response) once homeostasis has been restored.[207]

The physiological response to stressor application involves the activation of two interrelated systems, namely the sympathetic division of the autonomic nervous system and the HPA axis.[449] The sympathetic nervous system, which innervates vascular smooth muscle, kidney, gut and the adrenal medulla, provides a rapid, immediate response to stressor exposure.[412,449] Exposure to a stressor induces the release of NE from the dense fiber central network that includes the cerebral cortex, cerebellum, cingulate gyrus, thalamus, hippocampus, hypothalamus, amygdala, bed nucleus of the stria terminalis (BNST), the nucleus accumbens, and spinal cord.[91,137] This activation is followed by the peripheral release of NE and epinephrine from the adrenal medulla into general circulation and an increase in serum glucose.[207] The behavioral consequences of sympathetic activation include increased arousal, attention, memory, analgesia, and

suppression of feeding and reproductive activities.[207] From a physiological viewpoint, it is important to note that this increase in circulating epinephrine levels leads to the redirection of oxygen and nutrients to areas that have high energy demands in the organism (such as muscles).[207,412] As mentioned earlier, it is important to be able to shut the system down once it has completed its mission. This is accomplished by various feedback systems. Interestingly, noradrenergic and CRH neurons are inhibited by glucocorticoids, gamma-aminobutyric acid (GABA), ACTH and opioid peptides (released coincidentally with NE and epinephrine during the stress response).[449]

The second major system involved in the regulation of the stress response is the HPA axis.[308] Briefly, exposure to a stressor activates several forebrain structures, including the prefrontal cortex, hippocampus, amygdala, and septum, which converge on the paraventricular nucleus of the hypothalamus (PVN), the focal point of the stress response. This activation induces CRH release from axon terminals in the external zone of the median eminence into the hypophysial portal blood.[22,189,207] CRH subsequently induces the secretion of ACTH from the corticotroph cells in the anterior pituitary, which in turn stimulates the secretion of glucocorticoids from the adrenal cortex.[22,182,189,207,423] Glucocorticoid release, similar to that of epinephrine, is believed to enable the organism to engage an adaptive response to the threat at hand (i.e., increase arousal and redirect energy (released from glycogen stores) to required organs).[207] As with the sympathetic system, prolonged exposure of the organism to elevated glucocorticoid levels can lead to deleterious effects. Thus, an important component of this system is the inhibition of glucocorticoid secretion.[278] Rapid inhibition is accomplished through a fast feedback

system, whereby the rate of rise (as opposed to the absolute levels) in corticosterone levels is monitored and inhibition is achieved through the binding of glucocorticoids to specific cytoplasmic receptors found in the hypothalamus, and limbic structures such as the hippocampus.[278] Intermediate and delayed negative feedback works over a period of hours and even days, whereby the translocated glucocorticoid-receptor complex acts at the transcriptional level by suppressing CRH and pro-opiomelanocortin (POMC) gene expression, thus decreasing the secretory drive.[278] It would not be considered prudent for a system as powerful, important and potentially damaging as glucocorticoid secretion to depend on a single feedback system. A second modulatory system involves glucocorticoid receptors.[278] Type I, mineralcorticoid receptors have limited expression in the rat brain, located primarily in the hippocampus and septum. They have a high affinity, low capacity binding for glucocorticoids and, thus, appear to operate at low corticosterone concentrations, providing tonic inhibition.[278] On the other hand, type II, glucocorticoid receptors are more widely distributed in the brain, located in areas including the PVN, hippocampus, cortex, cerebellum and various brain stem nuclei.[278] They appear to be operative at higher hormonal concentrations after the mineralcorticoid receptors are saturated, and thus aid in the return to a state of homeostasis following a challenge.[278]

The HPA axis is not only a major regulator of the stress response but is also a key regulator of energy flow.[5,97] Thus, as alluded to earlier, abnormalities in the regulation and sensitivity of the HPA axis may be relevant in stress-related eating disorders such as anorexia nervosa.[423] The HPA axis represents one arm of the two-hormone system

(corticosteriods and insulin) that regulates energy balance.[97] An organism's response to stressful stimuli, regardless of the nature of the stressor, requires energy. As noted above, an important role of glucocorticoids (and epinephrine) is to mobilize and replenish energy stores (converting them to glucose) needed for brain and bodily functions during heightened activity.[295,470] Adrenalectomy, which reduces the availability of glucocorticoids, is known to decrease food intake and body weight, while peripheral and central administration of corticosterone stimulates feeding in adrenalectomized rats.[98,470] This effect appears to be mediated via the PVN, as corticosterone implants in this area increase intake (particularly that of carbohydrates), in adrenalectomized rats.[470] Also, glucocorticoid levels are increased in humans as well as rodents in response to both feeding and food deprivation.[98]

In conclusion, it is evident that exposure to stressors and the organism's subsequent response represent a major source of energy consumption that is mobilized through the activation of the sympathetic nervous system and the HPA axis. Assuredly, food intake is extremely sensitive to stressor exposure.[477] It has been repeatedly shown that the application of mild stressors (such as tail pinch) increase food intake, while more severe and prolonged stressor exposure (such as surgery, endotoxins, foot shock, crowding and restraint stress) decrease food intake.[477] In fact, a single exposure to a physiological (endotoxin) or psychological stressor (immobilization) has been shown to induce a prolonged reduction in food intake persisting beyond 24 hours.[477]

Overview of the Neurochemical Regulation of Feeding Behavior

One reason that animals eat is to satisfy their continuous requirements for energy.[316] Since food supply is not always continuous, its intake is controlled by a sophisticated regulatory system of separate, but interacting, mechanisms that maintain a balance between energy required and energy consumed.[316,499] The localization of food and meal initiation require that the organism be able to sense the presence of food. Thus, sight and smell are important in this regard.[59] The texture and taste of the food provide positive (or negative) cues regarding the nutritional value of the food and signal the organism to start (or not start) the process of ingestion.[59] Once ingestion has begun, the decision to terminate the meal occurs before the food actually gets digested or absorbed. Thus, one of the first satiety signals is gastric distention.[59] Signals acting on the intestinal mucosa, such as the long-chain fatty acid, oleate, also serve to decrease food intake.[59] Of most relevance for this thesis, are the gastrointestinal peptides that, when released from the small intestine, terminate feeding by either direct action on receptors within the gastrointestinal tract itself, or by sending signals to the central nervous system.[59]

Thus, feeding behavior involves complex interactions not only between anatomical structures (e.g. gut and brain), but also between numerous neuronal and endocrine systems utilizing neuromediators, including classical neurotransmitters and neuropeptides. The main focus of this thesis is on the role of neuropeptides in the regulation of food intake, and will be elaborated upon later. However, due to their role in feeding processes, classical neurotransmitters, including fast-acting amino acids such as GABA, and monoamines such as NE, 5-HT, DA[59] will also be briefly discussed. Table 1

gives an overview of some of the neurochemicals involved in the regulation of feeding behavior, including those that stimulate feeding (orexigenic agents) and those that suppress feeding (anorexic or satiety agents).

Table 1: Neurochemicals involved in the regulation of food intake:

Orexigenic Agents	*Anorexic/Satiety Agents*
Neurotransmitters:	**Neurotransmitters:**
Norepinephrine (NE) Gamma-aminobutyric acid (GABA)	Serotonin (5-HT) Dopamine (DA) Histamine
Neuropeptides	**Neuropeptides**
Neuropeptide Y (NPY) Beta-endorphin Dynorphin Galanin Melanocortin Agouti Related Protein Growth-hormone-releasing hormone Somatostatin Melanin-concentrating hormone Orexins (hypocretins)	Corticotropin Releasing Hormone (CRH) Urocortin (Ucn) Bombesin-like-peptides (BN-LPs) Cholecystokinin (CCK) Leptin Insulin Oxytocin Alpha-melanocyte-stimulating hormone (α-MSH) Cocaine-and amphetamine-regulated transcript (CART) Caerulein Calcitonin Enterostatin Vasoactive Intestinal Peptide Neurotensin 1 Sauvagine Insulin Growth Factor Calcitonin-Gene Related Peptide Glucagon-like-peptide-1,2 Thyrotropin Releasing Hormone Prolactin-releasing peptide Pro-opiomelanocortin (POMC)

A. Classical Neurotransmitters

As seen in Table 1, there are several neurochemicals involved in the initiation and termination of food intake. A detailed review of all of these compounds is beyond the scope of this thesis; however, a few of these agents will be discussed briefly.

GABA:

GABA, an inhibitory amino acid neurotransmitter is produced from glutamic acid and is widely distributed throughout the brain and spinal cord.[75] While GABA is most commonly known for its role in epilepsy and anxiety,[410,440] it also has the ability to stimulate food intake.[167,216,452] The GABA receptor is interesting, as it is formed from a multi-subunit transmembrane protein that consists of both a conducting pore embedded in the cell membrane and a transmitter binding site on the outer face of the membrane.[218] Two GABA receptors have been isolated, $GABA_A$ and $GABA_B$.[75] The $GABA_A$ receptor is comprised of at least three subunits - α, β, and γ.[218] All of the subunits bind GABA, although the α-subunit does so with the greatest affinity. Both the α- and β-subunits bind barbiturates, but only the γ-subunit binds benzodiazepines (antianxiety agents and muscle relaxants). Benzodiazepines and barbiturates act to enhance the GABA-induced chloride current. Interestingly, the presence of any one of the three ligands influences the binding of the other two. For example, a benzodiazepine will bind more tightly when GABA is bound to the receptor.[218] Important for this thesis is the fact that GABA agonists increase food intake, particularly that of palatable food, an effect thought to be mediated through the $GABA_A$ receptor although there appears to be some overlap in function between the two receptor systems.[28,35,36,191,289,363,442,451,453] For example,

exogenously administered $GABA_A$ receptor agonist, muscimol, and the $GABA_B$ receptor agonist, baclofen, dose-dependently elicit intense feeding.[451] The ventromedial nucleus accumbens shell appears to be an important site of action for the physiological role of GABA in food intake as administration of a selective GABA-transaminase inhibitor γ-vinyl-GABA (which increases levels of GABA) to this area elicited robust feeding in satiated rats.[451] It is possible that GABA exerts its effect by increasing the palatability of the food. In this regard, systemic administration of the benzodiazepine receptor agonist, midazolam, increased the ingestion of both sucrose and quinine solutions and, in taste reactivity testing, increased positive taste responses to both solutions (although quinine elicited fewer positive responses than sucrose).[167] Further evidence that benzodiazepines influence palatability is provided by a study that demonstrated a decrease in duration of drinking, total licks, and number of bouts for both sucrose and saccharin solutions following the administration of the partial inverse agonist Ro 15-4513.[191] Taken together, it is evident that GABA appears to have multidimensional effects in regulating/modulating ingestive processes.[35,37,191,217,228,239,240,277,324,386]

Norepinephrine

The monoaminergic signals, particularly those of NE, also influence food intake.[195,264,402,420] NE is synthesized in the dorsal vagal complex and the locus coeruleus, areas of the brainstem that project caudally to the spinal cord and rostrally to the hypothalamus (where, in the PVN, it is co-localized with NPY), thalamus and cortex. There is ample evidence that NE increases food intake.[420] Specifically, exogenous administration of NE into the PVN induces feeding, reduces energy expenditure, and

increases circulating levels of glucose, corticosterone and vasopressin.[420] Moreover, chronic administration of NE can produce substantial weight gain.[265,420] NE appears to exert its action through α_2 noradrenergic receptors, as the α_2 receptor inhibitor, idazoxan, was able to reduce food intake.[354] Furthermore, *in vivo* microdialysis at the PVN demonstrated that interstitial levels of NE were significantly higher in animals that consumed a large meal at dark onset, suggesting an important physiological role for NE in spontaneous food intake.[355] In addition, interstitial levels of NE have been shown to rise spontaneously during the first hour after dark onset, which correlates with the burst of feeding that rats exhibit at that time.[195] Finally, a putative interaction between NE and the newly characterized adiposity signal, leptin, has been suggested.[420] Genetically obese *ob/ob* mice (deficient in the leptin hormone[500]) display elevated levels of NE in the PVN, suggesting that leptin may reduce food intake, at least in part, by inhibiting NE release from this area.[420] These increased levels of NE in the PVN may account for the hyperphagia induced by leptin deficiency, which suggests that NE is an anabolic effector in the central nervous system.[420]

Dopamine

Similarly, DA appears to be important in the regulation of feeding; however, its role remains somewhat obscure or ambiguous.[420] Some of the discrepancies surrounding the effects of DA on food intake are attributable to DA's disruption of motor function, which can potentially interfere with feeding.[420] Notwithstanding the confounding motor effects, exogenous administration of the DA_1 agonist, SKF 38393, and DA_2 agonists, apomorphine or quinperole, has been shown to decrease food intake.[250] Administration

of these agonists also decreased the level of hypothalamic NPY, suggesting that the anorectic effects of these agonists may be mediated by NPY.[250] Another DA_1 agonist, A-68930, was shown to dose-dependently suppress the intake of a highly palatable diet by reducing the frequency of eating bouts.[4] In addition, the administration of both typical antipsychotics (e.g. chlorpromazine and haloperidol) or atypical antipsychotics (e.g. clozapine, olanzapine, risperidone and sulpiride) increases food intake.[225] Interestingly, the clozapine hyperphagia was reversed by the administration of a DA_1 and DA_2 agonist as well as quipazine, a $5\text{-}HT_{1B}$, $5\text{-}HT_2$ and $5\text{-}HT_3$ agonist.[225] *In vivo* microdialysis studies have revealed increases in the extracellular levels of DA at the central nucleus of the amygdala (CeA) and the medial prefrontal cortex (mPFC) in response to natural feeding (glucose and food pellet).[127,176] However, the effects of DA on food intake appear to be situation specific. Supporting this contention, midbrain DA neurons in monkeys are activated by unpredicted appetitive stimuli such as food, but not by fully predicted stimuli, and were depressed when a predicted reward was omitted.[325] Likewise, a voltammetry study done in rats trained to lever-press for food revealed increases in DA signaling levels in the nucleus accumbens in response to the initial few milk rewards.[393] The signal decreased during milk consumption and then increased again at the end of consumption, apparently in anticipation of the next reward.[393] Moreover, an increase in the rate of milk delivery initiated a decrease in the DA signal, an effect that was reversed by decreasing the flow of milk. Taken together, these results suggest that, at least in the nucleus accumbens, DA neurons are activated primarily in response to the incentive value of the stimulus.[393] This effect was noted again in the mPFC in regard to sensory specific satiety.[3] When rats are offered two consecutive meals of the same palatable

food, they eat very little of the second meal. However, if the second meal is composed of a novel food, they will eat almost as much of it as they did for the first meal. *In vivo* microdialysis revealed that DA efflux reflected the same pattern, suggesting that DA efflux may signal the incentive salience of the food reward being offered.[3]

Serotonin

A key issue in the study of feeding behavior is how the research relates to human problems such as eating disorders. In this regard, the neurotransmitter, 5-HT has been given thorough consideration.[87] The hypothesis that 5-HT was involved in feeding evolved from the knowledge that it was distributed in both the gut and the central nervous system.[46,433] Actually, 90% of the 5-HT found in the body is contained in the gut.[46] It was soon confirmed that drugs that increased 5-HT levels/activity in the brain were able to decrease food intake, whereas drugs that antagonized 5-HT activity often increased consumption.[46,433] Moreover, cerebrospinal fluid levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), have been shown to be low in underweight anorectic patients and increase once weight is regained.[87,488] Fenfluramine, which increases synaptic levels of 5-HT by both inducing its release and blocking its reuptake, dose-dependently suppresses food intake by reducing meal size without interfering with the latency to start eating or the frequency of meals.[433] Furthermore, 5-HT stimulation can inhibit the rate at which food is consumed.[433] These anorectic effects have been noted in a variety of feeding conditions in genetically lean and obese rats, baboons and humans.[433] In addition, the anorectic effect extends to indirectly acting 5-HT agonists such as the selective serotonin reuptake inhibitors, ORG 6582, fluoxetine, femoxetine, sertraline and

the 5-HT precursor 5-hydroxy-L-tryptophan.[433] The release of 5-HT activates both pre- and post-synaptic receptors.[46] 5-HT receptors can be divided into three subtypes, 5-HT_1, 5-HT_2, and 5-HT_3, all of which can be further subdivided. At present, there are at least 14 known 5-HT receptors.[46] The receptors most likely to mediate the 5-HT-regulated control of food intake are the presynaptic 5-HT_{1A} (activation of which increases food intake) and the postsynaptic 5-HT_{1B}, (5-$HT_{1D\beta}$ in humans) and 5-HT_{2C} receptors (activation of which induce anorectic action).[46,433] Importantly, microinjections of 5-HT agonists into the PVN decrease food intake, implicating this nucleus as a key regulator of its hypophagic effect.[433] Furthermore, moderate concentrations of 5-HT_{1B} receptors have been isolated in the dorsomedial (DM) and VMH hypothalamic nuclei, where they are located postsynaptically.[433] Notwithstanding, 5-HT may also be exerting its effects, at least in part, by interacting with other systems.[46] In this regard, an interaction with the cholecystokinin (CCK) system may be relevant. The hypophagic action of dexfenfluramine can be blocked by the CCK_A receptor antagonist, devazepide. Conversely, the anorexia induced by CCK-8 is reversed by 5-HT_1 and 5-HT_2 receptor antagonists.[46] It appears that CCK, released in response to the presence of food in the gut, stimulates its own receptors in the nucleus of the solitary tract (NTS), thereby activating the nearby serotonergic satiety system located in or near the PVN.[46] Moreover, 5-HT agonists attenuate NPY-mediated increases in food intake, and blocking 5-HT synthesis can increase levels of NPY in the PVN.[46] In addition, there is a possible interaction between NPY, leptin and the serotonergic systems.[46] Leptin is thought to increase levels of 5-HT and decrease levels of NPY within the PVN, thereby inducing a reduction in food intake.[46] Importantly, serotonergic agonists appear to be able to

selectively suppress appetite for energy-rich macronutrients, which makes them potentially useful targets for treatment of obesity.[46]

It is evident that there are multiple neurotransmitter signals regulating ingestive behavior and there exists "cross-talk" between these (and other) neurochemicals. As well, each neurotransmitter serves more than one (complementary) role. For example, as we have noted, NE plays a role in the organism's response to stressful stimuli, which involves the shift of energy from its stores to areas where it is needed, such as muscle. The fact that NE also stimulates food intake, particularly that of carbohydrates, appears to serve a complementary role for this transmitter, as carbohydrates are a fast way to restore energy. This cross-talk and multi-tasking can make it extremely challenging to tease out specific mechanisms involved in a particular behavior such as ingestion.

B. Neuropeptides:

There are several neuropeptides that are known to play an important role in feeding behavior. Neuropeptides typically range in size from 3 to 40 amino acid residues.[448] Larger molecules constitute proteins or hormones. The peptides of neuronal origin were coined as neuropeptides in the late 1960s, when it was first shown that several hypothalamic regulatory factors were, indeed, peptides.[448] Since then, it has become clear that many of the neuropeptides act to maintain physiological homeostasis, influencing behavior through the integration of the central nervous system and peripheral organs.[448] They accomplish this by acting as neurohormones (chemicals released by endocrine glands into the blood stream to act on distal targets), neurotransmitters

(chemicals released by the terminal buttons of neurons that communicate with other neurons in close proximity), and neuromodulators (chemicals that act like neurotransmitters except that they diffuse out of the synaptic cleft to influence many neurons).[448] Neuropeptides serve a wide variety of functions including the regulation of reproduction, growth, water and salt metabolism, temperature control, water intake, cardiovascular, gastrointestinal and respiratory control, exploration, memory, pain perception, affective states and, most important for this thesis, the control of food intake.[384,448]

As noted in Table 1, there are a number of neuropeptides that have a role in the regulation of feeding behavior and it is beyond the scope of this thesis to discuss each one in detail. There exists a wealth of information on the opioid system and CCK regarding their role in feeding behavior and I will discuss these peptides briefly, along with the newly characterized peptide, hypocretin/orexin. Most relevant to this thesis are CRH and BN-LPs and they will be discussed at greater length. Interestingly, these peptides are important not only in the regulation of food intake, but they also appear to be important in the mediation of the stress response. It is noteworthy that, while I am only looking at three peptides in detail, many other examples of neurochemicals with this "dual functionality" (role in both feeding and stress) can be found. This leads to the interesting and almost paradoxical concept that similar neuronal circuits and/or neurochemical substrates might subserve both feeding and stress.

Cholecytokinin

Various peptides that are secreted by the gut in response to the ingestion of food are known to relay signals to the brain, which then coordinates the termination of a meal. CCK is a gut-brain peptide initially isolated from the porcine duodenum as a 33 amino acid peptide.[33,280,448,469,499] CCK molecules of different sizes have subsequently been isoloated.[448] Variations in length of the active intestinal CCK molecule range in size from 58, 39, 33, 25, 18, 8, 7, to 5 amino acids.[448] In the nervous system, CCK is found predominantly as the C-terminal octapeptide (CCK-8). In the central nervous system, this neuropeptide appears to act as a neurotransmitter, as it is synthesized in neurons, is present in synaptic vesicles, is released through sodium- and calcium-dependent mechanisms, and is expressed in a region-dependent manner.[180,469] CCK binds to two receptor subtypes, CCK_A (located mainly in the periphery with scant distribution in the brain) and CCK_B (widely distributed in the central nervous system, particularly in DA rich areas, but also located in the stomach and vagus nerve).[469,499] As one of the most widely distributed neuropeptides, CCK has a role in a wide range of actions such as analgesia, thermoregulation, reproduction, memory processes, anxiety and motivation, but it is best known as an anorectic agent and putative satiety compound.[33,467,469,498,499] In 1973, Gibbs, Young and Smith[156] first demonstrated that intraperitoneal administration of CCK reduced food intake in rats.[33,144,180,345,438] Since then, it has been repeatedly shown that exogenous administration (both peripheral and central) of CCK reduces meal size and induces satiety in a variety of species including mice, rats, monkeys and humans.[92,108] Moreover, in more than 25 experiments under a variety of conditions, the administration of CCK antagonists before a meal increased the size of a meal, establishing a

physiological role for endogenous CCK in satiety.[438] In addition, CCK antagonists were able to block the satiating effects of stimuli that are known to induce the release of intestinal CCK, such as oleate, Intralipid and soybean trypsin inhibitor.[438] Peripherally, CCK appears to act in a paracrine manner, being released from CCK-containing cells in the duodenal mucosa diffusing into mucosa and submucosa to bind to CCK_A receptors on vagal afferent terminals.[438] Centrally, the brain stem appears sufficient to process the satiety signal received from the vagus as CCK reduces food intake in decerebrate animals, although forebrain structures may have a modulatory influence.[438] In addition, 5-HT and DA appear to be involved in the central processing of the CCK signal, as antagonists for each of these neurotransmitters attenuate the satiating effects of CCK.[438] In contrast, both insulin and estrogen, released in the periphery, enhance the satiating potency of CCK.[438]

Interestingly, many of the peptides that induce satiety appear to possess anxiogenic properties. In that regard, CCK appears to play a significant role in the mediation of anxiety and/or panic disorder.[280,469] In fact, exogenous administration of CCK elicits behaviors consistent with anxiety in both animals and humans.[55-57,181,280,281,389,435]

Opioids

As discussed earlier, the rewarding/hedonic value of food may be an important determinant of how much we eat. In that regard, the opioid system appears to be relevant.[381] Opioid agonists such as morphine are known to stimulate food intake in most

situations and the consumption of palatable (i.e. rewarding) foods such as sucrose, induces the release of endogenous opioids and/or alters their gene expression.[268,352,381] When forced to bar press for sucrose or sweetened alcohol reward, morphine treatment decreased responding.[352] Nonetheless, the opioid antagonist, naloxone, suppresses intake, including that of palatable foods, under a variety of conditions.[268,352] This effect appears to be mediated through μ opiate receptors since administration of the μ agonist (Try-D-Ala-*N*-Me-Phe,Gly-ol)-enkephalin (DAMGO) (but not σ or κ receptor ligands) is able to stimulate eating.[352] DAMGO-elicited stimulation of food intake has been demonstrated through microinjection into the CeA, PVN, nucleus accumbens, NTS, and VMH, suggesting potential sites of action.[160,268,352] Indeed, an opioid – opioid signaling pathway has been delineated from the CeA to the PVN.[160] Naltrexone microinjected into the PVN blocked CeA DAMGO-induced increase in food intake.[160] However, this pathway does not appear to be a reciprocal one in that microinjections of naltrexone into the CeA had no effect on PVN DAMGO-induced feeding.[160] Sucrose feeding appears to enhance opioidergic tone as evidenced by its ability to enhance the nociceptive effect of opioids such as morphine.[381] In fact, a recent study demonstrated that sucrose consumption increased endogenous opioid tone at the CeA as naloxone-induced c-Fos expression was up-regulated in rats that had been consuming sucrose solution for three weeks compared to rats that had only received plain drinking water.[381] Furthermore, altered opioid activity may be related to the pathophysiology of eating disorders.[352] For example, obese children display higher levels of β-endorphin than controls.[352] In addition, animal experiments have further revealed elevated β-endorphin levels in the pituitaries of genetically obese mice and rats compared to their lean counterparts.[268]

Finally, food deprivation increased the expression of prodynorphin mRNA in the CeA and LH with no change observed in the caudate nucleus, nucleus accumbens and the BNST and reduced levels of expression in the arcuate nucleus.[352] Taken together, these observations support an important role for endogenous opioids in the regulation of feeding behavior in general, and palatable food intake in particular.

Hypocretin/Orexin

A discussion of peptidergic agents involved in the regulation of feeding behavior would not be complete without at least a brief mention of the newly identified 130 residue excitatory peptide, preprohypocretin present in the hypothalamus.[368] It was soon recognized that preprohypocretin yields two peptides, hypocretin-1 and hypocretin-2, named for their hypothalamic origin and their structural similarity to secretin.[462] Almost simultaneously, Sakurai et al (1998) identified two hypothalamic peptides, which they referred to as orexin-A and orexin-B reflecting their ability to stimulate food intake when centrally administered.[368,462] It was soon realized that orexin-A and -B were identical to hypocretin 1 and 2, respectively.[368] Hypocretin-containing neurons are restricted to the hypothalamus, located in the perifornical nucleus, the DM and LH.[368] Despite the restriction of their cells to a small area of the hypothalamus, hypocretin projections have a wide distribution within the central nervous system.[368] Hypocretin fibers are found throughout the hypothalamus, in the locus coeruleus, septal nuclei, BNST, paraventricular and reuniens nuclei of the thalamus, the zona incerta, the subthalamic nucleus, the central gray, the substantia nigra, the raphe nuclei, the parabrachial area, the medullary reticular formation, the NTS and, less prominently, in cortical regions, the

anterior amygdaloid nuclei and the olfactory bulb.[368] It has subsequently been shown that the hypocretins can affect a fairly wide range of functions, including roles in hormone secretion, blood pressure, and rapid eye movement sleep.[462] Thus, it was concluded that the term orexin may be overly restrictive.[462] Nevertheless, several studies have alluded to the orexigenic effect of the hypocretins/orexins.[106,184,244,338,339,407,408,462] For example, intracerebroventricularly (ICV) administered orexin-A stimulates food intake in rats and/or feeding behavior in rats, mice, and goldfish.[397] In addition, injection of orexin-A into the LH stimulates food intake (an effect reduced by the orexin-A receptor antagonist, SB-334867-A[183]), and activates neurons associated with feeding (such as the PVN, DM, LH and perifornical area) and arousal.[116,244] Also, food restriction induces Fos expression in orexin-A-containing neurons of the LH and decreases the expression of orexin-2 receptor gene in the PVN.[251] However, the mechanism(s) regulating the effects of the hypocretin/orexins appear to be quite complex and are not fully understood.[203] For example, hypocretin stimulates the release of CRH, which is known to decrease food intake and pretreatment with the CRH antagonist, alpha–helical CRH or anti-CRH antiserum resulted in a significant increase of food intake by orexin, an effect that was blocked by the NPY Y1 receptor antagonist, 1229U91.[203] Thus, orexin's effect on food intake may be more complex due to orexin-CRH and orexin-NPY linkages.[203]

Thus, it is evident that the neurochemical regulation of ingestion is an intriguing phenomenon with complex underpinnings involving the intertwining of many peptides. To further complicate matters, each neurochemical appears to have several roles or effects. It is possible that such overlapping and redundancy may be vital in the regulation

of such a critical behavior such as feeding. If one system malfunctions, there are others to take over. On the other hand, the more complex the system is, the more chance there is of a malfunction.

Neuropeptide Y

NPY, a 36-amino-acid peptide widely distributed throughout the central nervous system and the peripheral nervous system, is the most abundant peptide present in the mammalian brain, the most potent orexigenic peptide known, and is one of the most conserved peptides in evolution.[20,97,149,214,266,390] NPY is involved in a number of physiologically relevant functions, including food intake, endocrine function (e.g. modulation of luteinizing hormone and CRH), cardiorespiratory parameters, circadian rhythms, and enhancement of cognitive function (e.g. learning and memory processes).[115] In addition, dysregulation of the NPY system is associated with pathological conditions such as obesity, depression/anxiety-related disorders and epilepsy/seizure.[115]

NPY distribution: immunoreactivity and receptors

In the hypothalamus, the arcuate nucleus appears to have the greatest density of cell bodies expressing NPY and there is strong evidence for a primary projection of these cell bodies to the PVN[266] and ventral hypothalamus, both important regions in the regulation of energy balance and neuroendocrine function.[448] In addition to the PVN and arcuate, several other feeding-relevant brain nuclei express the NPY gene, including the median eminence, VMH, DM, LH and medial preoptic area (mPOA).[266]

NPY is believed to exert its effects through interaction with a family of G-protein coupled receptors subtypes, Y_1, Y_2, Y_3, Y_4, Y_5, and y_6.[115] All of these receptors, with the exception of Y_3 have been cloned,[20] and, although the Y_1 and Y_5 receptor subtypes have been the most extensively studied,[214] all receptor subtypes are expressed in several species including man, with the notable exception of the y_6 receptor which is absent in the rat and not functional in man.[149,214] The Y_1-like receptor is highly expressed in the superficial layers of the cortex and thalamic nuclei. The Y_2 receptor is abundantly expressed in various parts of the brain, including the hippocampal formation (CA3), hypothalamus, in particular the arcuate nucleus, which provides NPY innervation to the PVN and DM, thalamus, amygdala and brainstem.[214,494] Y_2 mRNA-containing neurons have been located in the medial preoptic nucleus, the PVN and DM as well as the posterior hypothalamic nuclei, medial nucleus of the amygdala, parabrachial area, substantia nigra, paraventricular thalamic nucleus, NTS, lateral reticular nucleus, and locus coeruleus.[214,223] Since the characterization and cloning of other NPY receptor subtypes is recent and ongoing (NPY Y_3 has not yet been cloned[20,494]) there appears to be a paucity of information regarding the distribution of other NPY receptor subtypes. A discrete group of neurons within the NTS have been shown to respond only to NPY (and not peptide YY or any other analogs), indicating that these neurons fit the profile for NPY Y_3 receptors.[262] The same study demonstrated the presence of Y_1, Y_2, and Y_4 receptors in both the NTS and the neighboring area postrema.[262] Y_5 receptors have been detected in the PVN, suprachiasmatic nucleus, anterior hypothalamus, BNST, and the VMH.[122,489] Finally, the NPY y_6 receptor is written in lower case because it has not been found to be functional in rats or humans.[494]

The Role of NPY in feeding

It has repeatedly been shown that central administration of NPY dose-dependently stimulates food intake in both food deprived and sated rats, effects likely mediated via Y_1 and Y_5 receptors, although Y_2 receptors have also been implicated.[61,97,115,149,214,234] The orexigenic effect of NPY appears to particularly involve carbohydrate intake, although the animal's preexisting preference is important in this regard.[266] NPY also promotes energy storage in fat by decreasing sympathetic nervous system activity in brown fat and increasing insulin-stimulated glucose uptake in adipose tissue.[266] Other areas where NPY appears to affect energy balance and food intake are the perifornical area of the hypothalamus, VMH, and some brain stem regions.[266] Changes in energy balance produce significant alteration in NPY mRNA expression in both the arcuate and PVN. For example, food deprivation, particularly chronic deprivation, significantly increases NPY mRNA expression in the arcuate, accompanied by elevated levels of NPY-immunoreactivity in the PVN (which normalize once food is provided, suggesting that NPY may provide a signal to begin ingestion).[115,149,234,266] Factors that appear to increase levels of NPY expression include lactation (arcuate/median eminence complex) and intense voluntary running (arcuate, DM, mPOA, and LH).[266] In addition, push-pull perfusion methodology has revealed that, in schedule-fed rats (a paradigm that induces rats to consume a high intake of food in a short period of time), NPY is released from the PVN prior to food intake.[266] Further evidence for the importance of an arcuate/PVN pathway in NPY-induced feeding comes from the discovery that NPY concentrations were significantly higher in the arcuate and PVN in obese Zucker rats compared to their lean

counterparts.[31] This difference was evident by 30 days of life suggesting that it may be a contributing factor to the development of obesity in these animals.[31]

The orexigenic effect of exogenously administered NPY appears to reflect physiological function,[115] as central administration of the selective NPY Y_1 antagonist, BIBP3226, while it had no effect on regular chow consumption, significantly reduced the intake of a highly palatable diet as well as fasting-stimulated food intake.[221] Furthermore, administration of this antagonist blocked NPY-stimulated intake of regular rat chow.[221] Conversely, an NPY Y_1 and Y_3 agonist, [Leu^{31},Pro^{34}]NPY and a Y_5 receptor agonist, ($hPYY_{3-36}$) microinjected into the PVN increased the consumption of regular chow.[221] The NPY Y_1 antagonist, BIBP3226, was able to block the effect of [Leu^{31},Pro^{34}]NPY, but not that of $hPYY_{3-36}$.[221] Taken together, these findings suggest that NPY is functionally involved in the stimulation of feeding, that the PVN may be an important site of action, and that NPY displays properties that are functionally distinct from another potent orexigenic peptide, Peptide YY.

A potential mechanism of action for NPY's effect on feeding behavior may involve an interaction with other peptidergic and neurotransmitter systems. As previously discussed, DA and leptin agonists decrease hypothalamic NPY expression[46,250] and 5-HT agonists attenuate NPY-mediated increases in food intake, while blocking 5-HT synthesis increases levels of NPY in the PVN.[46] Furthermore, NPY stimulates the release of corticosterone and insulin, both of which increase food intake.[448] Interestingly, NPY also interacts, and is colocalized, with the NE system(s) in the hypothalamus.[448,506] Post

mortem studies have revealed that low doses of ICV administered NPY increased, and high doses decreased, tissue levels of NE in the hypothalamus, frontal cortex, hippocampus and striatum.[288] Furthermore, push-pull perfusion at the perifornical region of the hypothalamus demonstrated that NPY infusion induced a significant release and turnover of NE and DA during feeding.[341] Moreover, *in vivo* microdialysis at the vicinity of the PVN revealed that ICV injection of NPY (20 μg) significantly increased extracellular levels of NE, DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) during feeding behavior.[288] Taken together, these findings suggest that the regulation of food intake by NPY is highly complicated, involving many neuropeptide and neurotransmitter systems.

Not only can the central administration of NPY induce feeding in sated animals, it can also increase an animal's reward driven feeding.[131,266] Indeed, in tests using a progressive ratio schedule (i.e. increasing the number of lever presses required to obtain the next food reward), which more effectively measures motivation than simply measuring food intake, you see that this is the case.[266] Furthermore, NPY injected bilaterally into the perifornical hypothalamus stimulated intake of both regular powdered chow and sucrose as well as reward-related behaviors.[66] On the other hand, similar treatment at the nucleus accumbens induced reward-related behavior but not feeding.[66] Also, in the Geller-Seifter conflict test, NPY injection was able to markedly and dose-dependently increase punished responding for food reward.[186] Taken together these results suggest that NPY may increase the incentive salience of food rewards.

The Role of NPY in Stress and/or Anxiety

While the role of NPY in feeding behavior has been well established, there is also evidence that NPY plays an important role in the mediation and/or modulation of the stress response. As previously mentioned, NPY appears to be colocalized with NE, the prime activator of the sympathetic nervous system in response to stressful stimuli.[506] In addition, NPY induces vasodepressor and bradycardic responses in rats, and is associated with suppression of the release of transmitters such as glutamate and NE and stimulation of the release of arginine vasopressin (AVP) and oxytocin.[214] Furthermore, in rats, stressors such as exposure to footshock, cold stress and hemorrhage (but not immobilization stress) stimulate the release of NPY from sympathetic nerves and the adrenal medulla, thus increasing levels of the peptide into general circulation. This effect appears to be more potent in male rats compared to weight and age matched females, which may relate to the females' higher basal levels of NE and epinephrine.[506] The females' chronically elevated circulating adrenomedullary catecholamine levels may suppress NPY release through a presynaptic feedback mechanism thereby reducing stressor-induced NPY release.[506] Also, NPY release appears to be up-regulated by testosterone (in rats and humans) and down-regulated by estrogen (in humans).[506] In addition to the interaction with the catecholaminergic system(s), NPY appears to interact with CRH.[266] Centrally administered CRH or its antagonist attenuates and enhances NPY-induced feeding, respectively.[266] Furthermore, inactivation of CRH neurons at the PVN using the protein synthesis inhibitor, ricin A,[502] also enhanced NPY-induced feeding.[266] Finally, NPY increases release of corticosterone, the end product of HPA axis activation, while CRH increases NPY gene expression, transport, release, and receptor function.[266]

Taken together, this evidence points to the possibility that NPY is not only an orexigenic feeding agent, but may also be involved in the mediation of the stress response.

It was previously noted that many peptides that induce satiety appear to be anxiogenic in nature. Similarly, several of the orexigenic peptides appear to possess anxiolytic properties. For example, central administration of NPY and NPY receptor agonists, peptide YY, the NPY fragment 2-36, [Leu (31), Pro(34)]-NPY, and the NPY fragment 13-36 induced anxiolytic effects in the elevated plus maze and in the fear potentiated startle paradigm.[65] Additional evidence from animal studies further supports the notion that ICV administration of NPY reduces anxiety in rats.[63-65,471] NPY is also effective when administered directly into the CeA and the locus coeruleus.[222,223] Moreover, exposure of rats to anxiogenic stimuli such as electric footshock increased NPY immunoreactivity in the amygdala, nucleus accumbens and hypothalamus and decreased NPY immunoreactivity in the frontal cortex, an effect reversed by diazepam.[248] The anxiolytic effect of NPY agonists can be blocked not only by the NPY-Y_1 receptor antagonist (BIBP3226),[222] but also by the opioid receptor antagonist, naloxone, suggesting that NPY may impart its anxiolytic effects independent of the benzodiazepine receptor.[64] Finally, NPY knockout mice, when tested in locomotor monitors, elevated plus maze, inhibitory avoidance, acoustic startle, prepulse inhibition and hot plate paradigms, exhibited an anxiogenic- and hypoalgesic-like phenotype.[24] It is possible that the anxiolytic effect of NPY may be mediated through CRH,[63] as NPY antagonized the anxiogenic effect of CRH in a shock conflict paradigm (and the elevated plus maze) but produced little effect on its own.[63] In conclusion, it is evident that NPY may be important

not only in stimulating food intake, but may also be involved in the mediation of stress/anxiety responses.

Corticotropin Releasing Hormone

During the mid 1950s, Guillemin and Rosenberg[171] and Saffran and Schally[405] independently demonstrated the presence of an agent within the HPA axis that was able to stimulate the rate of corticotropin secretion by the pituitary gland.[474] Many years later, Wylie Vale and his associates characterized a 41-amino acid peptide that actually mediated the secretion of ACTH both *in vitro* (from the pituitary cell cultures) and *in vivo* (in plasma) from a variety of species including rat, sheep, monkeys, and humans,[107,396,474] and named it corticotropin-releasing factor (now more commonly known as CRH). CRH has an approximate 50% homology to another peptide, sauvagine, which had been isolated from the skin of the South America frog *Phylomedusa sauvagei* that had also been reported to induce the release of ACTH and β-endorphin. The more recent addition to this family of peptides is urocortin (Ucn).[206] Ucn has a 63% homology to fish urotensin I, a 43% homology to CRH, and a 35% homology to sauvagine.[99,206] Its mRNA and protein appear to be restricted to the Edinger-Westphal nucleus,[206] the hypothalamic area and a small population of neurons in the forebrain.[99] Even more recently, two additional peptides have been added to the Ucn/CRH family, namely the 38 amino acid peptides, Ucn II and Ucn III.[99,273] Centrally, Ucn II mRNA is highly expressed in the PVN, supraoptic nucleus and the arcuate, while Ucn III mRNA expression includes the rostral perifornical area of the hypothalamus, the posterior BNST, the lateral septum and the medial amygdaloid nucleus.[99] Similar to other members of the CRH family, Ucn

peptides have an active role in the modulation of endocrine, autonomic and behavioral responses to stressor exposure.[274]

Distribution of CRH Immunoreactivity in the Brain

CRH immunoreactivity is widely distributed in the central nervous system.[95] It became clear very early on that the major CRH-stained cell groups and pathways are within the limbic areas of the telencephalon, the hypothalamus and the brain stem that play a role in mediating autonomic and neuroendocrine responses.[464] Particularly relevant to both stress and energy balance is the discovery of immunoreactive cells within the parvocellular division of the PVN, which gives rise to a major CRH-containing pathway to the external zone of the median eminence.[95,247,464] Presumably, this pathway is involved in the mediation of the release of ACTH and β-endorphin via the hypothalamohypophyseal portal system.[464] Other areas containing immunoreactive CRH include the periventricular hypothalamic nuclei, the anterior hypothalamus (AH), the lateral preoptic-lateral hypothalamic continuum, olfactory bulb, the BNST, the amygdalar-hippocampal complex (an area important for stress adaptation), the substantia innominata, septum, Barrington's nucleus in the pons, cerebellum, cerebral cortex, hippocampus, and the nucleus accumbens.[95,99,206,247,374,437,464] Many of these regions project to the brain stem.[464] Some fibers in the periventricular system innervate the central grey, locus coeruleus, and the parabrachial nucleus.[206,464] Other fibers project from the posterior continuation of the medial forebrain bundle dorsally to the same regions and to the dorsal vagal complex.[464] An interesting phenomenon is the existence of an ultrashort positive feedback loop terminating at the parvocellular CRH perikarya of

the PVN that, presumably, controls CRH secretion during states of alarm.[114] Finally, CRH immunostaining was found in the dorsal raphe, locus coeruleus, the external cuneate nucleus, the medullary reticular formation and the thalamus.[95,247,317] It is noteworthy that a group of noradrenergic cells in the NTS sends fibers to the parvocellular PVN (thus potentially influencing the release of CRH in the median eminence) as well as other CRH containing nuclei, including the substantia innominata, the CeA, and mPOA.[464] The parabrachial nucleus, which receives massive input from the NTS (and is thus involved in the relay of visceral sensory information) and adjacent locus coeruleus also project to CRH-positive basal forebrain nuclei including parvocellular parts of the PVN, the BNST and the CeA.[464] Finally, a group of noradrenergic cells centered in the A_1 region of the ventrolateral medulla is part of the relay of visceral sensory information from the NTS to both the magnocellular (vasopressinergic) and parvocellular parts of the PVN, to the locus coeruleus and back to the dorsal vagal complex. CRH-stained neurons have also been found throughout the cerebral cortex, most prominently in limbic regions such as the prefrontal cortex, cingulate gyrus, the areas surrounding the rhinal fissure, and Ammon's horn.[464]

Distribution of CRH Receptors in the Brain

CRH receptor binding sites in the brain are generally consistent with the distribution of immunoreactive CRH.[107] Low to moderate concentrations of CRH binding sites are present in brain regions that contain CRH-immunoreactive cell bodies such as the PVN, the CeA, the BNST and the NTS, and may actually represent presynaptic autoreceptors.[107] CRH binding sites are present in the median eminence,

cortex, hypothalamus, hippocampal pyramidal cells, the parabrachial nucleus, amygdala, the caudate-putamen, the cerebellum, the facial and hypoglossal motor cranial nerve nuclei, and the ventral horn of the spinal cord.[107,206] CRH receptors are also distributed within the glomerular layer of the olfactory system, in the adjacent external plexiform layer and in the mitral cell body layer where the olfactory output neurons originate.[107]

Recently, two separate CRH receptor subtypes (products of distinct genes) have been cloned and characterized.[88,100,114,437] The CRH-R_1 is a 415-420 amino acid protein and exists in a single functional form (CRH-$R_{1\alpha}$) in the rat, although there are variations in the human.[100] The CRH-R_2 gene encodes three distinct functional subtypes: the 411-413 amino acid protein, CRH-$R_{2\alpha}$; the 431-438 amino acid protein, CRH-$R_{2\beta}$; and the 397 amino acid CRH-$R_{2\gamma}$.[88,100,437] While the CRH-R_1 displays similar binding for CRH and Ucn, the CRH-R_2 has an approximate 40-fold greater affinity for Ucn than CRH.[88,423] CRH-R_1 and CRH-R_2 are also differentially distributed within the organism, suggesting distinct functional pathways for the CRH-related systems.[88] CRH-R_1 is highly expressed in the pituitary corticotroph and is largely responsible for ACTH secretion.[2] CRH-R_1 receptors are also present in the cerebral cortex, olfactory bulbs, amygdala, and parts of the hippocampus, the cerebellum, brain stem and septum.[88,100] Low levels of this receptor are expressed in the hypothalamus, although exposure to immobilization stress induced the expression of CRH-R_1 in the parvocellular PVN of both male and female rats.[88,100,114] CRH-$R_{2\beta}$ is found predominantly in the periphery (heart, skeletal muscle, intestine, lung, kidney, and epididymis) and in cerebral arterioles and choroid plexus of the brain.[88,100,423] CRH-$R_{2\alpha}$ is expressed in neuronal tissue of the olfactory bulb, VMH,

mPOA, lateral septum, hypothalamus, hippocampus, dorsal and medial raphe and amygdala.[88,100] Interestingly, the pituitary does not express CRH-R_2 at all, suggesting that the CRH-R_1 is the principal receptor for CRH action in the pituitary.[423]

An interesting component of the CRH system includes the CRH-binding protein (CRH-BP), a 37 kDa secreted glycoprotein that binds CRH and Ucn with an equal or greater affinity than the CRH-R.[423] It was first isolated and characterized from human plasma. Interestingly, it is not found in the plasma of rats or mice, but is quite widespread in the rodent brain and pituitary. Specifically, *in situ* hybridization histochemical analyses have revealed that CRH-BP is expressed in layers II, III, V, VI of the cerebral cortex, dentate gyrus, CA1 and CA3 regions of the hippocampus, the amygdaloid complex, the BNST, olfactory bulb, and sensory relays associated with the auditory, olfactory, vestibula, trigeminal systems and several raphe nuclei.[382,423] Rat CRH and CRH-BP are co-localized in areas such as the olfactory bulb, BNST, CeA, mPOA, inferior colliculus, lateral dorsal tegmental nucleus and ventral part of the lateral septal nucleus. CRH-BP is also located in CRH target sites such as the anterior pituitary corticotropes. It has been postulated that CRH-BP functions as a negative regulator of CRH activity, both in the HPA axis and the central nervous system.[382,423] Indeed, acute restraint stress induces a 2-3 fold increase in pituitary CRH-BP. Furthermore, adrenalectomy decreased basal levels of CRH-BP mRNA to 8% of normal, suggesting that glucocorticoid secretion plays a positive feedback role in the expression of CRH-BP, thus providing an additional mechanism to maintain HPA axis homeostasis.[423]

The role of CRH in Stress and/or Anxiety

CRH has long been recognized as a key mediator of an organism's characteristic physiological (endocrine, autonomic and immune) response to stress and the maintenance of homeostasis.[114,374,437] Although CRH neurons are widely distributed in the central nervous system, the principal site of action for delivering CRH into the hypophysial portal system (and thus stimulating the HPA axis) is the PVN.[114] In fact, destruction of the PVN results in an attenuated ACTH and corticosterone response to stressor application.[114] Moreover, stressor applications such as restraint, foot-shock, cold swim, immobilization, ether inhalation, lipopolysaccharide or interleukin-1 administration all result in an up-regulation of the early gene, *c-fos*, and CRH gene expression in the parvocellular region of the PVN as well as the release of CRH into the pituitary portal system, stimulating hormonal release of ACTH from pituitary cortitrophs and corticosterone from the adrenal cortex.[114,188,207] Furthermore, stressor exposure induces alterations in CRH neuronal activity (either increases or decreases depending on the nature and intensity of the stressor) in various brain regions including hypothalamic nuclei (particularly the PVN), locus coeruleus, Barrington's nucleus, and the olfactory bulb.[114,188]

CRH has a number of behavioral effects, many of which depend on the baseline state of arousal of the animal.[107,206,437] In non-stressed animals, central administration of CRH or Ucn dose dependently increases locomotor activity, rearing and grooming in a familiar environment.[188,206,437] Furthermore, at low doses, CRH and Ucn are known to enhance learning and memory, whilst high doses impair performance.[206] Alternately, in a

novel (presumably stressful) environment, CRH and Ucn exert anxiogenic-like properties such as decreases in rearing and exploration in several paradigms, including the open field, the elevated plus-maze, the light-dark box, social interaction, and contextual fear conditioning.[100,206,437] CRH has also been shown to induce aggression, enhance acoustic startle reactivity, facilitate defensive burying, and produce both taste and place aversion.[427,437] Furthermore, central administration of CRH induces autonomic activation as reflected by increases in plasma levels of catecholamines, glucose, glucagon, AVP and elevations in mean arterial pressure, heart rate and oxygen consumption.[62,95,247,427,463,464]

Evidence that endogenous CRH is involved in these behavioral and endocrine responses to stressors comes from the anti-stress actions of CRH-receptor antagonists such as alpha-helical CRH_{9-41}, and D-Phe CRF_{12-41}.[188,206,437] Central administration of alpha-helical CRH attenuates the expression of anxiogenic-like behaviors such as stressor-induced reduction in food intake, fighting, reduction in open-arm exploration of the elevated plus maze and the suppression of exploratory behavior in the light dark box.[437] Furthermore, CP-154,526, a CRH-R_1 antagonist, blocks the effects of CRH on the acoustic startle reflex.[437] It has also been reported to have an antidepressant effect in rats exposed to inescapable foot shock.[437] Other CRH-R_1 antagonists have also been shown to possess anti-anxiety qualities, suggesting that the CRH-R_1 is involved in the stressor-induced behavioral changes seen in animal models of anxiety.[437] The functional role of the CRH-R_2 system is not yet well delineated.[437]

The CeA, known for its role in fear and anxiety, may be an important site of action for CRH.[206] Central (ICV) administration of CRH potentiates the acoustic startle response, an effect blocked by lesioning the CeA, but not the PVN.[206] Also, microinjection of CRH into the CeA stimulates locomotor and exploratory behavior in mice.[206] Interestingly, microinjection of alpha-helical CRH reverses social stressor-induced suppression of behavior in the elevated plus maze 100 times more potently than when the antagonist is administered ICV.[206] Furthermore, rats exposed to an acute stressor showed increases in CRH levels at the CeA assessed by *in vivo* microdialysis.[206]

Other evidence that blockade of the CRH system(s) has an anti-depressant/anti-anxiety role comes from studies utilizing antisense strategies.[227] When an antisense oligodeoxynucleotide corresponding to the CRH-R_1 was infused over a period of four days into the CeA of rats, the animals displayed a significant down-regulation of CRH-R_1, as well as anxiolytic behavior in the social defeat paradigm. A similar anxiolytic effect was seen using ICV infusion of the antisense probe, which resulted in a decrease in CRH binding in the hypothalamus and cortex. In contrast, no anxiolytic effect was seen using CRH-R_2 antisense probes, although these animals showed increased immobility in the forced swim test suggesting that CRH-R_2 may be involved in coping behaviors following acute stressor exposure.[227]

Further assessment of the role of the endogenous peptide can be achieved through the generation of the knockout mouse model.[337] It has recently been shown that the normally robust corticosterone response to restraint stress exposure was significantly

attenuated in CRH knockout mice.[337] Interestingly, this effect was different for males and females in that male CRH knockout mice were more profoundly affected.[337] These results suggest that, because other ACTH secretagogues such as AVP, oxytocin, and catecholamines could not compensate for the loss of CRH, an intact CRH system is required for a normal endocrine response to a stressor.[337] It is interesting to note that transgenic mice that over-express CRH display a heightened anxiogenic response, an effect reversed by the ICV administration of alpha-helical CRH.[88,447] One would expect that the CRH knockout mice would, therefore, display anxiolytic responses. However, the CRH knockout mice do not appear to exhibit aberrant anxiety-related behaviors.[337] Further, alpha-helical CRH and CP-154,526 were effective in blocking shock-induced freezing in both wild type and knockout mice models. Moreover, it has been found that CRH-R_1 knockout mice as well as mice over-expressing CRH-BP (presumably decreasing the bioavailability of CRH) display reduced anxiety-related behaviors under basal conditions in several behavioral paradigms.[88,337,423] Conversely, mice with inactivation of CRH-BP (thus increasing the availability of CRH) exhibit increased anxiety-like behavior.[337,423] Surprisingly, basal ACTH and corticosterone levels are unchanged in mice over- or under-expressing CRH-BP.[423] These findings suggest that another CRH-like molecule, such as Ucn, may be involved in the anxiogenic behaviors attributed to CRH anxiety through the CRH-R_1.[88,337]

Recently, in order to investigate the potential role of CRH-R_2 in the stress response, the CRH-R_2 knockout mice genotype was generated by three independent laboratories.[88] Studies using this mouse genotype have been ambiguous.[227] However, it

has been noted that these mice display abnormalities in the function of their HPA axis. Specifically, the mutant mice exhibit a robust ACTH response following stressor exposure that declines more rapidly than that of wild type mice, and a more prolonged elevation of corticosterone levels compared to their wild type counterparts.[88] Thus, CRH-R_2 is potentially involved in the recovery phase of HPA axis activation following exposure to a stressful stimulus.[88]

The role of CRH in Feeding

In addition to being a key stress peptide, CRH has also been implicated in the control of food intake.[88,267] Central administration of CRH and, more potently, Ucn, has been shown to suppress food intake in rodents. This effect could be secondary to stressor-like effects.[164,187,198,199,206,246,337,437,445,465] Conversely food intake appears to play a regulatory role in HPA responsivity in that food-deprived animals show a blunted ACTH response to stressor exposure.[5] Furthermore, fluctuations in immunoreactive CRH levels in relation to feeding status have been observed in several brain regions known to be relevant to feeding behavior.[311,378]

It has been observed that stressful stimuli are able to both increase and decrease feeding behavior.[335] Tail pinch stress increases feeding presumably through the stressor-induced release of endogenous opioid peptides.[335] On the other hand, immobilization/restraint stress reduces food intake for up to four hours following restraint and, depending on the time of day at which the stressor is applied, throughout the ensuing 24-h period and up to several days following treatment.[477] In this context, ICV

administration of CRH (as well as ACTH[483]) is able to dose dependently decrease food intake (including intake stimulated by other orexigenic peptides such as NPY, dynorphin, muscimol, insulin and NE[187,267]), decrease gastric acid secretion,[107] and increase self grooming.[119,335] The PVN may be an important site in the mediation of this anorexic effect.[206] CRH has been shown to selectively decrease food intake when administered into the PVN but not into a number of other nuclei known to be involved in the regulation of feeding behavior.[247] Other studies have demonstrated that CRH and/or Ucn not only diminished food intake, but also attenuated weight gain and increased energy utilization.[423] These effects appear to be gender dependent in that chronic administration of CRH resulted in decreased weight gain and food intake, reduced body fat and protein content in males, but not in females.[423] However, responses to pharmacological doses of a peptide do not necessarily imply a physiological role in that behavior.

Confirmation of a physiological role for CRH in stressor-induced feeding came with the development of the competitive CRH antagonist, alpha-helical $CRF_{(9-41)}$. Alpha-helical CRH was able to partially block the reduction in food intake induced by CRH as well as the reduction in food intake due to restraint stress.[246] Moreover, pretreatment with alpha-helical CRH has been shown to attenuate restraint-induced anorexia, enhance the hyperphagia induced by NPY, reduce the latency to begin eating and increase the duration of feeding induced by tail pinch.[187] In addition, ICV treatment with antisense phosphorothioate oligonucleotides, directed against the CRH mRNA induced short-term increases in cumulative food intake compared to missense controls.[202]

Although the central administration of CRH clearly suppresses food intake, recent studies using CRH knockout mice suggest that CRH is not necessary in the mediation of stressor-induced anorexia.[337] CRH knockout and wild type mice display similar basal levels of food intake. Upon exposure to stressors such as restraint, turpentine abscess, surgical stress, or systemic administration of interleukin-1, lipopolysaccharide, or a serotonergic agonist, both the knockout and wild type mice display similar decreases in food intake followed by a similar rate of recovery when treatment was discontinued. Likewise, adrenalectomy had similar effects on each genotype. Thus, as with the mediation of the stress response itself, it would appear that another CRH-like peptide may be involved in the mediation of stressor-induced anorexia.[337]

Studies exploring the relative importance of the CRH-R_1 and CRH-R_2 receptor subtypes in stressor-induced anorexia have been ambiguous, but the story is now starting to become clearer.[81] It has been speculated that the CRH-R_2 is the primary mediator of the effect of CRH on feeding.[88] Nonetheless, recent studies using knockout mouse strategies indicate that these two receptor subtypes may play biphasic roles in feeding behavior.[81] It appears that the CRH-R_1 may mediate the anorectic effect of Ucn from 2-4 hours after treatment, while the CRH-R_2 may be involved in late-phase (6-10 hour) suppression of food intake.[81] Following central administration of Ucn, CRH-R_2 knockout mice return to normal intake levels more quickly than their wild type counterparts.[81,88] In comparison, Ucn-treated CRH-R_1 knockout mice experience more hypophasia in the late but not early phase.[54,81,88] In rats, emotional stress can be induced using a communication box whereby the test rat is surrounded on all sides by, and able to communicate with,

other rats that are being exposed to a stressor.[198] Hotta et al[198] demonstrated that such a stressor is able to induce a reduction in food intake, an effect that was abolished by a selective CRH-R_1 antagonist, thus highlighting the importance of the CRH-R_1 in the modulation of anxiety.[198] The dense expression of CRH and CRH-R_1 in the amygdala suggests that this nucleus has an important role in coordinating the effects of stressors on feeding.[81]

Once again, the CRH-BP plays an interesting role. Food deprivation has been shown to increase CRH-BP mRNA levels in the dorsal part of the mPOA and basolateral amygdala in both lean and obese Zucker rats. Furthermore, in the pituitary, CRH-BP gene expression was significantly higher in the lean Zuckers compared to their obese counterparts and was decreased by 24-h food deprivation only in the lean rats.[423] In the lean rats, these increases in CRH-BP (which would reduce the bioavailability of CRH) may be acting to reduce energy expenditure and stimulate food intake.[423] In addition, CRH-BP over-expressing mice (MT-CRH-BP transgenic mice), display increased weight gain, beginning at three months of age for the males and 8-12 months of age for the females.[423] At this point it is not clear whether the weight changes are the result of increased food intake or decreased energy expenditure, as food intake has not been systematically measured in these animals. Interestingly, CRH-BP deficiency decreased weight gain in male (with a trend toward decreased food intake), but not in female (up to 9 weeks of age) mice.[423] Taken together, these findings indicate that free CRH and/or Ucn are associated with sexually dimorphic effects on weight gain, food intake and energy balance. Considering that the ACTH and corticosterone levels in these animals

are unaltered, it would appear that these effects are not being mediated by the HPA axis but, rather, by some yet to be determined central mechanism.[423] It is important to note that results obtained from gene knockout strategies need to be interpreted with caution.[100] Genetic changes, when induced early in development, may be accompanied by compensatory adaptive changes. Thus, observations of these genotypes may be the result of alternate gene expression rather than the targeted gene mutation.

In summary, CRH, which has been classically thought of as a "stress" peptide, appears to be involved not only in the mediation of the organism's response to stressor exposure, but also in the regulation of energy resources and food intake. Thus it would appear that the same neurochemical mediator may be subserving these two seemingly diverse stimuli. What is not clear at this point is whether food intake is being regarded, at least at some level, as a stressful event or whether CRH does, in fact, have a much broader role than previously envisioned.

Bombesin-like peptides (BN-LPs)

Bombesin (BN), a 14 amino acid peptide, was first isolated in 1970 from the skin of the European discoglossid frog, *Bombina bombina*, and has been shown to have a number of pharmacological actions.[8] Subsequently, several other BN-LPs of mammalian origin have been isolated, including the 27 amino acid peptide, gastrin-releasing peptide (GRP_{1-27}).[67,293] The carboxyl-terminal decapeptide of GRP is identical to that of BN except for a histidine/glutamine substitution at position 8 from the carboxyl terminal.[310] The discovery of GRP was soon followed by the characterization of additional BN-LPs,

bearing structural resemblance to litorin and ranatensin (see Table 2), including neuromedin B (NMB_{1-32}, NMB_{2-32}, and NMB_{23-32}) and GRP_{18-27}, (also known as neuromedin C).[78,310,320,322,333,348]

Table 2: Representative members of each BN-LP subfamily[78,320,322,323]

Bombesin subfamily	
BN	pGlu-Gln-Arg-Leu-**Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2**
GRP_{1-27}	Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-**Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2**
GRP_{18-27}	**Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2**
Alytesin	pGlu-Gly-Arg-Leu-Gly-Thr-**Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2**
Ranatensin subfamily	
NMB_{1-32}	X-Val-His-Ser-Arg-**Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**
NMB_{23-32}	**Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**
Ranatensin	pGlu-Val-Pro-**Gln-Trp-Ala-Val-Gly-His**-Phe-**Met-NH_2**
Ranatensin R	Ser-Asn-Thr-Ala-Leu-Arg-Arg-Tyr-**Asn-Gln-Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**
Ranatensin C	X-Glx-Thr-Pro-**Gln-Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**
Litorin	pGlu-**Gln-Trp-Ala-Val-Gly-His**-Phe-**Met-NH_2**
Rhodei-Litorin	pGlu-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**
Phyllolitorin subfamily	
Leu^8-Phyllolitorin	pGlu-Leu-**Trp-Ala-Val-Gly**-Ser-Leu-**Met-NH_2**
Phe^8-Phyllolitorin	pGlu-Leu-**Trp-Ala-Val-Gly**-Ser-Phe-**Met-NH_2**
Thr^5-Leu^8- Phyllolitorin	pGlu-Leu-**Trp-Ala**-Thr-**Gly**-Ser-Leu-**Met-NH_2**

Note: Structural homology of BN-LPs at the decapeptide C-terminal region (in bold). "X" represents the amino acid chain preceding the sequence indicated.

Distribution of BN-LP immunoreactivity

BN-LPs are widely distributed throughout the brain and gastrointestinal tract of many mammals including humans.[292,307,485] BN/GRP immunoreactivity is virtually absent in fetal brain and very early in development, but reaches adult levels by postpartum day 21.[358] In the gut, BN-LP immunoreactivity has been localized in nerve fibers of the myenteric plexus, submucosal plexus, and the mucosa of the stomach.[292] In the nervous system, BN-LP immunoreactivity has been detected in neuronal cell bodies and/or terminals of the hypothalamic and medullary/brainstem nuclei involved in the regulation of autonomic function and sensory systems.[90,292,358] Principally, high densities of BN-LP immunoreactive cell bodies have been isolated in the ventral and medial parvocellular PVN, which are known to project to the dorsal vagal complex.[90] Other hypothalamic areas expressing BN-LP immunoreactivity include the median eminence, AH, VMH, LH, suprachiasmatic nucleus and mPOA.[159,292,358] The medulla/brain stem/spinal cord is another important area that contains a high density of BN-LP immunoreactivity, specifically within the NTS, the substantia gelatinosa, lemniscus medialis and central gray of the spinal cord and the stria terminalis. Significant BL-LP immunoreactivity has also been detected in the amygdaloid complex, particularly in cell bodies, perikarya, and fibers of the CeA and lateral amygdala, and lateral septal nucleus. Finally, these peptides are also present in the thalamus, olfactory bulb, nucleus reticularis gigantocellularis, hippocampus, cingulate cortex, striatum, olfactory tubercle, frontal cortex, nucleus accumbens, and anterior pituitary.[78,157,172,292,296,323,329,332,358,504]

The first detailed localization of NMB using *in situ* hybridization with a very sensitive and specific cRNA probe was done in 1990, allowing for a direct comparison of GRP mRNA and NMB mRNA distribution within the central nervous system.[485] In the forebrain, a strong hybridization signal was found for prepro NMB mRNA in the olfactory bulb and the dentate gyrus. Moderate signals were isolated in the accessory olfactory bulb, pyramidal cell layer of Ammon's horn field CA3, the CeA, in an undescribed area between the AH and the septum, and between the fornix and septal nucleus. As well, a moderate signal was detected in the brain stem (principal sensory nucleus of the trigeminal, dorsal motor nucleus of the vagus, tegmental nucleus, nucleus incertus, hindbrain raphe, NTS, and dorsal motor nucleus of vagus). Furthermore, NMB (like GRP) is present in all major components of the HPA axis, including the hypothalamus, pituitary and adrenal glands.[113,211,321,330] A weaker signal for NMB was seen in the BNST, nucleus accumbens shell, lateral habenular nucleus, arcuate nuclus, mPOA, supramammillary nucleus, nucleus of the trapezoid body, nucleus of the lateral lemniscus, dorsal part of the area postrema, parabrachial nucleus, facial nucleus, median raphe, gigantocellular reticular field and lateral reticular nucleus. GRP mRNA was more widely distributed in the forebrain (presubiculum, parasubiculum, and pyramidal cell layer of the subiculum). Strong signals were found in the dentate gyrus and the cortical amygdaloid nucleus. A moderate signal was detected in the cortex, layers II and III, and a weak signal was observed in layers V and VI. Moderate signals were also detected in the olfactory nucleus, entorhinal area, presubiculum, pyramidal layers of Ammon's horn fields CA2 and CA3, medial, lateral and basomedial nuclei of the amygdala, amydalohippocampal area, medial geniculate nucleus, suprachiasmatic nucleus, the

mPOA, inferior colliculus, medial NTS and the parabrachial nucleus. Weaker signals were detected in the tenia tecta, lateral entorhinal area, pyramidal layer of Ammon's horn field CA1, anterior and basolateral areas of the amygdala, medial septal nucleus, nucleus of the diagonal band, magnocellular preoptic nucleus, posterior intralaminal nucleus, mPOA, anteroventral periventricular nucleus, PVN, posterior hypothalamic area, dorsal column nuclei, spinal trigeminal nucleus, central gray region, locus coeruleus, interfascicular nucleus, paranigral nucleus, rostroventrolateral reticular nucleus, and the substantia gelatinosa of the spinal cord.[485]

Distribution of BN-LP receptors

Upon release into the synapse, NMB and GRP bind to a family of G-protein coupled receptors, BB_1 (NMB-preferring) and BB_2 (GRP-preferring), respectively. Binding sites appear early in embryonic development in the parietal cortex, hippocampus and nucleus accumbens and may be important for early organization and maturation of the central nervous system.[150,157] The BB_2 cell surface receptor has a molecular weight of 43 to 45 kD, a size that is increased to 75-85 kD by glycosylation.[350,448] The BB_2 receptor contains seven putative membrane-spanning domains and is a member of the G protein-coupled receptor family.[448] Ligand binding results in stimulation of phospholipase C, protein kinase activation, Ca^{++} mobilization and tyrosine phosphorylation of various intracellular proteins.[350,403] The BB_1 receptor (molecular weight of 43kD) is a member of the G protein-coupled receptor family and is also glycosylated.[448]

Recently, two additional BN-like receptors have been isolated, BN receptor subtype 3 (BRS-3, or BB_3) and BN receptor subtype 4 (BB_4).[350] To date, no endogenous ligand for the BB_4 receptor has been found.[6] Similarly, the BB_3 receptor, which is a 399-amino-acid protein, has been dubbed an orphan receptor as no endogenous ligand has yet been identified.[403] The BB_3 receptor appears to be quite distinct from the other receptors[6] and is present only in testes, the pregnant uterus, a few brain regions, human lung,[350] breast and epidermal cancer cell lines.[403] Notwithstanding, it does appear to be important in the regulation of glucose metabolism, energy balance and the maintenance of blood pressure as BB_3 deficient mice become obese, develop hypertension and diabetes.[403]

In the periphery, BB_1 and BB_2 receptors are distributed in the gastrointestinal tract, particularly in the circular muscle layer of the gastric fundus and antrum and submucosal layer of the duodenum, jejunum, and ileum, as well as in the longitudinal and circular muscle layers of the ascending, transverse, and descending colon.[333] BB_1 and BB_2 receptors display a distinct binding pattern within the gastrointestinal tract. For example, these receptors in the esophageal muscularis mucosa show a higher affinity for NMB and have thus been classed as BB_1 receptors, while BN-like receptors identified in the pancreas display higher affinity for GRP and probably represent BB_2 receptors.[333] Both receptor subtypes are suspected to mediate the satiety signals; however, the peripheral site(s) of action for the initiation of the BN-LP signal remain to be identified.[310] In that context, the celiac artery may be important. Kirkham et al[237] found that significantly lower doses of BN were required to suppress food intake when the peptide was administered directly into the celiac artery compared to intraperitoneal,

superior mesenteric artery or inferior vena cava injections.[237] They suggested that receptors perfused by the celiac artery might be mediating the satiety-like effect of peripheral endogenous BN-LPs.

Generally speaking, BN-like receptors are widely distributed in various brain regions.[308] High to moderate densities of receptors are found within the amygdala, hippocampus, locus coeruleus, parabrachial nucleus and NTS.[308] Other areas where BN-like receptors have been localized include the hypothalamus (particularly in the suprachiasmatic nucleus, PVN, LH, VMH, dorsomedial hypothalamus, arcuate nucleus, median eminence and preoptic area), deep layers of the cerebral cortex, striatum, thalamus, olfactory bulb, nucleus accumbens, olfactory tubercle, cingulate and parietal cortex, medial forebrain bundle, caudate putamen, ventral pallidum, rhinal cortex, basomedial and central, outer molecular layer of the dentate gyrus, periaqueductal gray, BNST, dorsal parabrachial nucleus, facial nucleus, and paragigantocellular reticular nucleus.[90,314,329,358,367,372,497,503] The BB_1 and BB_2 receptors are differentially distributed.[275] For example, BB_2, but not BB_1 receptors appear to be localized in the arcuate nucleus.[275] BB_2 receptor mRNA predominates in limbic structures such as the amygdala and hippocampus.[308] In addition, there are high levels of BB_2 receptors in the rostral hypothalamus, particularly in the suprachiasmatic nucleus and high levels of BB_1 receptors in the dorsal raphe nucleus.[372] Interestingly, both BB_2 and BB_1 receptor mRNAs exist in brainstem structures such as the NTS and at the area postrema.[308] Finally, BB_1 receptors are also located at the dorsal raphe, where they appear to regulate the release of 5-HT at various forebrain structures.[308]

It is noteworthy that, in the central nervous system, BB_1 and BB_2 receptors are more widely distributed than BN-LPs.[329] Areas of close overlap include the interstitial nucleus of the stria terminalis, the mPOA, AH and suprachiasmatic nucleus, the periventricular and PVN, the central medial thalamic nucleus, CeA, facial nucleus and the NTS. Areas that have high concentrations of receptors but low BN-LP immunoreactivity include the olfactory bulb and tubercle, the caudate putamen, the hippocampus, and the locus coeruleus. The cortex has a moderate density of receptors but no BN-LP immunoreactivity. It is possible that these receptors must be interacting with other structurally similar peptides yet to be identified.[329]

Although caudal brain structures appear to be sufficient for the suppression of food intake by BN, other brain nuclei also appear to be involved and it is not yet clear how the endogenous peptide signal is transmitted within the central nervous system.[310] Studies using retrograde and anterograde tracing methods reveal that the NTS and/or the dorsal motor nucleus of the vagus receive projections from the prefrontal cortex, BNST, CeA, the PVN, arcuate nucleus and posterolateral areas of the hypothalamus.[478] As mentioned above, the parvocellular portions of the PVN provide a major source of BN immunoreactive terminals located at the NTS/dorsal motor nucleus of the vagus complex.[90] Major forebrain connections to the NTS/dorsal motor nucleus originate in the lateral prefrontal area, and major subcortical projections originate from the CeA and the PVN.[478] The CeA, PVN, NTS/dorsal motor nucleus of the vagus connection is particularly relevant because of the intimate relationship of this pathway to both feeding behavior and stress responses.[310]

The role of BN-LPs in feeding

It had long been suspected that satiety might be mediated by peptides released from the gut in response to ingested food. Interest in BN's relationship to feeding arose naturally from the observation that this family of peptides was widely distributed in the mammalian gut.[152] Today, there are numerous reports that support the conclusion that BN-LPs dose-dependently suppress food intake and contribute to satiety processes.[90,151,152,155,292,307,310,314,334,340] Interestingly, BN administration does not lessen the animals' initial interest in food, suggesting that the effect is not simply due to sickness or malaise.[39,132,310,340] Indeed, humans injected intravenously with BN report less hunger and early fullness without any other significant side effects.[340] Animals administered exogenously with BN consume normal amounts of foods in the initial five minutes (i.e. comparable to the saline treated animals). However, they stop eating (i.e. experience satiation) sooner than saline-injected animals and increase their intermeal interval (experience increased satiety), thereby reducing their overall food intake. Furthermore, peripheral administration of BN does not affect drinking behavior. Since eating and drinking require similar motor activity, it would appear that these effects are not due to motor impairment. Finally, when the animals do stop eating, they engage in normal postprandial activities such as grooming and exploring.[151] Thus, the satiety-like effects of BN-LPs appear fairly specific, mimicking those seen during spontaneous satiety.

It has long been apparent that the actions of BN-LPs are centrally mediated. While both peripheral and central administration of BN (and related peptides) suppress

food intake, the effects are much more potent when the peptides are administered directly into the brain.[133,153,209,313] In terms of the relative potency, administration of BN itself engenders the most potent effects followed by centrally administered GRP, then NMB_{1-32} and, least potent, NMB_{23-32}.[310] BN itself has an amidated C-terminal that makes it relatively insensitive to enzymatic degradation, while the endogenous forms of the peptide, GRP and NMB, are subject to fairly rapid degradation.[310] Unlike systemic administration, when BN is administered centrally, intense and sustained grooming is seen, suggesting that the suppression in food intake following ventricular administration could be confounded by competing behaviors. However, the effects on ingestion can be differentiated from those on grooming. For instance, when exquisitely low doses of BN are administered into the 4th ventricle or the NTS, suppression of feeding can be achieved without a concomitant increase in grooming.[209,310,333] Finally, knockdown of BB_1 and BB_2 receptors using continuous infusion of mixed antisense agents directed against the respective mRNAs eliminated the suppressant effects of BN on food intake compared to control animals (vehicle, mismatch, or scrambled).[379]

Physiological effects seen following administration of pharmacological doses of a peptide do not necessarily imply a role for *endogenous* BN-LPs in those processes. If endogenous BN-LPs are actually involved in satiety mechanisms, one would expect that the blockade of their effects by antagonists would increase feeding. In fact, the BN receptor antagonist, [Leu^{14}, psi 13-14]-BN, did increase food intake but only in sated animals. This effect was much more potent when the drug was administered into the 4^{th}, compared to the 3^{rd} ventricle.[314] The lack of effect in food deprived animals was thought

to be due to a ceiling effect.[310] In other words, food deprived animals consumed so much food that they were physically unable to consume more. Another approach to clarifying the functional role of endogenous peptides is to study animal models that lack the specific peptide receptor (for example, receptor knockout mice). It has been shown that in mice lacking the GRP receptor, BN is unable to suppress feeding.[178] However, these animals, as well as NMB receptor deficient animals, developed normally and did not display any ingestive abnormalities.[178,349,484] Although this finding seems paradoxical, it is more than likely that parallel or compensatory mechanisms may have taken over this essential function.[310]

The neural circuit(s) governing the satiety effects of BN-LPs remains poorly understood.[310] Several mapping studies involving microinjection of BN into various brain sites, point to certain central nuclei in mechanism governing BN-LP effects.[133,154,208,209,252,455] Early studies found that microinjections of BN into various regions of the hypothalamus, including the LH, PVN, DM, VMH, as well as the amygdala, reduced food (but not water) intake.[154,252] These effects were site specific in that similar injections into the anterior and posterior hypothalamus, septum, ventral tegmental area, reticular formation and the 4th ventricle were not effective. The lack of effect in the 4th ventricle was surprising as others have found 4th ventricular injections to be exquisitely sensitive to the effects of BN,[133] suggesting that hind brain nuclei may be involved in the action of BN-LPs. Indeed, the NTS (a caudal brain nucleus) proved to be highly sensitive to the behavioral effects of BN including its suppression of food intake.[208,209] Furthermore, ablation of the NTS abolished the effects of 4th ventricularly

injected BN and attenuated the effects of peripherally administered BN.[255] In addition, injection of BN into the 4th ventricle is fully able to suppress food intake in decerebrate rats indicating that the caudal brain stem is sufficient to mediate the feeding suppressant effect of BN.[134] Several forebrain nuclei are probably also involved in the mediation of BN's feeding suppressant effects, however these may be upstream to the final output pathways involving an intact brain stem.

The ingestion of food is a peripheral process and, thus, if feeding and satiety are governed by central mechanisms, there must be a communication link between the gut and the brain. Typically, satiety occurs prior to digestion or absorption of a meal. Accordingly, it is logical to assume that, upon ingestion, certain peptides are secreted from the gut, which then, via endocrine, neurocrine and/or paracrine modes of action, signals the brain to terminate eating.[154,155,310] This putative gut-brain axis remains to be identified.[310] It is not likely that these peptides cross the blood-brain barrier,[23] although it is possible at areas where the barrier is more porous such as the area postrema.[23,310]

Once again, lesion studies have been undertaken to try to establish the neural pathway between the peripheral site of action of BN and the brain.[154] Adrenalectomy, hypophysectomy, bilateral lesions of the ventromedial or dorsomedial hypothalamus, spinal cord section at the sixth thoracic vertebra and subdiaphragmatic vagotomy all failed to eliminate the satiety effect of peripherally administered BN in rats.[154] One possible conduit by which BN-LPs may be mediating its action is through the vagus nerve, which is known to mediate the action of other satiety peptides such as CCK.[213] A

breakthrough study revealed that, although subdiaphragmatic vagotomy was not sufficient to block the satiety effects of systemically administered BN,[456] a more complete neural disconnection of the gut from the brain did block BN-induced satiety.[456] This procedure involved initially cutting the spinal cord at the level of the sixth thoracic vertebra and performing a dorsal rhizotomy at the third, fourth, fifth, and sixth thoracic cord segments. Following this initial procedure, BN was still effective in suppressing food intake in these animals. A second operation was performed to bilaterally dissect the subdiaphragmatic vagal nerve. The combination of spinal-visceral neural disconnection and bilateral subdiaphragmatic vagotomy abolished the effect of BN on first meal size. Thus, it appeared that the gut-brain communication link involved neural rather than endocrine link(s).[310]

The possibility of an alternate neuronal signaling network for BN-LP's peripheral effects on food intake has also been explored. Neonatal animals were injected subcutaneously with capsaicin, a neurotoxin commonly found in chili peppers.[319] This treatment produces a deafferentation of the unmyelinated (C fiber) or thin myelinated (Aδ fiber) axons, which desensitizes a subset of primary sensory neurons including both vagal and spinal fibers while sparing efferent pathways.[310,319] Upon reaching adulthood, capsaicin treated animals were completely insensitive to the feeding-suppressant effects of systemically, but not centrally, administered BN.[310,319]

It is clear that exogenous administration of BN suppresses food intake by reducing the size of the meal and by increasing the intermeal interval. However, before

we can say with certainty that BN-LPs play a role in the physiology of feeding, it needs to be established that *endogenous* peptide levels actually vary in accordance with food consumption. Indeed, endogenous tissue levels of BN-LP immunoreactivity have been shown to change rapidly at specific brain sites and gut in response to deprivation and ingestion of a meal.[224,307,378] In fasted animals, the spontaneous ingestion of a meal induced increases of BN-LP immunoreactivity at the PVN, arcuate nucleus, DM, and in the antrum of the stomach. However, changes in tissue peptide levels do not necessarily constitute release of the peptide into the synapse but, rather, could simply be a reflection of the rate of synthesis and/or biodegradation.[310] A more dynamic study using push-pull perfusion at the PVN revealed that the increased tissue levels at that nucleus did, in fact, represent release of BN-LPs in response to ingestion. During the meal, BN-LP immunoreactivity levels dropped significantly, potentially disinhibiting food consumption. Cessation of food intake was then coupled with a sharp rise in peptide release.[377] These results support the hypothesis that BN-LPs do indeed provide a signal in the PVN to induce and/or maintain satiety.

The role of BN-LPs in stress and/or anxiety

Although BN has been studied primarily from the vantagepoint of its ability to induce satiety, recent evidence suggests that this family of peptides may also play a role in the mediation and/or modulation of the stress response.[308]

There are several lines of evidence to support a role for BN-LPs in the mammalian stress response. Based on their distribution pattern, BN-LPs appear to be

well positioned to influence the stress response including that of the HPA axis. Moreover, it has become well established that exogenous administration of BN and its mammalian counterparts, GRP and NMB, markedly elevates the circulating levels of ACTH and corticosterone.[308] This effect appears to be mediated via the BB_2 receptor complex as central administration of a competitive and specific BB_2 antagonist blocked the effect.[142] However, one cannot rule out BB_1 involvement due to the unavailability of highly selective BB_1 antagonists. The data concerning endocrine responses following peripheral administration of BN-LP agonists have been inconsistent as intravenous injection of GRP and/or BN had either no effect or induced significant elevations in plasma ACTH and corticosterone levels.[308] These discrepancies are likely due to methodological differences in either the species being tested or of the dosages used.[308] With respect to sympathetic activity, central administration of BN dose-dependently increased plasma levels of epinephrine and NE as well as mean arterial blood pressure and plasma glucose levels.[308] These effects appear to be centrally mediated and independent of the HPA axis as hypophysectomy did not prevent BN-induced hyperglycemia and adrenalectomy did not prevent BN-induced rise in plasma catecholamines.[308] The NTS and/or VMH and LH are potential sites of action, as microinjection of BN into the NTS produces a dramatic elevation in circulating catecholamine levels whereas similar treatment in the VMH and LH induces hyperglycemia.[308]

In addition to the neurochemical and endocrine findings, there is also behavioral support for the BN-LPs' role in the stress response.[308] Although data are not available for

the BN-LPs' effects in tests utilizing standard animal models of anxiety such as the elevated plus maze, light-dark box, or fear potentiated startle, several other observations suggest a similarity between BN-induced and stressor-induced behaviors.[308] For example, when rats are offered a highly palatable snack in a familiar environment, they readily consume the snack; however, when offered the same snack in a novel environment, rats will display a prolonged latency to initiate consumption and a diminution in the amount of food consumed, an effect that can be reversed with anxiolytic treatment.[308] Thus, it was of interest to note that the effect of the novel environment can be attenuated with the administration of the mixed BB_1/BB_2 receptor antagonist, PD 176252, suggesting that the BN family of peptides might play a role in the mediation of anxiety.[308] As previously discussed, BN-LPs potently reduce food intake and have been actively investigated as a satiety peptide. However, it is possible that stressor-induced anorexia recruits circuits using BN-LPs as well. In support of this contention is the ability of the CRH receptor antagonist alpha-helical CRF to block BN-induced suppression of food intake.[308]

Finally, exogenous administration of BN or GRP induces several behaviors that are also associated with the stress response. Central (but not peripheral) administration of BN induces grooming behavior, similar to that seen following stressor application.[249,208,308] Both the administration of BN-LPs and the application of a stressor induce the reduction of exploration in a novel (presumably stressful) environment and increased exploration in a familiar environment.[367,306,308] Lastly, just as stressor exposure has an antinociceptive effect, likewise the microinjection of BN into the periaqueductal

gray matter produced an antinociceptive effect in both the hot plate and tail flick tests.[367,308]

Another approach to ascertaining an association between BN-LPs and the stress response would be to assess whether stressor exposure functionally affects the endogenous BN-LP system(s).[308] There are several pieces of evidence suggesting that this is indeed the case. For example, immobilization stress is associated with site-specific alterations of endogenous levels of BN-LPs at the hypothalamus and medulla.[230,308] In addition, the density of BN-LP binding sites was increased in stress relevant regions including the NTS, arcuate nucleus and PVN following acute exposure to immobilization stress.[230,308] As previously discussed, post-mortem tissue analyses can be difficult to interpret as they represent a "snapshot in time" and can fail to detect dynamic changes in turnover if, for example, the alterations of peptide utilization were offset by compensatory changes in peptide synthesis or degradation.[308] In an attempt to assess dynamic changes in the actual release of BN-LPs in response to stressor exposure, *in vivo* push-pull perfusion experiments were performed. Results confirmed that mild stressor exposure (air puff) was associated with increased interstitial levels of both GRP and NMB at the anterior pituitary.[229] Together, these findings suggest a functional role for BN-LPs in the mediation and/or modulation of the stress response.

Although the neuronal mechanisms underlying the stress-relevant BN-induced effects remain to be fully elucidated, there is evidence that BN-LPs stimulate the HPA axis by acting through other ACTH secretagogues, in particular, CRH and/or AVP.[308] In

support of this contention, it has been shown that central administration of the CRH antagonist, alpha-helical CRF, attenuated the BN-induced rise in plasma ACTH, NE, epinephrine, glucose and corticosterone levels.[232,308] It has been further demonstrated that GRP administration dose-dependently stimulated ACTH release and potentiated the ACTH-stimulating effects of CRH and AVP, an effect blocked by CRH and/or AVP antisera.[308,351] In addition, *in vitro* studies using isolated perfused and mixed anterior pituitary cells, BN or GRP enhanced CRH-induced ACTH release, an effect completely blocked by the BB_2 receptor antagonist D-Tyr^4 BN(6-13) propylamide, indicating that BN-LPs may exert their effects by binding to BB_2 receptors on anterior pituitary cells.[14,308] As BN-LPs appeared to mediate their effects through central CRH and/or AVP neurons, it was of interest to determine the anatomical locus (loci) of these interactions. Using the micropunch brain survey approach, Kent et al[231] were able to demonstrate that ICV administration of BN (0.25 and 0.5 μg) significantly decreased levels of CRH immunoreactivity at various hypothalamic and extrahypothalamic brain sites, including many stress-relevant regions such as the VMH, AH, PVN, NTS and CeA. In contrast, BN administration was associated with a significant increase in the endogenous levels of AVP at the PVN and the median eminence/arcuate nucleus. Moreover, more direct push-pull perfusion experiments revealed that BN administration evoked the release of immunoreactive CRH and AVP from the median eminence/arcuate nucleus and increased interstitial CRH and AVP levels at the anterior pituitary.[231,308]

In summary, evidence suggests that CRH and BN-LPs play important roles in both the stress response and feeding behavior. It is interesting that some of the

modulators of feeding behavior are very similar to those mediating the stress response. It may be that stressors, in order to facilitate an adaptive response, deploy mechanisms normally used to terminate food intake. On the other hand, these peptidergic responses may be activated by the organism to draw attention to cues or events of physiological significance, such as those associated with the availability and/or the ingestion of food. This observation suggests the possibility that both stress and feeding are being subserved by overlapping, redundant peptidergic systems.

Overall Objectives

The overall objective of this thesis was to examine the pattern of utilization or release of BN-LPs and CRH at specific brain loci during 1) various phases of food ingestion, 2) the presentation of anticipatory cues paired with an appetitive event, and 3) exposure to stressors. Some peptidergic linkages between 'stress' and 'satiety' responses will also be explored. These studies could potentially expand our understanding of the neurochemical mechanisms regulating the initiation, maintenance and/or termination of a meal. Furthermore, they may also provide insight into the role of peptidergic and endocrine systems in mediating physiological and psychological responses to appetitive (food) and aversive (stressor) events.

In an attempt to attain the overall objectives of this thesis, we have addressed the following six specific objectives or questions:

CRH and BN-LPs are released in response to both aversive and appetitive events: Neurochemical approach

1) Are BN-LPs and/or CRH released at the CeA in response to biologically salient events such a meal ingestion or stressor exposure? Given that postmortem tissue level analyses provide only a "snapshot in time" with equivocal interpretation about peptide utilization, we decided to a more dynamic *in vivo* approach. Thus, the first series of experiments were aimed at validating the use of *in vivo* microdialysis in monitoring the release of peptides from the CeA of freely behaving animals, and

assessing whether the release of CRH and BN-LPs in the amygdala was influenced by stressor exposure or by feeding.

2) Is feeding associated with altered peptide availability at the anterior pituitary? In Chapter II, we examined, through the use of *in vivo* push-pull perfusion, the effects of palatable snack intake on interstitial levels of CRH, GRP, NMB, AVP and corticosterone at the anterior pituitary. Furthermore, we also examined whether the animals' prior stress history (previous exposure to an acute or chronic stressor) influenced the feeding-associated fluctuations in the interstitial levels of CRH, GRP, NMB, AVP, and corticosterone.

3) Are "anticipatory" cues associated with changes in CRH, BN-LP and 5-HIAA levels at the medial prefrontal cortex? In contrast to the role of central dopaminergic pathways in the control of reward motivated behaviors (e.g. food consumption and drug abuse), little is known about the potential role of BN-LP's and CRH in the anticipation of a food reward. Experiments presented in Chapter III assessed the potential role of DA, 5-HT, CRH and BN-LPs in the "anticipation" and subsequent ingestion of a palatable snack (a natural reward). Based on previous findings, mPFC was selected as the neuroanatomical target, with special attention to the potential lateral bias in the neurochemical responses. Specifically, this set of experiments assessed changes in DA, 5-HT, CRH and BN-LP within the right and left mPFC in response to the 1) "anticipation" and 2) consumption of a palatable "snack".

Factors that influence an organism's response to stressor exposure also impact feeding behavior: Neurochemical and Behavioral approaches

4) Do short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding responses in adult rats and, if so, are these effects gender dependent? This set of experiments was predicated by the findings that neonatal rats separated from their dam during early developmental phase, display altered responsiveness to stressors in adulthood. The aim of the next set of experiments was to characterize the effects of early manipulations of mother-pup interactions on ingestive responses among male and female rats. Specifically, we assessed the effects of neonatal handling and maternal separation on 1) the developmental changes in weight gain, 2) anxiety responses, 3) propensity to consume palatable "snack" food and 4) the feeding-suppressant effects of various satiety peptides (CCK, BN, and amylin).

5) Does gender affect the propensity to consume a palatable snack? Research over the past few decades has highlighted the fact that, among other things, there may be gender differences in feeding behavior. Thus, the purpose of the next set of experiments was to 1) examine the consumption of both "bland" rat Chow and a palatable high carbohydrate "snack" over the course of the females' estrus cycle, 2) investigate potential gender differences in both rat Chow and palatable "snack" intake and 3) compare the total intake of male and female rats in the absence and presence of a palatable snack.

6) **Do genetically variant rat lines with altered stress-reactivity display differences in carbohydrate snack consumption? Are these differences associated altered stress- and/or pharmacological-responses, and/or altered endogenous peptide levels? In the final section, we investigated potential differences between Fast and Slow rats with respect to (1) their appetite for palatable snacks (Graham Wafer and sucrose), (2) stressor- (novel environment) induced suppression of snack intake, in the absence and presence of the anxiolytic diazepam, and (3) regional variations in the endogenous CRH and BN-LP levels across various brain regions.**

These six sets of experiments constitute the chapters of this thesis and are presented in journal format, either published or prepared for publication. They are presented in an order that creates a logical flow as opposed to the order in which they were carried out. The general discussion that follows the experiments represents an overall view of the material from the perspective of peptidergic mechanisms governing ingestive behavior. Attempts are also made to reconcile the role of CRH and BN-LPs in feeding behavior, with their role in the mediation of the stress response.

Preface to Chapter I

It is established that CRH system(s) is (are) activated by stressful stimuli. There is also considerable evidence to support the notion that BN-LPs play an important role in the modulation of the stress response. Furthermore, it has been shown that both CRH and BN-LPs play a role in the regulation of feeding behavior. Previous research in our laboratory has demonstrated that tissue levels of BN-LPs and CRH fluctuate at feeding-relevant brain sites including hypothalamic nuclei and the CeA in relation to the animals' feeding status. However, the mechanisms contributing to such tissue level changes are inconclusive and open to interpretation, as post mortem tissue levels can be influenced by several processes including the rate of release, synthesis and/or biodegradation. Furthermore, they are static measurements reflecting a "snapshot in time" and may not reflect dynamic fluctuations. We attempted to obtain an unequivocal mechanistic view of the dynamics of *release* of CRH and BN-LPs at the CeA in response to both aversive (restraint) and appetitive (spontaneous food intake) events, by deploying the *in vivo* microdialysis technique.

CHAPTER I

Aversive as well as appetitive events evoke the release of corticotropin releasing hormone and bombesin-like peptides at the central nucleus of the amygdala.

Abstract

There is wide agreement that CRH system(s) within the brain is (are) activated by stressful stimuli. There is also mounting evidence for the role of BN-LPs in the mediation of the stress response. To date, however, the extent to which other stimuli increase the activity of these peptidergic systems has received little attention. In the present investigation we validated and used *in vivo* microdialysis sampling followed by *ex vivo* radioimmunoassays, to monitor the release of CRH and BN-LPs during appetitive (food intake) and stressful (restraint) events. It is demonstrated for the first time that the *in vivo* release of CRH and BN-LP(s) at the CeA, was markedly increased by both stressor exposure and by food ingestion. In fact, the meal-elicited rise of CRH release was as great as that associated with 20 min of restraint stress. Paralleling these findings, circulating ACTH and corticosterone levels were also increased in response to both food intake and restraint. Contrary to the current views, these results indicate that either food ingestion is interpreted as a "stressful" event by certain neural circuits involving the CeA, or that the CRH and/or BN-related peptidergic system(s) may serve a much broader role than previously envisioned. Rather than evoking feelings of fear and anxiety, these systems may serve to draw attention to events or cues of biological significance, such as those associated with food availability as well as those posing a threat to survival.

Introduction

There is considerable evidence suggesting that CRH, a hypophysiotropic peptide comprising 41 amino acids,[474] plays a fundamental role in stress reactivity. In particular, stressors reliably enhance the expression of CRH mRNA in hypothalamus,[26,238,245,413,473] and ICV administration of CRH elicits a constellation of behavioral, physiological, and endocrinological changes similar to those produced by stressors.[118] Conversely, CRH antagonists attenuate the behavioral effects of CRH, as well as those elicited by stressors.[165,185] In addition to hypothalamic CRH, it seems that central amygdaloid CRH changes are also intricately involved in orchestrating the response to stressors. In this respect, amygdaloid CRH manipulations predictably affect behaviors indicative of anxiety,[118,215,466] although these effects may be more closely aligned with a fear response than nonspecific anxiety,[263] Moreover, stressors have been shown to affect CRH mRNA levels and the levels of CRH within the amygdala.[370] In this respect, various aversive conditions (such as withdrawal from alcohol, cocaine or cannabis) influence the levels and/or turnover of CRH at the CeA.[370,398]

The HPA axis activation associated with stressors, is an exceedingly robust phenomenon, so much so that glucocorticoid changes have been taken to reflect the presence of stressors. Contrary to this dogma, however, there is reason to believe that alterations of HPA activity may be influenced by appetitive stimuli, just as aversive events elicit such effects. For example, Dallman and associates[97,419] as well as Shiraishi et al[430] have reported that the HPA responsiveness to stressors was influenced by whether the animals were food deprived or sated. Furthermore, food consumption itself, as well

as other rewarding stimuli also promotes glucocorticoid secretion.[369] In the case of humans, cortisol response to food occurs prior to the food actually being absorbed from the gastrointestinal tract.[7,219,474] As well, electrophysiological evidence supporting the view that information processing involving the cortex-amygdala-LH contributes to the control of feeding behaviors. In particular, it was demonstrated that some amygdala neurons responded to cues associated with food and that the degree of responsiveness varied with the apparent affective significance of the stimulus.[139] The suggestion has indeed been offered that the amygdala contributes to the processes by which sensory stimuli gain motivational and emotional significance.[140,210,444,505] In effect, neurons of the CeA may react to the salience or significance of emotional stimuli, rather than simply the negative or positive attributes of these stimuli.

Like CRH, the BN-family of peptides may be part of a constellation of responses to both stressors and to feeding. For instance, endogenous levels of BN-LPs in several brain regions vary during the course of a meal,[307,378] and exogenous administration of BN suppresses food intake[151,314] Interestingly, BN administration markedly elevates circulating ACTH levels and this effect can be blocked by pretreatment with a CRH antagonist.[315] Moreover, stressors influence BN-LP immunoreactivity in several brain regions, particularly the hypothalamus.[230] Thus, there is reason to believe that BN-LPs modulate the HPA response to both appetitive and aversive stimuli.

In the present investigation we demonstrate that, in response to food intake, as in response to stressors, plasma ACTH and corticosterone levels are increased. Moreover, it

is shown for the first time that the release of CRH and BN-LP(s) in the amygdala, as assessed by *in vivo* microdialysis, was markedly increased by both stressor exposure and by feeding. It is suggested that the CeA, and particularly CRH and/or BN-LP neurons within this region, is fundamental in mediating the response to emotionally laden events, irrespective of whether they are negatively or positively charged.

Materials and Methods

Subjects and surgical procedures

Male Sprague-Dawley rats weighing approximately 350-450 g (n = 50) were individually housed in standard clear plastic cages, and were maintained on a 12-h light-dark cycle (light phase: 0630-1830 h). Animals had free access to Purina Lab Chow and water. All experimental procedures followed the guidelines of the Canadian Council on Animal Care, and were approved by the Research Ethics Committee of the University of Ottawa. Rats were anesthetized (60 mg/kg pentobarbital intraperitoneal) and stereotaxically implanted with a 20 gauge guide cannula containing a removable 24 gauge obturator, aimed at the CeA. The placement coordinates (obtained from Paxinos and Watson, 1986[362]) with level skull were anterioposterior = -2.3 mm, dorsoventral = -7.0 mm, and lateral = ±4.2 mm. The guide cannula protruding from a custom manufactured Delrin pedestal was secured to the skull with 4 screws and dental cement. After surgical recovery (a minimum of 7 days), animals were transferred to individual testing chambers and allowed to acclimate for 2 days prior to testing. The testing chambers comprised of Plexiglas cages (25 x 35 x 34 cm) with a stainless steel grid floor. Food (powdered Purina Lab Chow) was available through a 3 cm hole centered in a short

tunnel (6.5 x 6.5 x 10 cm) protruding from the cage. A food cup located beneath the hole was positioned atop an electronic scale, permitting continuous monitoring of food consumption.

Carotid cannulation for blood sampling

For some experiments, rats were implanted with carotid arterial catheters under aseptic conditions. The carotid artery was exposed by blunt dissection, and a small incision was made using a 23 gauge needle. The catheter, consisting of silicone tubing (Dow Corning) and polyethylene (PE-50) tubing, was inserted into the artery, ligated to the vessel, tunneled subcutaneously, and exteriorized at the neck level. During sampling, the cannula was connected to remote syringe, using a tethering jacket and a swivel assembly, which permitted animals to move about freely in their cages during the experiment.

In vivo microdialysis

Two hours prior to testing, rats were briefly anaesthetized with halothane, and the obturator within the guide cannula was replaced with a microdialysis probe. The concentric microdialysis probe had 2.5 mm of active membrane (250 μm outer diameter) of regenerated cellulose (6000 molecular weight cut-off; Spectrum Medical Industries, CA), that protruded into the central nucleus of the amygdala. Each probe was secured with a retaining screw and connected via a polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel and a 2.5 ml infusion syringe (Hamilton) attached to a pump (Harvard, model 22). Microdialysis probes were perfused at the rate of 2 μl/min, with

filtered Kreb's Ringer Phosphate (KRB) solution consisting of (in mM) 2.7 K^+, 145 Na^+, 1.35 Ca^{++}, 1.0 Mg^{++}, 150 Cl^-, 0.05 ascorbate, pH 7.4[327] and BSA (0.1%). Upon collection, each sample (40-60 µl) was immediately frozen on dry ice and stored at –80°C until radioimmunoassay analyses. The efficiency of the microdialysis probes in terms of peptide recovery, was assessed *in vitro* as follows: probes were first submerged in a plain KRB solution and flushed with perfusion medium (KRB with 0.1% BSA) at the rate of 2 (l/min for 1 hour. They were then switched to tubes containing either [^{125}I-Tyr^0]CRF or [^{125}I-Tyr^4]BN in KRB. The average peptide recovery for each probe was 3.3 ± 0.6 % for CRH and 9.2 ± 0.15 % for BN, over 5 successive sampling periods with negligible variability. When the solution bathing the probes was changed from one containing one of the ^{125}I-labeled peptides to that of plain KRB, there was only a slight carry-over effect on the first sample (< 0.6%) and then dropping to an average of 0.15%. Reinsertion of the probes into the solution of iodinated peptide was accompanied by recovery of counts in the perfusate to their level originally seen with that probe, within the first sampling period.

Radioimmunoassays (RIAs)

The detection and quantification of CRH was achieved through a solid-phase high sensitivity adaptation/modification[285] of the double antibody liquid phase radioimmunoassay originally described by Vale and colleagues.[475] BN-LP(s) were detected using a similar solid-phase radioimmunoassay.[378] Briefly, protein A/G (Calbiochem-Novabiochem Corp., La Jolla, CA)-coated Immulon®-4 wells (Dynatech Laboratories, Inc., Chantilly, VA) were incubated with anti-CRH serum (rC70 kindly

provided by W. Vale) or anti-BN serum (α-BN2 kindly provided by Dr. T. Moody) for 2 hours at 20°C. Samples, standards (reconstituted in the KRB solution, ranging from 0.05 to 250 fmol/well) or blanks were incubated for 24 h at 4°C. Next, 25 µl assay buffer containing 5000 - 6000 CPM [^{125}I-Tyr^{0}]rCRF (Amersham Canada Ltd., Oakville, ON) or [^{125}I-Tyr^{4}]BN (iodinated in house, as per Salacinske et al.[409]) was added to each well and incubated for an additional 24 hour period at 4°C. Finally, the wells were rinsed, separated and their residual radioactivity counted in a gamma-counter (Cobra® II Auto-gamma, Meriden, CT). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically about 0.1 and 2 fmol/well for CRH and BN, respectively.

The specific anti-CRF serum used in the study recognized CRH_{1-41} and displayed negligable cross-reactivity with other related peptides[475] including urotensin 1 and urocortin (data not shown). The BN antibody used in the RIAs recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with amphibian BN (100%) and certain mammalian BN-LPs including GRP_{1-27} (110%), and GRP_{18-27} (neuromedin C) (82%), but only weakly with GRP_{1-16}, NMB-10, NMB-32 or substance P (≤0.1%).[332] We have shown in the past that the major source of BN-LP immunoreactivity from the hypothalamus is attributable to GRP.[307]

To verify the identity of the CRH immunoreactive material detected in the dialysates, probes positioned within the CeA (n = 3) were perfused at the rate of 2 µl/min for 8 hours. The total dialysate volume (960 µl) collected over ice, was split in two

aliquots (480 µl each) and freeze dried for two distinct high performance liquid chromatography (HPLC) analyses. The freeze dried samples were reconstituted and separated by a reverse-phase HPLC system consisting of Spectra-Physics gradient pump (P-2000; Spectra-Physics Analytical, San Jose, CA) and a C-18 Vydac® column (model 218TP54, 250 x 4.6 mm, 5µ, C18, 300-Å pore size). The column was equilibrated with mobile phase "A" (10% acetonitrile (AcN) with 0.1% TFA, in H_2O) and peptides eluted with mobile phase "B" (90% AcN with 0.1% TFA, in H_2O), using a linear gradient (from "A" = 100% to "B" = 100%, over 50 minutes), at a flow rate of 1ml/min. Authentic CRH standard (rat/human CRH_{1-41}, Peninsula laboratories, CA) was run under identical conditions and the absorbence of the elutant monitored at 214 nm (Applied Biosystems absorbence detector model 783A). The peak elution time for synthetic CRH was 31.8 minutes, and that of CRH-immunoreactive material (ir-CRH) contained within the dialysate eluted in fractions collected over 31-32 minutes, coinciding with the elution time of authentic CRH. The major peak of BN-LP immunoreactive material eluted in fractions collected over 17-18 minutes and coincided with the elution time of synthetic GRP_{1-27} (Peninsula laboratories, CA) of 17.4 minutes, whereas, the smaller peak (preceding or shouldering the major peak) that eluted with fractions collected over 14-16 minutes, corresponded with elution time of synthetic GRP_{18-27}.

The identity of eluting peptides was replicated and verified using a) the same HPLC conditions but a different microdialysis sample and b) a second, distinct set of HPLC conditions. This alternate HPLC procedure utilized a different C18 column (Jupiter® 250 x 4.6 mm, 5µ, C18, 300-Å pore size; Phenomenex, Torrance, CA) and the

following elution conditions: the system was equilibrated with mobile phase "A" (10% AcN with 0.1% TFA in H_2O) and peptides eluted with mobile phase "B" (90% AcN with 0.1% TFA in H_2O), using a linear gradient (from 100% "A" to 100% "B" in 100 minutes) at a flow rate of 1 ml/minute. The eluting fractions were collected every 30 seconds and freeze dried for subsequent radioimmunoassay analyses. Under this set of conditions, synthetic CRH eluted at 50.3 minutes and the major peak of endogenous CRH immunoreactive material from the dialysate was contained within fractions collected over 50-51 minutes. BN-LP immunoreactivity from the sample eluted at 26.5 minutes (minor peak) and 29 minutes (major peak), once again corresponding to retention times of synthetic GRP_{18-27} (25.9 minutes) and GRP_{1-27} (29.2 minutes), respectively (data not shown). These results are consistent with the assertion that the CRH immunoreactive material in the amygdaloid dialysate represents authentic CRH whereas BN-LP immunoreactive material represents GRP_{1-27} and/or GRP_{18-27}.

Plasma ACTH and corticosterone measurements

Corticosterone and ACTH levels were measured using commercial radioimmunoassay kits (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

Experimental design and procedures

Thirty rats with probes aimed at the CeA participated in the initial experiment (stress study). The probes were continually perfused with KRB, and dialysates pooled every 30 minutes throughout the experiment. After collection of 5 baseline samples, rats in the "stress" group (n = 20) were manually restrained for 20 minutes. The restraint

procedure consisted of a rat, situated on the floor of the test chamber, being lightly grasped about the shoulder/forelimbs by an experimenter's gloved hand. Thus, the ability of the animals to move about was prevented. Dialysate samples continued to be collected (every 30 minutes) for 2.5 hours after which the animals were again restrained (for 20 minutes) and dialysates collected for an additional 2.5 hours. The control or "no stress" group (n = 10) underwent identical collection procedure, however they were not stressed and remained undisturbed in the test cages throughout the experiment. In addition to the dialysate samples, blood (100 μl) was collected from a tail nick immediately prior to restraint, just prior to release from restraint, and again just prior to the end of the second restraint period. Plasma samples were stored at –80°C for subsequent ACTH and corticosterone analyses.

A second experiment (feeding study) using 10 rats, assessed central amygdaloid CRH and BN-LP fluctuations associated with various spontaneous ingestive states in animals with *ad libitum* access to food (powdered Purina Lab Chow). Probes were inserted into the guide cannula at 1400 hours (4 hours before dark onset) and were continually perfused with KRB solution. Commencing 2 hours after probe insertion, dialysates were pooled over every 30 minutes (over 5 hours). The food cup was positioned atop an electronic scale, thus permitting continuous monitoring of food consumption. When an animal initiated a meal the 30-min dialysate sampling period began anew. A meal was defined as consumption of a minimum of 0.3 g during the 30-minute sampling period. The meals ranged in size from 0.3 to 3.1 g (mean (S.E.M. = 1.26 ± 0.12 g) and the first meal usually occurred within 90 minutes of dark onset. The

30-min period prior to meal initiation was considered as the preprandial period, and the 30-min interval preceding this was considered as the baseline. The postprandial period was the 30-min period following meal termination, during which no food was consumed. If during this time a new meal was initiated, then the postprandial was considered as the subsequent 30-min period.

Whereas the preceding experiment assessed peptide and endocrine variations in the context of spontaneous meals, a separate experiment evaluated plasma ACTH and corticosterone levels before and after presentation of a palatable snack (Graham Wafers). This experiment was conducted during the light-phase of the diurnal cycle (1000-1200 hours) among animals that had previously experienced this particular type of snack (on 4 occasions, to reduce any neophobic responses). Rats (n = 8) equipped with a carotid artery catheter, had blood samples drawn at 20, 10 and 0 minutes prior to food presentation. Rats were then presented with the familiar palatable food (Graham Wafer; 16 g) for a 10 minute period, during which all animals readily consumed some food (mean ± S.E.M. = 3.40 ± 0.84 g). Blood samples were drawn 5 minutes after meal initiation (i.e. during the 10 minute meal), and then again at 15, 30 and 60 minutes after meal presentation/initiation. Plasma samples were stored at –80°C for subsequent ACTH and corticosterone determinations.

Histology

At the end of each experiment, animals were perfused under heavy sodium pentobarbital anesthesia and the brains extracted, sectioned, and stained for histological examination.

Statistical Analyses

In all microdialysis experiments, only data from correctly positioned probes (verified histologically) were included for statistical analyses (i.e. excluding values from animals with misaligned or "off site" probes). From animals included in the statistical analyses, there were occasional missing values (replaced by the harmonic mean), due to accidental sample loss, assay error or flow problems, contributing to variations in sample size. As in the case of most microdialysis studies assessing other central neurotransmitters (such as dopamine), appreciable inter-individual and inter-experiment variability was noted in the present experiments with respect to baseline interstitial CRH levels. This variability may have stemmed from genuine differences between animals, as well as technical aspects related to the microdialysis procedure, including variability in the relative peptide recovery of individual microdialysis probes, subtle differences in probe placements, as well as the variability associated with individual RIAs. Accordingly, in the present investigation, the baseline values of each subject were averaged and defined as 100%. All values were then expressed as a percentage of the average baseline values. Repeated measures analysis of variance (ANOVA) with sample blocks (baseline, post-stress 1, post-stress 2) and samples nested within each block treated as within measures, was performed independently for both CRH and BN-LP.

Post-hoc comparisons were conducted using Tukey's tests. In the feeding study (Experiment 2) all values were expressed as a percentage of baseline. For some animals, multiple values were available for particular feeding state(s), and these values were averaged, prior to repeated measures ANOVA analysis. The baseline, preprandial, prandial and postprandial periods were treated as a within factor.

Plasma concentrations of ACTH and corticosterone values were derived from all animals (irrespective of probe position) before and after the two stressor presentations, and were analyzed by repeated measures ANOVA. In the food intake study, values from all animals with patent blood sampling cannula, were subjected to repeated measures ANOVA to compare both the ACTH and corticosterone concentrations at various times prior to food presentation (20, 10 or 0 minutes) and at the various times during or following food presentation (5, 15, 30 or 60 minutes).

Results

Restraint stress-induced release of CRH and BN-LPs at the amygdala

Data from animals with correctly positioned probes (n = 10) revealed that the interstitial CRH varied as a function of the Stress condition (restraint or no stress) x Blocks (baseline, stress1 and stress 2) x Samples (time) interaction $F_{8,144} = 4.72$, $p<0.01$. Levels of BN-LP varied as a function of Stress condition x Samples interaction ($F_{4,56} = 9.37$, $p<0.01$). The comparisons of the means of the simple effects comprising these interactions revealed that, among non-stressed rats, both CRH and BN-LP levels were stable throughout the session. The initial stressor application resulted in an immediate

(within the initial 30 minutes) and sustained (over 2.5 hours) rise in the interstitial levels of CRH (see Figure 1). Upon the second stressor application, a further increase in the release of CRH was evident, which continued to rise for the duration of the test period.

In a parallel control "no stress" group, animals with probes correctly positioned at central nucleus (n = 7), CRH release did not fluctuate significantly over time. The stress effect appeared to be specific to the central nucleus, as there was no significant change in CRH release from "off site" probes with detectable levels of the peptide (n = 5 from total of 10 "off site" probes; data not shown).

The stressor also promoted an immediate rise in the release of BN-LPs. Compared to CRH release, this response was slower in onset and less pronounced, but continued to increase progressively over the entire session (see Figure 2). Upon stressor re-exposure, a further increase was noted, which persisted to end of the test period (2.5 hours). As in the case of CRH, this response was stressor related, as non-restrained animals did not show significant variations in the interstitial levels of BN-LPs. Yet, in non-stressed animals a modest, non-significant increase of BN-LPs was evident near the end of the session, a few hours prior to dark onset, raising the possibility that circadian factors may also have contributed to BN-LP rise in stressed animals. It is of interest to note that the fluctuations of BN-LPs were not as site-specific as those of CRH, in that some "off site" probes with detectable BN-LP immunoreactivity levels (n = 6), showed some elevation in the release of BN-LP immunoreactivity, but not until much later (1.5 hours after second stressor application) (data not shown).

Effect of acute restraint episodes on circulating ACTH and corticosterone levels:

As expected, the plasma ACTH and corticosterone concentrations were significantly elevated by the stressor treatment ($F_{9,23} = 29.88$ and $F_{9,22} = 100.85$, respectively) $p<0.01$. The multiple comparisons indicated that relative to basal ACTH concentrations (50.1 ± 9.6 pg/ml) elevations of the hormone were seen immediately after the first (395.4 ± 63.9 pg/ml) and second stressor exposure (274.3 ± 96.9 pg/ml). Likewise, compared to baseline (9.5 ± 2.3 (g/100 ml), plasma corticosterone levels were increased both after the first and the second stressor exposures (25.8 ± 2.6 and 23.5 ± 1.8 (g/100 ml, respectively). It is noteworthy that the levels of ACTH and corticosterone observed after the first and second restraint periods were comparable to one another, whereas the interstitial levels of CRH and BN-LP were significantly higher following the second stress episode, compared to the first. As a baseline blood sample was not taken immediately prior to the second stress period, the relative magnitude of the hormonal changes cannot be determined.

Meal-elicited release of CRH and BN-LPs at the amygdala:

Figure 3 shows the interstitial CRH (upper panel) and BN-LPs (lower panel) immunoreactivity levels as a percent of baseline, prior to, during and following ingestion of a spontaneous meal. Both peptides varied significantly over the test session ($F_{6,18} = 21.62$ $p<0.0001$ for CRH and $F_{6,18} = 9.11$, $p<0.007$ for BN). The multiple comparisons revealed that during the preprandial period, neither peptide varied from baseline. However, during ingestion and during the postprandial period, interstitial levels of both

CRH and BN-LPs were markedly increased. Indeed, the meal-elicited rise of CRH was as great as that associated with the first restraint period (cf. Figures 1 and 3).

The positions of probes on target as well as those misaligned (and thus excluded from analyses) were assessed. This response appeared to be specific to the CeA, as the "off site" probes failed to show meal-related fluctuations in release of CRH and/or BN-LPs (data not shown).

Plasma ACTH and corticosterone, from samples taken before and after palatable food consumption (mean = 3.7 ± 0.7 g) were both found to vary over the course of the session, ($F_{6,36} = 3.91$, $p<0.01$ and $F_{6,30} = 6.74$, $p<0.01$, respectively). The multiple comparisons confirmed that ACTH levels were significantly increased during ingestion and 15 minutes after initiation of consumption, and declined thereafter (see Figure 4). The rise of corticosterone was slower than that of ACTH, and was significant at 15 and 30 minutes after meal initiation, and then declined at 60 minutes. It is noteworthy that while the extent of the CRH and BN-LP increase induced by food ingestion was comparable to that noted during a similar period following restraint exposure, the meal-elicited rise of ACTH was of a smaller magnitude than that elicited by the stressor, while both treatments provoked comparable elevations of corticosterone.

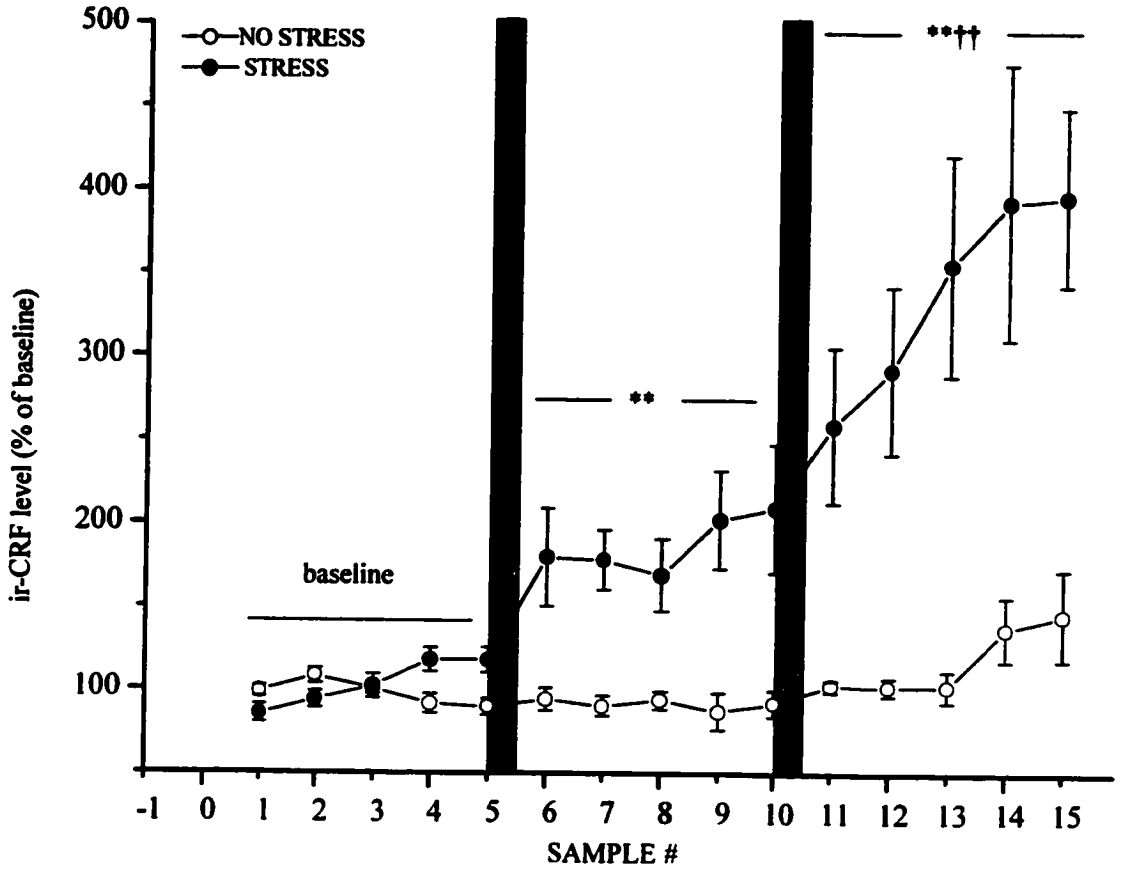


Figure 1: Restraint stress induced release of CRH at the central nucleus of the amygdala as measured by *in vivo* microdialysis. Dialysate samples were collected uninterrupted throughout the experiment, and samples pooled every 30 minutes. Following collection of 5 baseline samples, rats in the "stress-group" (n = 8-10) were hand-restrained (solid circles) for 20 minute episodes on two separate occasions, depicted by shaded vertical bars (stress 1 and stress 2). The rats in the "no stress" (control) group (n = 5-7) were left undisturbed throughout (open circles). The 5 baseline values from each of the subjects were averaged and defined as 100%. All values were then expressed as a percent of that baseline. Basal CRH values were 2.77 ± 0.11 and 2.31 ± 0.39 fmol/sample for the "stress" and "no stress" groups, respectively. ** Significantly different from baseline at $p<0.01$; †† Significantly different from stress 1 at $p<0.01$.

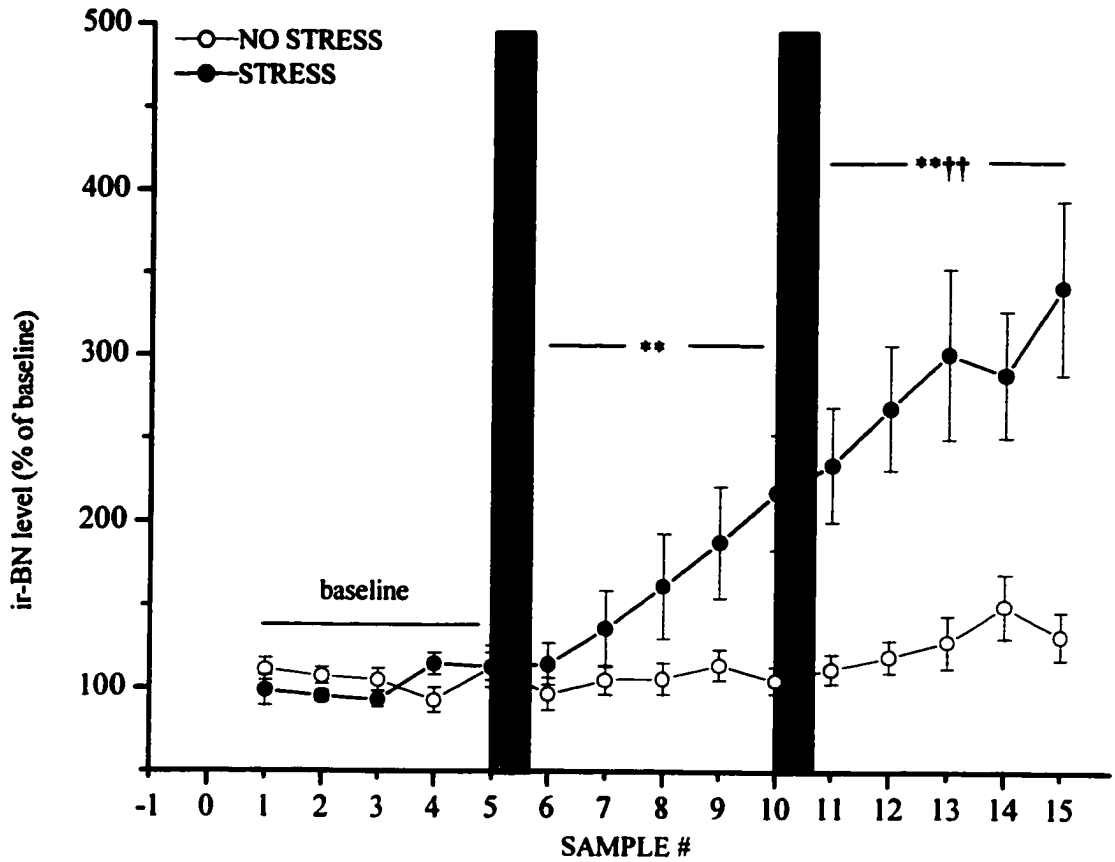


Figure 2: Restraint stress induced release of BN-LPs from the CeA, as measured by *in vivo* microdialysis. Dialysate samples were collected uninterrupted throughout the experiment, and samples pooled every 30 minutes. Following collection of 5 baseline samples, rats in the "stress group" (n = 10) were hand-restrained (solid circles) for 20 minutes episodes on two separate occasions, depicted by shaded bars (stress 1 and stress 2). The rats in the "no stress" (control) group (n = 7) were left undisturbed (open circles). The 5 baseline values from each of the subjects were averaged and defined as 100%. All values were then expressed as a percent of that baseline. Basal values for BN immunoreactive peptides were 3.01 ± 0.42 and 3.28 ± 0.33 fmol/sample for the "stress" and "no stress" groups, respectively. ** Significantly different from baseline at $p<0.01$; †† Significantly different from stress 1 at $p<0.01$.

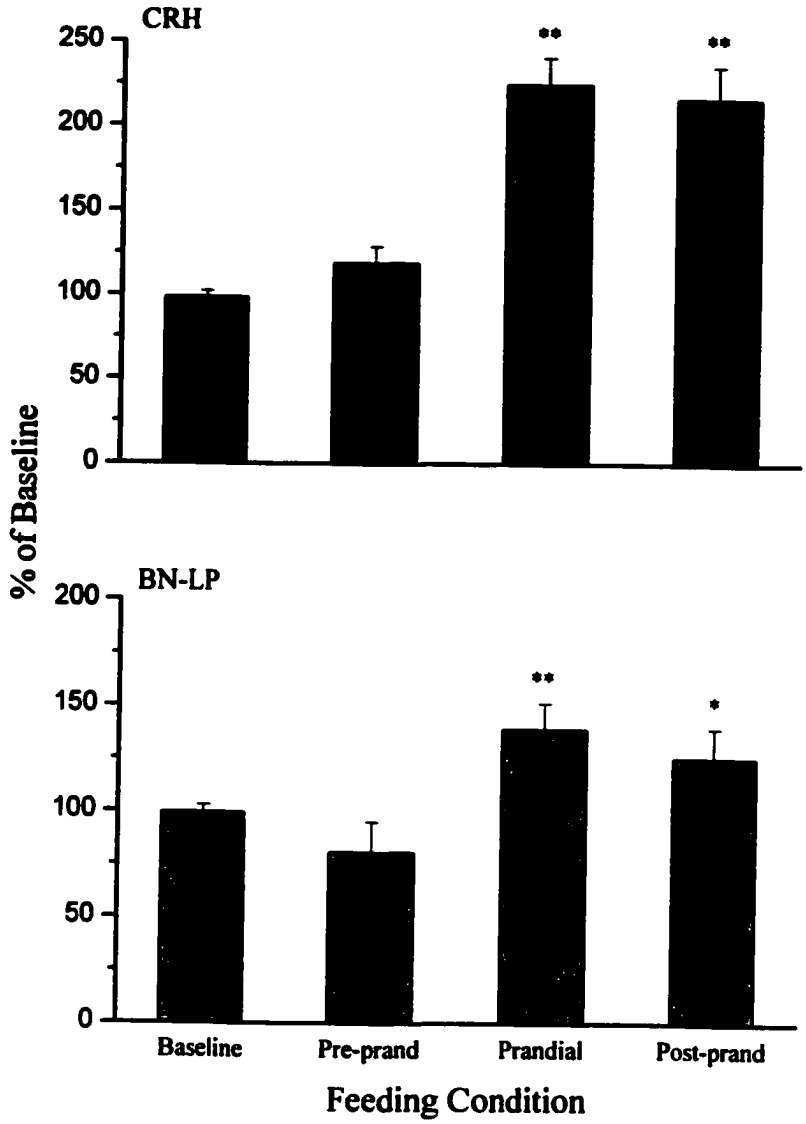


Figure 3: Meal-elicited release of CRH and BN-LPs at the CeA. Microdialysate samples were collected continually and pooled every 30 minutes, for 5 hours. The quantity of food ingested during each 30 minutes bin was noted. The 30 minute period prior to meal initiation was considered as preprandial period, and the 30 minute sample preceding this was considered as the baseline. The postprandial period was the 30-minute period following meal termination. The baseline value was defined as 100% and all other values expressed as a percentage of this value. The meal-related CRH changes are presented in the upper panel, whereas the changes in BN-LPs are shown in the lower panel. The basal values for CRH and BN-LPs were 0.95 ± 0.12 and 0.77 ± 0.07, respectively. *,** Significantly different from control at $p<0.05$ and 0.01 respectively.

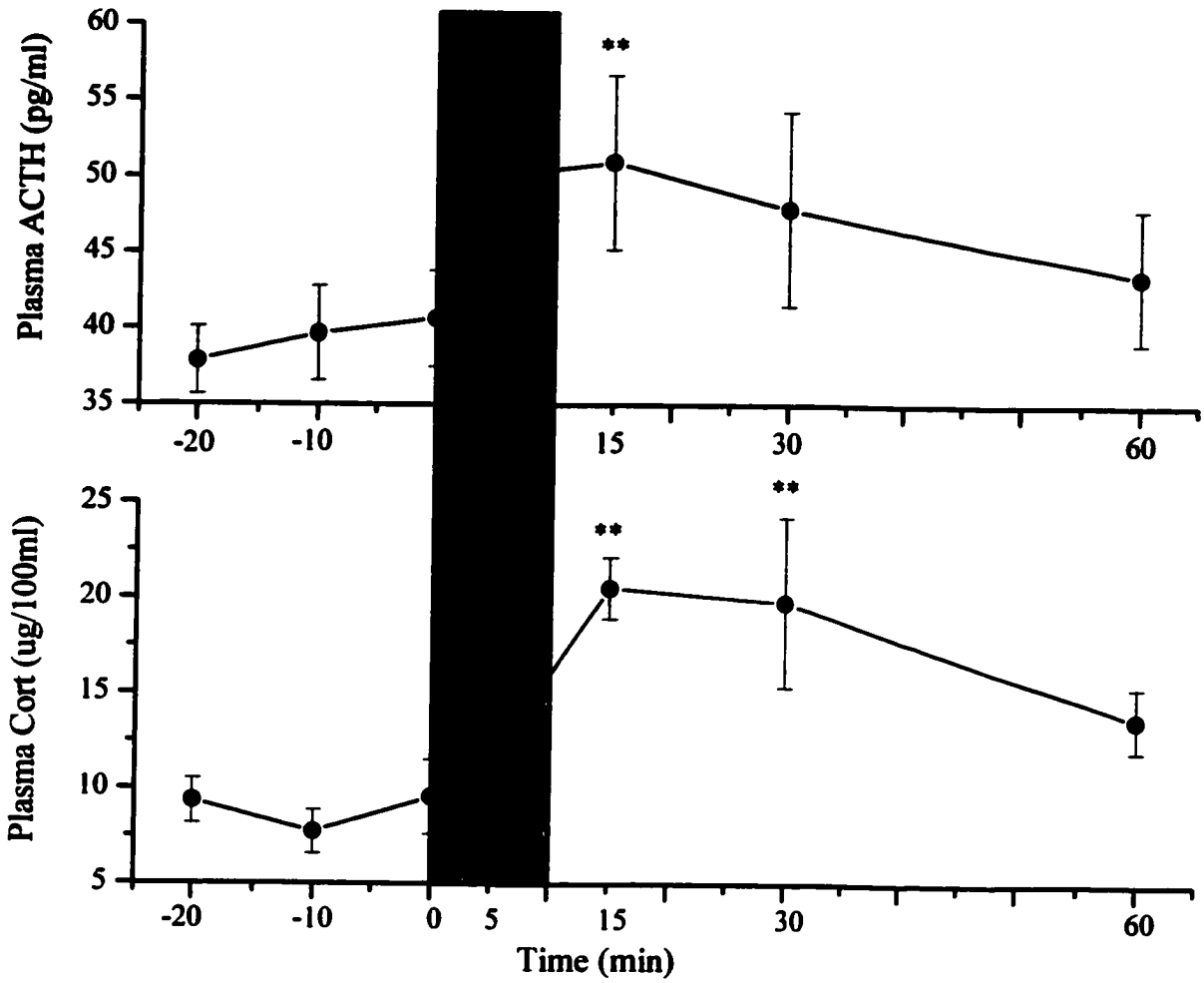


Figure 4: Effects of food ingestion on circulating ACTH and corticosterone levels. Rats equipped with carotid cannula were used in this study. Blood samples (400 µl) were drawn remotely, at 20 min, 10 min and just prior to food presentation (0 min). Rats were then offered Graham Wafers for 10 minutes (depicted by shaded bar). Blood samples were collected at 5, 15, 30 and 60 minutes following food intake. Plasma ACTH levels are expressed as pg/ml and presented as mean ± S.E.M. (upper panel). Plasma corticosterone levels are expressed as µg/100 ml and presented as mean ± S.E.M. (lower panel). ** Significantly different from control at $p<0.01$.

Discussion

Most studies that have assessed the effects of stressors on central CRH have done so using relatively indirect techniques, such as measurement of mRNA expression, immunohistochemistry or postmortem tissue level analyses.[245,414,472] The former two approaches are indicative of potential variations of CRH, but do not necessarily reflect actual peptide release. Moreover, these techniques, like postmortem analyses of CRH content, do not permit evaluation of dynamic within subject variations that may occur over the course of a treatment regimen. In the present investigation, we demonstrate the feasibility of *in vivo* microdialysis, combined with highly sensitive solid-phase radioimmunoassays, in the measurement of interstitial levels of CRH and BN-LPs. Furthermore, using HPLC fractionation of brain dialysates and external standards, in combination with radioimmunoassay and ultraviolet detection, respectively, we demonstrate that the CRH immunoreactivity within the dialysates corresponds to authentic CRH. In addition, through similar fractionation and analyses we identified the BN-LP immunoreactive material in the dialysate to represent GRP_{1-27} and/or GRP_{18-27}, the mammalian counterparts of amphibian BN.[486] In both instances, this was further confirmed through the use of two different columns and gradient conditions, wherein the variations of the retention times for the respective peptide standards was associated with a corresponding shift in immunoreactive peaks eluting from the dialysate samples.

Stressor exposure in the present investigation produced a pronounced and sustained increase in the release of CRH at the CeA. This increase was evident soon after stressor onset and was still pronounced and stable 2.5 hours afterwards. Earlier studies

had demonstrated that *in vivo* CRH release was elevated by stressors such as restraint or drug (alcohol or cannabinoid) withdrawal.[370,398] In these experiments, the detection and quantification of CRH involved incorporation of anti-CRF serum directly into the medium perfusing the microdialysis probes. In the present investigation, CRH changes were observed using a simpler, more direct method that did not require spiking the perfusion medium with the anti-CRF serum, and yielded comparable basal interstitial levels of CRH. In both the Pich et al (1995)[370] and in the present study, marked CRH elevations were evident in response to 20 minutes of restraint stress. While the magnitude of the effects reported by Pich et al[370], were more pronounced, they also were more transient (40 minutes vs. 2.5 hours in the present study). These differences may have been related to several factors. In the Pich et al[370] study, inclusion of the CRH antibody into the dialyzing medium may have created a greater positive gradient for CRH from the interstitial fluid, resulting in a sharpened, temporally limited CRH peak. Alternatively, the dynamics of CRH may have been influenced by the cues associated with the stressor. Specifically, in the report by Pich et al[370], the restraint was applied in a novel cage (thus the "stressful environment" comprised both the novel situation coupled with restraint) after which the rat was returned to it's home cage ("safe environment"). In contrast, in the present study, the entire procedure was conducted in the rat's home cage. Thus it is possible that the otherwise neutral home cage cues may have taken on secondary aversive (or "stressful") qualities, hence leading to more protracted peptide variations. This supposition is consistent with the view proposed by Lee and Davis (1997),[263] who suggested that the amygdala plays a fundamental role in the fear response (being elicited by an identifiable stimulus and subsiding with the offset of this stimulus),

whereas the BNST, a primary target of the amygdaloid projections, is more closely aligned with more generalized anxiety states. In effect, applying the stressor in the animal's home cage resulted in the sustained CRH release even when the primary stressor was terminated.

Paralleling the CRH changes, release of BN-LPs was also induced by the stressor, a finding commensurate with our earlier postmortem results.[230] The changes in BN-LPs developed relatively slowly over the post-stress period. Since interstitial BN-LP immunoreactivity levels increased progressively throughout the 2.5 hours following the initial stressor, it is unclear whether the further rise following the second stressor actually reflects the consequences of the second restraint episode or a sustained effect of the initial restraint period. Nonetheless, the fact that increased release of BN-LPs continued over time following termination of restraint, while the elevations of CRH release were relatively stable, raises the possibility that BN-LPs may be important in subserving sustained emotional changes associated with a stressor experience. Of course, as in the case of CRH changes, the possibility can not be dismissed that environmental cues associated with the stressor and the peptide alterations are related to one another. In fact, since BN promotes ACTH release and this effect is prevented by pretreatment with CRH antagonists,[315] the possibility ought to be considered that BN-LPs provoke ACTH elevations by stimulating CRH release. The potential physiological role of BN-LPs at the CeA, particularly with respect to CRH release, remains to be elucidated. It is noteworthy that while the variations in ACTH, corticosterone and BN-LPs were evident within 30 minutes of stressor initiation, the increase in BN-LP immunoreactivity was not evident

until approximately 1 hour after stressor onset. Accordingly, it is not likely that the plasma ACTH and/or corticosterone changes were causally related to variations of BN-LPs within the amygdala.

It has been suggested that the activation of HPA neurons may be a fundamental response to stressors. In this context, limbic regions, particularly the amygdala may be essential in determining the response to 'processive' stressors (requiring interpretation by higher brain structures) whereas 'systemic' stressors (involving immediate physiologic threat) may affect HPA activity through non-amygdaloid mechanisms.[189] Indeed it has been suggested that the amygdala may be important in the formation, consolidation and expression of those events that have been associated with aversive stimuli,[101] and may activate the HPA axis via amygdaloid inputs to the hypothalamus.[166,305] Although not discounting these views, the present results make it clear that appetitive stimuli also influence not only HPA activity but also the release of CRH and BN-LPs at the CeA. While restraint was clearly more effective in provoking the ACTH changes, the two treatments were roughly comparable in elevating circulating corticosterone levels. Similar prandial and/or postprandial ACTH and/or cortisol elevations have also been reported in human studies.[7,136,219] Moreover, indirect analyses of CRH, vasopressin and oxytocin, indicated that among deprived animals, the presentation of water may provoke increased release of these peptides from the median eminence.[399] Interestingly, in these animals, the frustration of being presented with empty water bottles also had similar effects. Thus, these peptidergic changes may reflect general arousal rather than the response to aversive stimuli.

Although the CeA has long been considered to contribute to emotional responses, the view has also been expressed that this brain region plays an essential role in complex processes such as attention, secondary reinforcement, reward, and social behavior.[101] In fact, in the present investigation *in vivo* CRH and BNLP immunoreactivity changes were as pronounced following an appetitive stimulus as those elicited by the aversive event. Clearly, the amygdala is not uniquely responsive to aversive stimuli, and CRH and BN-LPs are released in response to appetitive events as well. This observation is reminiscent of the catecholaminergic responses noted at the prefrontal cortex and/or nucleus accumbens. Although once believed to be stress-specific, the catecholaminergic systems were subsequently found to be activated by stimuli with positive valence as well, including reinforcing drugs,[495] food[190,393,468] or sex.[491] This led to the suggestions that catecholaminergic signals contribute to specific cognitive functions and/or arousal,[393] or to processes related to attention and learning.[468,492] It is of interest to note that glucocorticoids too are secreted in response to stressful as well as rewarding events, and have been suggested to represent a biological substrate of reward. According to this contention, glucocorticoids may play a role in counteracting the aversive effects of external threats or insults, allowing for a better coping.[369] As in the case of brain dopamine circuits, we believe that the CRH system(s) may serve a much broader role than previously envisioned. Rather than evoking feelings of fear and anxiety, this system may serve to draw attention to biologically significant events (or cues) such as those associated with food availability as well as those posing physical threat.

The curious observation that an aversive event (such as acute restraint) as well as an apparently appetitive circumstance (such as meal ingestion) would both enhance the release of "stress" peptides (CRH and BN-LPs) at the amygdala and "stress hormones" (ACTH and corticosterone) into general circulation, may have an alternate explanation. It could be argued that at some central level, meal ingestion might be interpreted as a "stressful" event. Although at first blush this may appear counterintuitive, observation of various species suggests that feeding related activities can indeed be threatening to the organism, often requiring vigilance and/or aggression. For instance, acquisition of a meal by many carnivorous species may require hunting of the prey, a physically challenging and dangerous situation. Furthermore, during ingestion, the organism may need to aggressively protect its food from others (e.g. a growling dog protecting his steak bone), or protect itself from attack during this vulnerable time (e.g. a bird at a feeder eating cautiously, vigilant about a potential attack from cohorts or birds of prey). Finally, the ingestion of a meal may signal imminent flooding of the system with nutrients and/or toxins that may threaten homeostasis. Thus, any or all of the events associated with feeding can potentially be deemed stressful. In the case of humans, the relatively plentiful conditions and structured social environment may have resulted in the brain evolving effective mechanism(s) to suppress and/or modulate the perception of stress associated with food ingestion. According to such a model, it is conceivable that disruption of the mechanism(s) balancing the positive and negative (stressful) attributes of food ingestion may be associated with disorders affecting food intake such as anorexia nervosa, bulimia nervosa, depression or obesity.

Preface to Chapter II

The preceding investigation demonstrated that exposure to a stressor and spontaneous ingestion induced the release of both CRH and BN-LPs at the CeA. Moreover, plasma levels of ACTH and corticosterone were elevated in response to the ingestion of a palatable snack. It is known that the CeA and the HPA axis are intimately and reciprocally connected. Thus, in the next set of experiments were designed to assess whether the increased release of CRH and BN-LPs at the CeA in response to food intake translated into an increased availability of these peptides at the anterior pituitary. Due to the probe size and technical difficulties, microdialysis can not be performed at this structure. Thus we used *in vivo* push-pull perfusion technique to assess the dynamics of meal-associated peptidergic fluctuations at the anterior pituitary. The peptides assessed included CRH, BN-LPs (NMB and GRP), as well as AVP (which works in concert with CRH to affect HPA activity).

It has been demonstrated that acute or chronic exposure to certain stressful events can induce protracted changes (including phenotypic changes) in peptidergic expression. Furthermore, it has been demonstrated in our laboratory, that both acute and chronic exposure to restraint stress induced changes in basal as well as stressor (air-puff)-induced elevations of CRH, AVP, NMB, and GRP. Thus, the second objective of this experiment was to determine whether prior stressor exposure history (acute exposure (followed by the passage of time) or chronic stressor exposure), influenced pituitary CRH, AVP, NMB, GRP and/or corticosterone responses to the ingestion of a palatable snack.

CHAPTER II

Are meal-related pituitary peptidergic fluctuations influenced by prior stress history? Impact of chronic and acute stressor manipulation on GRP, NMB, CRH, and AVP at the anterior pituitary.

Abstract

To date, the neural, neurocrine or endocrine mechanisms governing the initiation, duration and cessation of a meal remain only partially understood. We had demonstrated that CRH and BN-LPs were released at the CeA in response to both aversive (restraint) and appetitive (spontaneous ingestion) events.[311] Since CRH-positive neurons within the CeA project to hypothalamic nuclei,[185] it would be expected that changes in CRH release at the CeA might be reflected in altered HPA axis activity. In fact, significant increases in plasma ACTH and corticosterone did accompany the ingestion of a palatable snack.[311] In this study, we monitored *in vivo* interstitial levels of CRH, AVP, and BN-LPs (GRP and NMB) at the anterior pituitary in response to the presentation and consumption of a palatable snack (Graham Wafers). Since stressor-exposure can elicit phenotypic changes in neurons secreting CRH and AVP (and hence HPA functioning), we also examined whether previous exposure to acute or chronic restraint influenced the snack-induced fluctuations in interstitial levels of GRP, NMB, CRH, and AVP at the anterior pituitary. Results showed significant elevations of interstitial levels of GRP, NMB and CRH, but not AVP, following snack consumption. Furthermore, in general, basal interstitial levels of CRH, AVP, GRP, and NMB at the pituitary were significantly elevated in animals previously exposed to either repeated episodes (14 sessions) or a single episode of restraint stress followed by the passage of time (14 days), in comparison to non-stressed controls. Basal corticosterone levels at the pituitary were also significantly increased in animals exposed to chronic restraint compared to animals exposed to acute restraint followed by the passage of time. Prior exposure to stress appeared to blunt the snack-induced rise in interstitial peptide levels. Taken together, these findings provide further

evidence for an interrelationship between the systems governing feeding behavior and those mediating the stress response. This study also suggests mechanism(s) by which previous stress history may come to influence subsequent neurocrine or endocrine responses to food, which may lead to a greater understanding of stressor-related feeding disorders such as anorexia nervosa and bulimia.

Introduction

Neurochemical mechanisms governing feeding behavior in general, and palatable food intake in particular, remain largely unknown.[97,446,482] An association between the system(s) governing food intake and the HPA axis (one of the key system mediating the stress response) has long been recognized.[5,97] Moreover, meal ingestion is associated with activation of the HPA axis, as reflected by enhanced glucocorticoid secretion.[98] Further, exposure to stressful stimuli often affects ingestion, although the direction of such change is variable, influenced by the type, intensity and duration of the stressor.[17,123,360,432] Animals subjected to a protracted (3-hour) episode of restraint displayed a significant reduction in food intake.[404] Meanwhile, application of a shorter period of restraint stress had no effect on the ingestion of a palatable snack. However, sessions of restraint stress (1-hour) applied chronically (5 days per week over 40 days) increased palatable snack and chow intake.[432] It is interesting to note that clinical research has revealed that abnormalities in HPA functioning (impaired regulation and sensitivity of the HPA axis) are implicated in eating disorders such as anorexia nervosa and obesity.[49,318,364]

One of the principal peptidergic regulators of the HPA axis is the 41-residue polypeptide, CRH. During HPA activation, CRH, which is synthesized in the parvocellular region of the hypothalamic PVN,[22] is released into the hypophysial portal blood from axon terminals in the external zone of the median eminence.[189,207] CRH subsequently stimulates the secretion of ACTH from the pituitary corticotroph cells, which in turn, stimulates the secretion of glucocorticoids (cortisol in humans and

corticosterone in rats) from the adrenal cortex.[22,182,189,207,423] Interestingly, not only is CRH considered to be a key stress peptide, it has also been implicated in the control of food intake.[88,267] Exogenous, central administration of CRH is known to induce anxiogenic-like behaviors including the suppression of food intake in normal rodents, suggesting that CRH modulates stressor-induced reduction in food intake.[164,187,198,199,246,337,437,445,465] In addition, pretreatment with alpha-helical CRH attenuated restraint-induced anorexia, enhanced the hyperphagia induced by NPY, reduced the latency to begin eating and increased the duration of feeding induced by tail pinch.[187] Furthermore, fluctuations in immunoreactive CRH levels in relation to feeding status have been observed in several brain regions known to be relevant to feeding behavior such as the LH, VMH, and CeA.[311,378]

In addition to CRH, other peptides can also influence HPA activity. AVP is colocalized with CRH in parvocellular neurons of the PVN[71,342] and is co-secreted with CRH into portal circulation in response to certain stressful stimuli. Although AVP is a weak ACTH secretagogue on its own, it shows strong synergistic action with CRH.[259] Interestingly, exposure to a variety of chronic stressor regimens or even a single stressor episode followed by the passage of time induces a progressive increase in the number of CRH neurons co-expressing AVP, which project from the PVN to the external zone of median eminence.[26,102-105,414] It is thought that under such conditions, non-AVP-producing neurons are changed to the AVP co-producing phenotype resulting in the shift from a CRH-dominated to an AVP-dominated signal.[282,472] As AVP appears capable of modulating the secretion of ACTH and, thus, the activity of the HPA axis, this shift in

AVP/CRH ratio has potential implications for energy balance and, consequently, feeding behavior. Although there are only a few studies that have examined the role of AVP in the regulation of feeding behavior, it has been shown that intraperitoneal injection of AVP suppressed food intake in both goats and rats, an effect reversed by an AVP receptor antagonist.[259] Furthermore, in rats that were food deprived for 48 hours, concentrations of AVP were significantly reduced in the PVN (parvocellular subdivision) and in the supraoptic nucleus.[71] Also, a hypersecretion of AVP has been observed in the cerebrospinal fluid of underweight anorexia nervosa patients.[109]

Although both CRH and AVP are regulators of the HPA axis, the mechanism(s) triggering their release have not been fully determined.[231] Several other neuromodulators are co-produced in parvocellular CRH neurons of the PVN, some of which are co-localized within CRH nerve terminals at the external zone of the median eminence.[472] However, little is known about the physiological relevance of these other substances. GRP, a mammalian analogue of the BN family of peptides, is one such peptide co-localized with CRH within the external zone of the ovine median eminence.[14,159] BN-LPs are well recognized for their ability to suppress food intake and induce a state of satiety.[117,133,152,173,310] More recently, BN-LPs have been shown to induce HPA activation, possibly through interaction with CRH and/or AVP.[230-232] Central administration of BN or GRP has been shown to dose-dependently increase plasma ACTH, corticosterone, epinephrine, NE and glucose, effects attenuated or blocked by both GRP and CRH receptor antagonists.[142,232,351,376] Furthermore, central BN administration induces site specific alterations in the turnover of CRH and AVP as well

as the *in vivo* release of CRH and AVP at the median eminence/arcuate nucleus, with subsequent increases in the availability of these peptides at the pituitary.[231] Finally, the application of stressful stimuli altered BN-LP (GRP) immunoreactivity in several brain regions.[230]

In addition to GRP, NMB (another mammalian analog of BN) also appears to be involved in the regulation of food intake as well as in HPA activation. Specifically, central administration of NMB is known to suppress food intake.[310] Although at present, the neuroendocrine effects of central NMB administration remain unexplored, subcutaneous administration has been shown to elevate plasma ACTH and corticosterone levels in rats.[286] Furthermore, NMB (like GRP) is present in all major components of the HPA axis, including the hypothalamus, pituitary and adrenal gland.[113,211,321,330] In fact, high levels of NMB mRNA are present in the anterior pituitary, suggesting an endocrine or paracrine function for this peptide.[200] In the context of the paracrine function, NMB may serve as a modulator of CRH and/or AVP within the anterior pituitary.

It was previously demonstrated in our laboratory that CRH as well as BN-LPs are released at the CeA in response to aversive (restraint) or appetitive (spontaneous ingestion) events.[311] Since CRH-positive neurons project from the CeA to hypothalamic structures,[185] it was of interest to explore whether changes in CRH release at the CeA would affect the HPA axis. In fact, significant increases in plasma ACTH and corticosterone did accompany the ingestion of a palatable snack.[311] In the current investigation we were interested in determining whether the increased release of CRH

and BN-LP at the CeA in response to food intake translated into an increased availability of these peptides at the anterior pituitary. Furthermore, we also wanted to determine if prior stress history impacted upon the pattern of peptidergic availability at the anterior pituitary induced by the consumption of a palatable snack. Specifically, we exposed animals to the following conditions: (1) no stress, (2) an acute episode of restraint followed by a stressor-free period (14 days), or (3) a daily episode of restraint over a 14-day period. We then assessed, via push-pull perfusion, the *in vivo* availability of CRH, AVP, GRP, NMB, and corticosterone at the anterior pituitary under basal conditions and following the ingestion of a palatable snack.

Materials and Methods

Subjects

Male Sprague-Dawley rats, weighing 325-400 g, were obtained from Charles River (St. Constant, Quebec). Animals were individually housed and maintained on a 12 hour light/dark cycle (lights on at 0600) in a temperature controlled (23°C) environment with 60% relative humidity and had *ad libitum* access to food (Purina Rat Chow) and water. All experimental procedures followed the Canadian Council on Animal Care guidelines and were approved by the Research Ethics Committee of the University of Ottawa.

Experimental Protocol

Animals were randomly divided into three treatment groups and exposed to (1) no stress (control group), (2) acute stress: a single 20-min restraint stress episode, followed

by 14 days of no stressor exposure or (3) chronic stress: exposure to a daily 20-min restraint-stress episode for 14 days. Immediately following the last stressor episode, the rats from the chronic restraint group were anaesthetized (60mg/kg, pentobarbital, intraperitioneal) and placed in a stereotaxic instrument with level skull. A 20-gauge guide cannula containing a removable 24-gauge stainless steel stylette was implanted, aimed at the anterior pituitary using placement co-ordinates:[362] 5.0 mm posterior to bregma, 0.9 mm lateral to the midline and 0.5 mm dorsal to the sphenoid bone (at the base of the skull). The guide cannula (which protruded from a custom designed Delrin™ pedestal) was then secured to the skull with four stainless steel screws and acrylic dental cement.

Following a 5-day recovery period and 48 h prior to testing, animals were transferred to individual test cages made from clear Plexiglas (25 x 35 x 34 cm) with stainless steel grid floor, for acclimatization.

Push Pull Perfusion

On the day of testing, the stylette was replaced with a push-pull probe aimed at the anterior pituitary. The push-pull probe consisted of an outer (pull) 24-gauge stainless steel cannula that protruded 0.4 mm beyond the end of the permanent guide cannula, and an inner (push) cannula (glass silica) protruding 1.2 mm beyond the end of the pull cannula and into the anterior pituitary. Probes were connected by polyethylene tubing (PE-20 and PE-10) to two independent, pre-equilibrated peristaltic pumps (Minipuls 3, Gibson, Middleton, WI) via a dual channel swivel assembly (Instech Laboratories,

Horsham, PA). The system was flushed at a rate of 15 µl/min with filtered KRB solution consisting of (in mM): K^+ 2.7, Na^+ 145, Ca^{2+} 1.35, Mg^{2+} 1.0, Cl^- 150, (pH 7.4),[327] and BSA (0.1%). Three baseline samples were collected, and then animals were presented with a Graham Wafer snack (~3.5 g of Christie's Honeymaid®) for 10 minutes followed by the subsequent collection of 5 more samples. Samples (150 µl) were collected every 10 minutes, immediately frozen on dry ice and stored at –80°C until radioimmunoassay analysis.

Histology

Following completion of the experiment, rats received an overdose of pentobarbital. India ink (1 µl) was then delivered through the push-pull probe (into the perfusion site) for verification of probe placement. The pituitary gland was removed and placement was verified visually under a dissecting microscope. Only the data from rats with confirmed position at the anterior pituitary were included in the data analysis.

Radioimmunoassays:

Solid-phase RIAs were used for the detection and quantification of CRH, AVP, and BN-LPs (GRP and NMB).[285,311] Corticosterone was also measured in the perfusates using a micro-adaptation[51] of the commercial radioimmunoassay kit (ICN pharmaceuticals, Canada). Briefly, protein A/G (Calbiochem Corp, La Jolla, California) coated Immulon-4 well plates (Dynex Technologies, Chantilly, VA) were coated with CRH, AVP, BN or NMB antibody (1:100,000 final dilution) for 2 hours at room temperature. The plates were rinsed three times with wash buffer (0.15 M phosphate

buffered saline, pH 7.4). Calibrating standards for CRH (0.05 to 250 fmol), AVP (0.0025 to 125 fmol), GRP (0.25 to 512 fmol), and NMB (3.37 to 690.5 fmol) were prepared using pure peptides dissolved in KRB solution. Blanks, standards and samples were applied into designated wells and incubated at 4°C for 24 hours. Next, 25 µl of KRB solution containing 5000-6000 CPM of [^{125}I-Tyr^{0}]rCRF, [^{125}I-Tyr^{0}]AVP (Amersham, Canada), [^{125}I-Tyr^{0}]NMB (NEN Life Science Products, Boston, MA) or [^{125}I-Tyr^{0}]BN (iodinated in our laboratory according to Salacinski et al.[409]), was added to each well and incubated at 4°C for an additional 24 hours. Following incubation, the plates were rinsed three times with wash buffer. Individual wells were then counted for residual bound radioactivity using a gamma-counter (Canberra Packard, Cobra II Autogamma, model D5002).

The specific anti-CRH serum (rC70, kindly provided by W. Vale, Salk Institute, LaJolla, CA) recognizes CRF_{1-41} and cross-reacts poorly with other related peptides including urotensin 1 and Ucn.[475] The anti-AVP serum (Phoenix Pharmaceuticals, U.S.A.) recognizes [Arg^{8}]-Vasopressin and cross-reacts 100% with [Arg^{8}]-vasotocin, 38% with [Lys^{8}]-vasopressin, and 0% with other peptides. The BN antibody (α-BN_2, kindly provided by T. Moody, NCI, Rockville, MD) recognizes the C-terminal of BN and cross reacts strongly with GRP_{1-27} (110%), BN (100%) and GRP_{18-27} (82%), but only weakly with NMB-10, NMB-32 or substance P (<0.1%). The NMB antibody (α-NMB, kindly provided by T. Moody, NCI, Rockville, MD) recognizes the C-terminal region of NMB (NMB-10), with which it cross-reacts 100%. It also cross-reacts with NMB-30

(104%) and NMB-32 (105%), but shows negligible cross reactivity with GRP, BN, or substance P.

Statistical Analysis

A summary of all effects was done using mixed measures ANOVA with Stressor Treatment as the between group factor and Sampling Period as the within group factor. Basal differences in peptide concentrations of GRP, NMB, CRH, AVP (pmol x 10^{-3} /10 µl), and corticosterone (pg/15µl) concentrations were determined using ANOVA with stressor treatments as the between group factor. To determine the effect of the ingestion of a submaximal quantity of Graham Wafer on peptide and corticosterone levels, basal GRP, NMB, CRH, AVP and corticosterone values for each subject were averaged over the first two baseline samples and defined as 100%. All values were then expressed as a percentage of that baseline. The peptidergic and hormonal changes associated with snack ingestion were analyzed independently for each group, using mixed measures ANOVA with each sample compared to the 3rd baseline value. In all cases, the Huynh Feldt correction was used. Post hoc analysis was done using Tukey HSD ($p<0.05$).

Results

Effect of prior stress history on basal interstitial levels of GRP, NMB, CRH, AVP and corticosterone at the anterior pituitary

The resting or basal interstitial levels of CRH, AVP, GRP, NMB and corticosterone (expressed as pmol x 10^{-3}/10µl) are depicted in Figures 1a, 2a, 3a, 4a, and 5a, respectively. Although there was no interaction between the three groups

(representing distinct stressor exposure history), the ANOVA revealed significant effects of Stressor Treatment on interstitial levels of CRH ($F_{2,21} = 32.67$, $p<0.001$), AVP ($F_{2,27} = 9.54$, $p<0.01$), GRP ($F_{2,19} = 7.63$, $p<0.01$), NMB ($F_{2,22} = 23.28$, $p<0.001$) and corticosterone ($F_{2,22} = 5.15$, $p<0.05$). Tukey HSD multiple comparisons of the group effect indicated that, compared to either non-stressed controls or acutely stressed animals, basal levels of CRH, AVP, GRP, NMB and corticosterone at the anterior pituitary were markedly elevated in rats previously exposed to restraint stress although these group differences varied according to the different peptides. For CRH, only the animals that had received a single (acute) prior stressor episode had elevated basal levels of the peptide whereas the animals that had been chronically stressed displayed basal levels that were comparable to those of the Control (previously non-stressed) rats. On the other hand, for AVP, the Chronically stressed group had significantly higher basal levels of peptide compared to both the Control and Acutely stressed groups. Although both stressor histories were associated with elevated basal levels of GRP, the differences only reached statistical significance for the Acute stress group. In the case of NMB, rats previously exposed to either Chronic or Acute restraint stress conditions, had significantly higher interstitial levels at the anterior pituitary compared to control rats. Finally, rats exposed to the Chronic stress condition had significantly higher interstitial levels of corticosterone compared to the animals exposed to Acute restraint stress followed by the passage of time.

Effects of palatable snack intake on interstitial levels of CRH, AVP, GRP, NMB and corticosterone

Analysis of absolute peptide levels revealed that exposure to a palatable snack induced only modest changes in peptide levels, which did not appear to vary differentially according to prior stress history. However, analysis of absolute levels may be misleading when basal levels are very low and prior treatment (exposure to acute or chronic stress) induced significant differences in these levels. Thus, peptide levels were assessed as a percent change from their respective baselines.

Analysis of the percent scores indicated that the exposure to a palatable snack significantly influenced peptide concentrations. Interstitial levels of CRH, AVP, GRP, NMB and corticosterone for the three groups of animals (non-stressed Controls, those exposed to a single (Acute) episode of restraint followed by a 14-day stressor-free period, and those exposed to 14 sessions of restraint (Chronic) stress) are shown in Figures 1b, 2b, 3b, 4b, and 5b, respectively. ANOVA revealed a significant Stress Treatment condition x Sample (time) interaction for CRH ($F_{3,32} = 3.22$, $p<0.05$) and GRP ($F_{4,38} = 3.78$, $p<0.05$). Although there was no interaction effect for NMB, there was a significant main effect of Sampling period ($F_{4,84} = 6.62$, $p<0.001$). For AVP and corticosterone, ANOVA revealed no significant effects of Sampling period.

Exposure to, and subsequent ingestion of, the palatable snack induced a significant increase in the interstitial levels of CRH during the first ingestive sample in the Control group (see Figure 1b). Interestingly, the availability of CRH appeared to

increase post ingestively in some of the chronically stressed rats, however this did not reach statistical significance. Further, ingestion of the palatable snack increased interstitial levels of NMB in the Control group (see Figure 4b); an effect that was immediate in onset and protracted in duration. During the first post ingestion sampling period, the Control group displayed significantly greater levels of interstitial NMB compared to the other two groups. This effect was maintained during the second sampling period post ingestion, but the difference was only significant between the Control and Acute Stress groups at that time. Snack presentation and ingestion also increased interstitial levels of GRP, and once again this effect was seen only in the Control animals (see Figure 3b). Interestingly, this increase was more delayed than that of NMB, appearing only at the 4^{th} sample post injection and continuing to the end of the sampling period. Furthermore, when compared to the previously Stressed animals, post ingestion levels of GRP in the control animals were significantly greater during the 4^{th}, 5^{th}, and 6^{th} sampling periods.

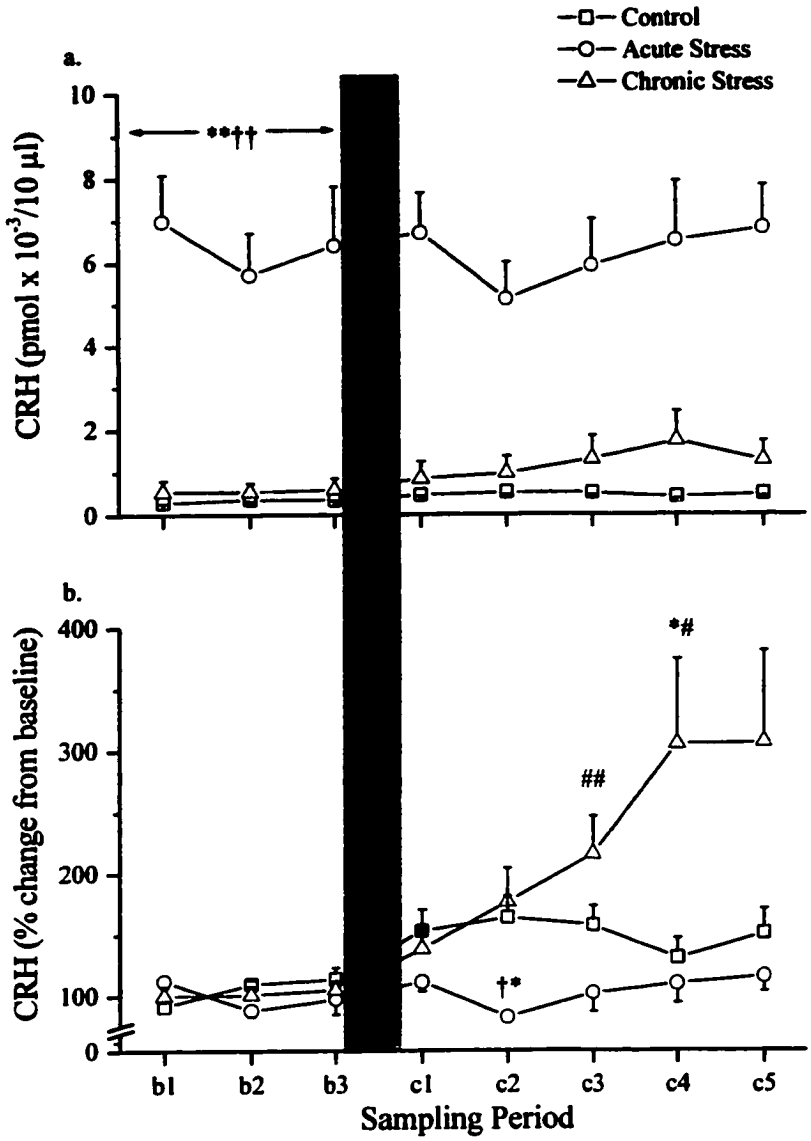


Figure 1a: Basal and snack-induced changes in interstitial CRH levels at the anterior pituitary. Each value represents interstitial levels of immunoreactive CRH (± SEM) (expressed as pmol x 10^{-3}/10µl) under basal conditions, during (c1) and following (c2 onward) snack ingestion in previously non-stressed rats (Controls; squares), and those exposed to a single restraint episode followed by a 14-day stressor-free period (Acute; circles), or 14 daily sessions of restraint (Chronic; up-triangles). **,††Significantly greater relative to non-stressed controls and to chronically stressed groups, respectively ($p<0.01$).

Figure 1b: Illustrates the same data (as in Fig. 1a) except that the values are expressed as a percentage of their respective basal scores. Solid symbols represent points that are significantly greater relative to the last baseline sample of the respective treatment groups ($p<0.008$). *,†,##Significantly greater relative to non-stressed Controls, Chronically stressed rats and those exposed to a single prior session of restraint (Acute) followed by a 14-day stressor-free period, respectively ($p<0.05$ and 0.01 respectively).

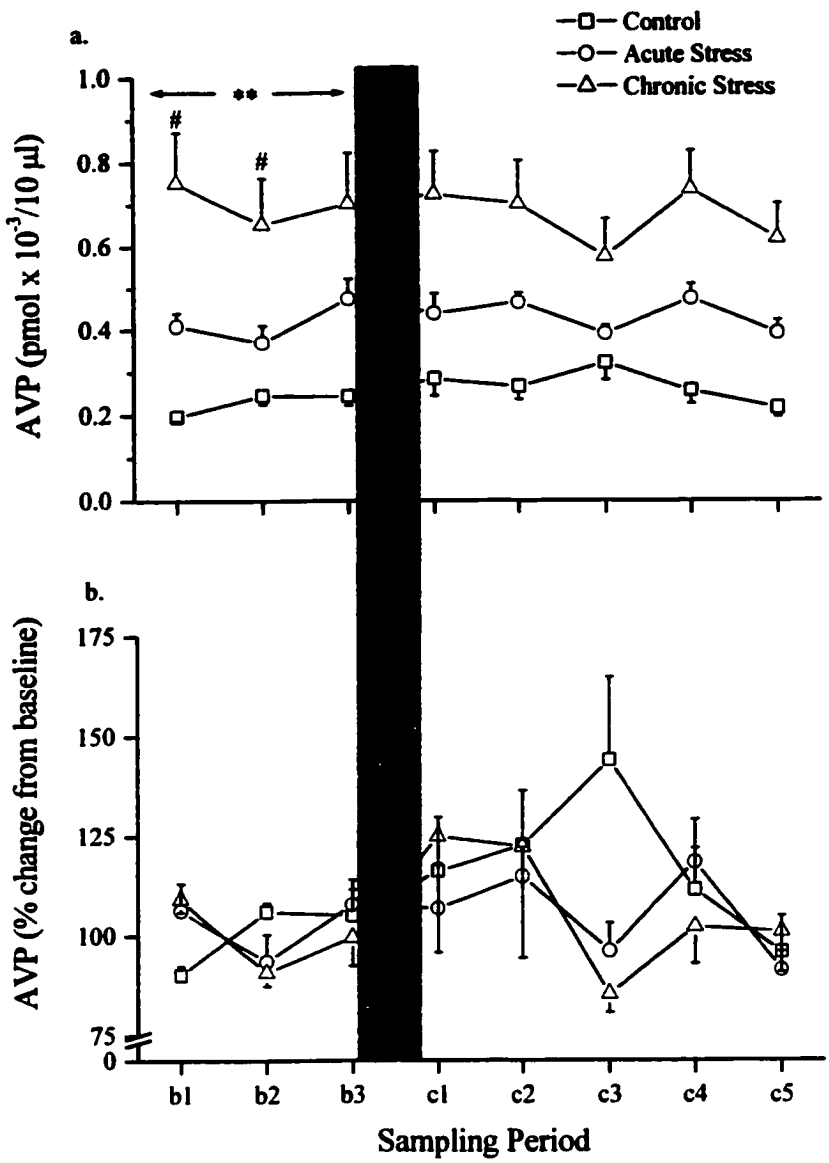


Figure 2a: Effect of differential stress history on basal and snack-induced AVP changes at the anterior pituitary. Each value represents interstitial levels of immunoreactive AVP (± SEM) (expressed as pmol x 10^{-3}/10µl) under basal conditions, during (c1) and following (c2 onward) snack ingestion in previously non-stressed rats (Controls; squares), and those exposed to a single restraint episode followed by a 14-day stressor-free period (Acute; circles), or 14 daily sessions of restraint (Chronic; up-triangles). **,#Significantly different relative to non-stressed control rats and to those exposed to a single prior session of restraint (Acute) followed by a 14-day stressor-free period, respectively ($p<0.01$ and 0.5 respectively)

Figure 2b: Illustrates the same data expressed as a percentage of their baseline scores. The ingestion of a palatable snack had little if any impact on interstitial levels of AVP.

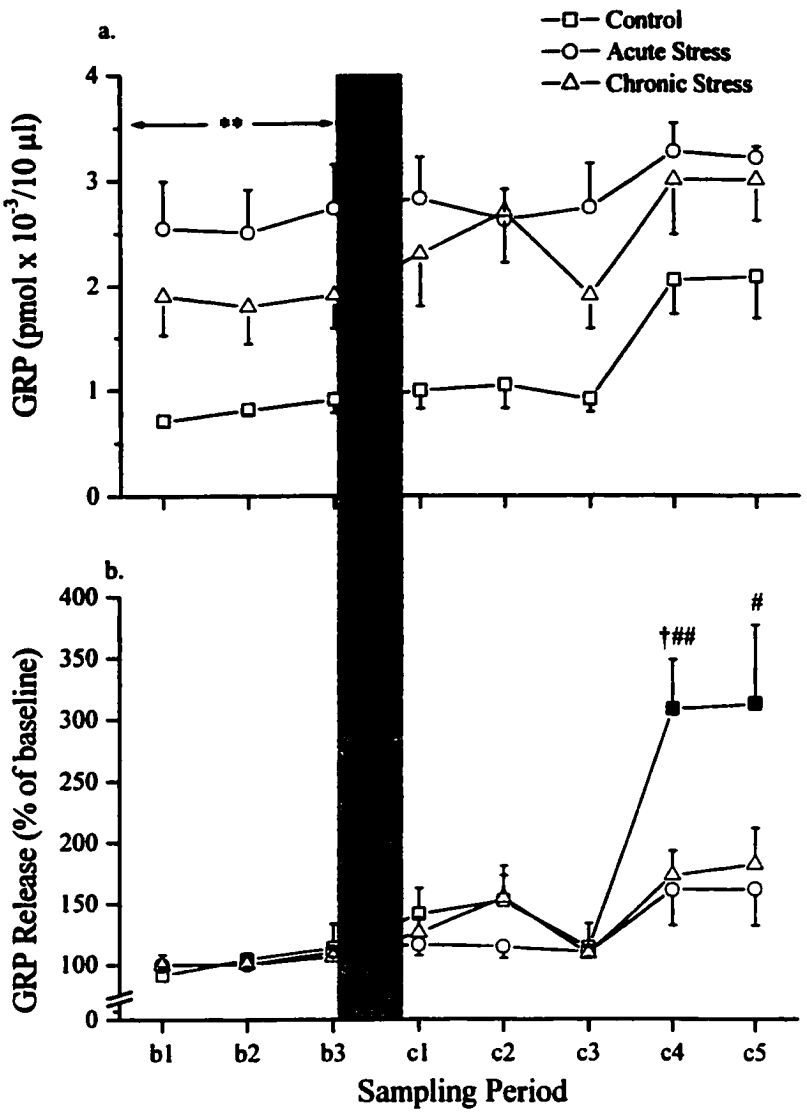


Figure 3a: Effect of differential stress history on basal and snack-induced GRP changes at the anterior pituitary. Each value represents interstitial levels of immunoreactive GRP (± SEM) (expressed as pmol x 10^{-3}/10µl) under basal conditions, during (c1) and following (c2 onward) snack ingestion in previously non-stressed rats (Controls; squares), and those exposed to a single restraint episode followed by a 14-day stressor-free period (Acute; circles), or 14 daily sessions of restraint (Chronic; up-triangles). **Significantly different relative to non-stressed Control rats, $p<0.05$ and 0.01 respectively.

Figure 3b: Illustrates the same data expressed as a percentage of their baseline scores. Solid symbols represent points that are significantly greater relative to the last baseline sample of the respective treatment groups, $p<0.008$. †,#,##Significantly different from animals previously exposed to chronic restraint stress and acute stress followed by a 14-day stressor-free period respectively, $p<0.05$ and 0.01 respectively.

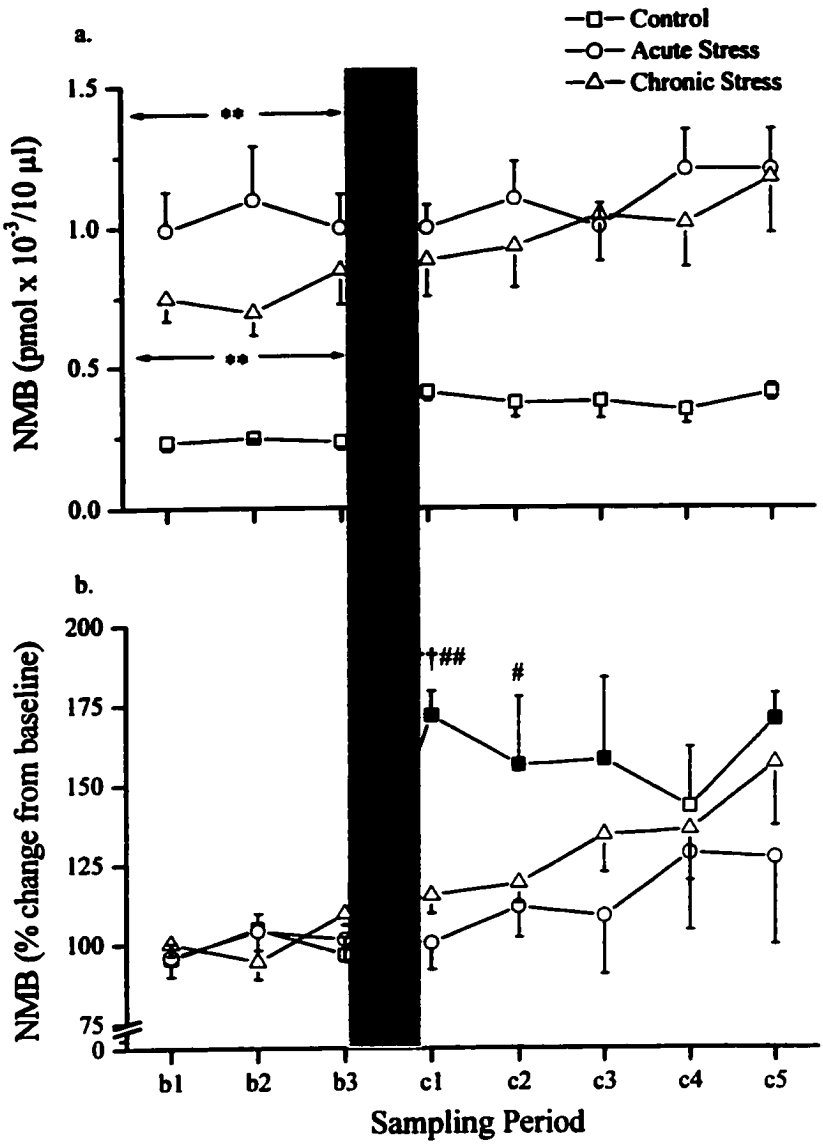


Figure 4a: Effect of differential stress history on basal and snack-induced NMB changes at the anterior pituitary. Each value represents interstitial levels of immunoreactive NMB (± SEM) (expressed as pmol x 10^{-3}/10µl) under basal conditions, during (c1) and following (c2 onward) snack ingestion in previously non-stressed rats (Controls; squares), and those exposed to a single restraint episode followed by a 14-day stressor-free period (Acute; circles), or 14 daily sessions of restraint (Chronic; up-triangles). *,**Significantly different relative to non-stressed control animals, $p<0.05$ and 0.01, respectively.

Figure 4b: Illustrates the same data expressed as a percentage of their baseline scores. Solid symbols represent points that are significantly greater relative to the last baseline sample of the respective treatment groups, $p<0.008$. ††Significantly different relative to rats previously exposed to a chronic stressor $p<0.01$. #,##Significantly different relative to rats previously exposed to an acute stressor followed by a stress-free period $p<0.05$ and 0.01 respectively.

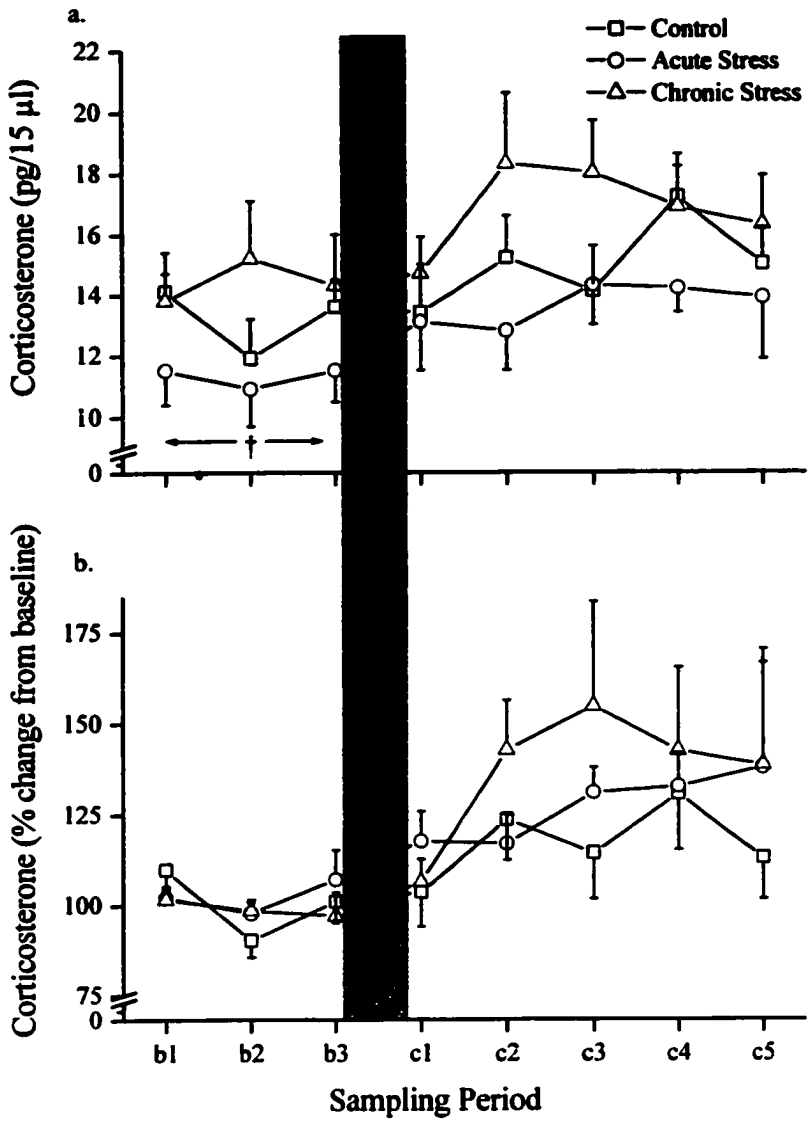


Figure 5a: Effect of differential stress history on basal and snack-induced corticosterone changes at the anterior pituitary. Each value represents interstitial levels of immunoreactive corticosterone (± SEM) (expressed as pg/15µl) under basal conditions, during (c1) and following (c2 onward) snack ingestion in previously non-stressed rats (Controls; squares), and those exposed to a single restraint episode followed by a 14-day stressor-free period (Acute; circles), or 14 daily sessions of restraint (Chronic; up-triangles). †Significantly different relative to rats previously exposed to a chronic stressor $p<0.05$.

Figure 5b: Illustrates the same data expressed as a percentage of their baseline scores.

Discussion:

The primary objective of the current investigation was to assess whether ingestion of a palatable snack was associated with altered peptidergic availability at the anterior pituitary. Results showed significantly elevated interstitial levels of BN-LPs (GRP and NMB) and CRH, but not AVP or corticosterone at the anterior pituitary following consumption of a palatable snack (Graham Wafers). The second objective was to determine if prior stress history influenced the basal and/or feeding-induced changes in the availability of these peptides at the anterior pituitary. In general, results showed that interstitial levels of these peptides were substantially elevated in animals previously exposed to chronic restraint or acute restraint followed by passage of time, compared to naïve non-stressed controls. Furthermore, prior stress history altered the peptidergic responses to the ingestion of the palatable snack. Together, these findings provide further evidence for an interrelationship between the systems governing feeding behavior and the stress response.

In control animals, the ingestion of a palatable snack was associated with increased interstitial levels of NMB, GRP and CRH at the pituitary gland. Although the availability of AVP and corticosterone was slightly increased following consumption of the snack, these increases did not reach statistical significance. These findings are consistent with previous observations in our lab showing the release of CRH and BN-LPs at the CeA following spontaneous food intake.[311] Moreover, we have previously demonstrated increased tissue levels of BN-LPs in the median eminence/arcuate region associated with food intake.[378] While the interpretation of altered tissue levels is tenuous,

it is possible that the increased BN-LPs at the median eminence/arcuate represent increased release and/or synthesis at this region, which in turn could translate into an increased availability of these peptides at the anterior pituitary.

It is of interest to note that, while interstitial levels of both NMB and GRP were significantly increased following snack consumption, their kinetics were distinct. The increased NMB availability was immediate in onset and dramatically large in magnitude, while that of GRP was relatively delayed and smaller in magnitude. This differing temporal profile makes sense in light of what is known with respect to the distribution of these peptides. For example, mRNA expression of NMB and NMB receptors is concentrated in the anterior pituitary.[200] In contrast, GRP mRNA expression is much more prominent at hypothalamic structures such as the PVN.[485] Thus, the immediate increase in interstitial NMB at the pituitary could represent local release, whilst the more delayed increase of GRP may be attributable to upstream release, most likely from hypothalamic structures. The functional significance of the increased availability of these peptides at the pituitary remains elusive. It is well known that exogenous administration of BN-LPs (including GRP and NMB) suppress food intake.[151,152,154,155,249,252,255,292,310,450] This effect is antagonized by the blockade of BB_1 (using a selective NMB receptor antagonist, BIM-23127) and/or BB_2 receptors (using high affinity GRP receptor antagonists)[256,257] or central administration of anti-BN antiserum.[313] It has been shown that the anorectic action of GRP, while abbreviated in comparison to BN (due to a more rapid enzymatic processing of this endogenous peptide family), was more pronounced than that of NMB_{1-32} and NMB_{23-32}.[310]

In addition to the increased availability of BN-LPs, we also observed elevated interstitial levels of CRH following snack consumption. Although this peptide is generally associated with the mediation of the stress response, there is clear evidence that CRH is also involved in the regulation of ingestive behavior.[81,259,316,392] Again, the functional significance of this increased availability of interstitial CRH remains speculative. CRH is known to be the principal regulator of the HPA axis.[188,207,449] In addition to CRH (and AVP), it is becoming clear that the BN family of peptides, including GRP and NMB may also be capable of regulating the HPA axis functioning.[230-232] The HPA axis is also critically involved in the regulation of energy balance within the organism;[5,97] glucocorticoids (corticosterone in the rat and cortisol in the human) promote increased release of glucose from energy stores.[207,449] It would appear paradoxical that during nutrient or energy intake, mechanisms are being set into motion to release energy from the body's stores. However, it is possible that, at some level, feeding can be interpreted by the organism as being stressful. Alternatively, some energy expenditure of internally stored energy supply may be necessary for preparing the organism to consume, metabolize, transport and store newly acquired raw materials. Finally, these peptidergic responses may be activated to draw attention to external events or cues of biological significance, such as those associated with food availability or a threat to survival.[311]

Food intake is known to increase plasma levels of corticosterone in rats and cortisol in humans.[241,311,400] Therefore, we expected that, in the current investigation, the ingestion of a palatable snack would likewise induce an increase in interstitial levels of

corticosterone at the pituitary. This, however, was not the case as only a modest (statistically non-significant) rise in interstitial corticosterone was observed in this area, following snack intake. While this finding is not consistent with other reports, it may be related to the fact that corticosterone is not constitutively expressed at the pituitary, but rather has to come from circulation, and the dynamics may be different.

It was interesting that the animal's stress history had a significant impact on the feeding-related peptidergic availability at the anterior pituitary. For instance, the snack-induced increase in the availability of GRP and NMB was markedly blunted in animals previously exposed to either a chronic or acute stressor (followed by passage of time). Also, animals exposed to an acute episode of stress followed by a stress free period displayed a blunted CRH response to the snack intake, while those chronically stressed showed a tendency towards a delayed and protracted increase. These results are interesting but difficult to explain. It is noteworthy that the animals exposed to a single episode of stressor followed by passage of time displayed high basal levels of CRH, GRP and NMB. Further, animals exposed to a chronic stressor exhibited elevated basal levels of GRP, NMB and AVP. Thus, in all cases where basal levels of the peptide were high, snack-induced changes were minimal. This can be interpreted in different ways. If the availability of the peptide is already high, perhaps even a minimal increase in that availability is physiologically significant. Alternatively, perhaps the peptidergic system is already running at maximal capacity and simply cannot increase further. In either case, it is clear that previous stress history has a significant effect on peptide availability and subsequent response to ingestion of a palatable snack.

It should not be surprising that variables that alter HPA reactivity would impact on peptidergic responses to food intake. Indeed, this is consistent with previous findings. For example, dysregulation of the HPA axis, particularly increased activation, has been implicated in eating disorders such as anorexia and obesity.[25,32,161,199,446] Animal studies have shown that, while the effect of stressors on food intake does tend to be variable, many stressors, including restraint stress, induce anorexia.[17,404] Again, this response may depend on the type, severity and intensity of the stressor as well as the macronutrient used in testing. For example, rats that have been acutely stressed consumed a similar amount of fruit loops relative to controls, while chronically stressed animals showed an increased consumption, an effect that has been reversed with diazepam.[123,432]

In terms of basal levels, as mentioned earlier, previous stressor exposure was associated with substantial increases in interstitial availability of peptides at the anterior pituitary. The enhanced availability of AVP following chronic restraint is consistent with previous findings demonstrating that chronic stressor exposure is associated with increased AVP expression (and stores) in CRH expressing neurons.[472] However, the dramatic increase in basal levels of CRH at the pituitary following a single stressor exposure was unexpected and interesting. It is important to keep in mind that the nature of the stressor may be relevant. Restraint stress is thought to be a processive stressor as it involves both emotional and cognitive processing of an adverse experience. Acute restraint stress has been shown to increase c-fos mRNA in CRH-producing neurons,[204] and CRH mRNA in the parvocellular neurons of the PVN.[373] However, these changes were not apparent after chronic stressor application.[373] Glucocorticoid feedback may be

involved in the adaptation to the chronic stressor due to the fact that this habituation did not occur in adrenalectomized animals with corticosterone replacement at daily trough levels.[373] In the current investigation, basal levels of interstitial corticosterone at the pituitary in the chronically stressed rats, while not different from control animals, were elevated in comparison to the acutely stressed animals. While it remains highly speculative, it may be possible that the higher levels of corticosterone seen in the chronically stressed rats are imposing a greater feedback on the HPA axis in these animals compared to the acutely stressed group. However, plasma corticosterone levels were not measured in this study, making conclusions only tentative.

In summary, the most important finding of this investigation is the fact that, in the Control animals, interstitial levels of NMB, GRP and CRH were increased in the anterior pituitary in response to the consumption of a palatable snack. In a separate study, it had been demonstrated in our laboratory that exposure to air puff stressor elicited a significant rise in the availability of NMB, GRP, CRH, AVP and corticosterone at the anterior pituitary in the control animals. It is interesting (and consistent with our observations at the CeA[311]) that, with the exception of AVP and corticosterone, two apparently diverse stimuli such as stressor exposure and palatable food intake appear to be subserved by similar neurochemical substrates. These findings once again appear to highlight the possibility that feeding is regarded, at least at some level, as stressful. Alternatively, these peptides may serve a much broader role than previously envisioned. In either case, the results of the current report should provide new insights into the

mechanism(s) governing feeding behavior as well as stressor-induced eating disorders such as anorexia nervosa, bulimia and obesity.

Preface to Chapter III

The first chapter established that levels of the "stress" peptide, CRH, as well as those of the "satiety" BN-LP(s), increased at the CeA in response to both appetitive (feeding) and aversive (restraint) events. Chapter two demonstrated that the availability of CRH and the BN-LPs, NMB and GRP, were elevated at the anterior pituitary in response to the ingestion of a palatable snack. This supports the contention that some common neurochemical mechanisms are deployed mediating the response to stressors as well as food intake. However, the noted peptidergic alterations could reflect responses to physiological events (e.g. presence of food in the gut) or to associated psychological factors (e.g. cephalic processing of information). In this context, evidence has increasingly been pointing to the importance of the mPFC in the regulation of reward system(s). It is of interest that human as well as animal research seems to indicate that the mPFC responses appear to be asymmetrical (or laterally biased). In the next experiment, rats were trained using auditory (60-dB white noise), visual (sight) and olfactory cues (smell of the Graham Wafer snack) to expect or "anticipate" the presentation of a palatable snack. We again used *in vivo* microdialysis to assess the release of CRH and BN-LP (GRP), as well as DA, DOPAC, 5-HT and 5HIAA at the left and right mPFC, in response to anticipatory cues paired with palatable snack presentation.

CHAPTER III

"Anticipatory" cue-associated changes in CRH and 5-HIAA levels at the medial prefrontal cortex

Abstract

In the study of ingestive behaviors, it may be important to consider the neurochemical variations associated with the anticipatory phase of a meal or palatable snack. In the current investigation, we trained rats using auditory (60-dB white noise), visual and olfactory cues (sight and smell of a Graham Wafer snack) to expect or "anticipate" the daily presentation of a palatable snack (Cue Relevant group). Two control groups were similarly trained, but, for one group, the snack was discontinued at least two weeks prior to testing (Extinction group), and for the other group, the snack was never paired with the cues (Cue Irrelevant group). *In vivo* microdialysis sampling, followed by RIA and HPLC analysis was used to monitor the release of CRH, BN-LPs, 5-HIAA, DA and DOPAC at the right and left mPFC in response to the "anticipatory" cues and subsequent consumption of the snack. It was observed that the release of CRH at the mPFC was markedly increased in response to the anticipatory cues, which was laterally biased (right greater than left). 5-HIAA availability was also increased in response to snack consumption. The release of BN-LP (GRP), which was most prominent in the left mPFC, was of a similar magnitude in both the Cue Relevant and Extinction group of animals, but only reached statistical significance in the Extinction group. Behaviorally, Cue Relevant animals were more vigilant during the "anticipatory" sampling period. These results indicate that these systems may serve to allocate salience and/or incentive reward value to biologically significant stimuli.

Introduction

In examining the neurochemical substrates of ingestive behaviors, it may be important to distinguish the neurochemical variations associated with consumption from those related to the anticipatory (or preparatory) processes associated with a meal or a palatable snack (i.e., incentive motivation). A large body of evidence highlights the importance of central dopaminergic pathways extending from the ventral tegmental area to the nucleus accumbens septi in the control of behaviors motivated by reward, such as food consumption and drugs of abuse.[27,42,44,365,369,393,394,496] However, the potential contribution of peptidergic (or non-catecholaminergic) system(s) to such processes remains unknown.

Over the past few years, evidence has been pointing to the importance of the mPFC in regulating the dopaminergic reward system in particular, and the reward system in general.[496] This brain region is recognized for its role in higher cognitive functions such as attention, memory and learning, personality, intellect and decision-making processes.[206,479] Studies in animals and humans suggest that the mPFC (among other areas) becomes activated in response to pleasurable and rewarding stimuli (e.g., food, sex, drugs of abuse and music) as well as during the anticipation of reward.[43,93,242] Furthermore, much of the mPFC supports responding for rewarding electrical brain stimulation.[38,385] Anatomically, the prefrontal cortex is intricately connected to the hypothalamus, amygdala, ventral striatum, midbrain periaquaductal gray, and sympathetic and/or parasympathetic motor nuclei in the brainstem and spinal cord.[69,434,459,479] As well, prefrontal terminals synapse with dopaminergic and GABA-

ergic neurons in the ventral tegmental area which project back to the prefrontal cortex and nucleus accumbens, respectively.[76,77] These ventral tegmental area projections to the mPFC may have an important role in the temporal organization of motivational and coping behavior.[394,459] Taken together, the mPFC likely contributes to the monitoring of the internal and external milieu, and the modulation of adaptive behavioral and autonomic responses.[458,459,479]

While aversive events effectively increase neurochemical responses within the mPFC,[163,459] it also appears that the mPFC may be activated in response to appetitive situations.[127,385,393,394] For instance, in animals trained to lever press for food reward, the dopaminergic voltammetry signal decreased when animals were allowed to consume their sweetened condensed milk reward.[394] Interestingly, delaying the reward increased the DA signal, which then decreased again upon presentation and consumption of the reward. The DA signal also increased when the reward duration was unexpectedly short. These results suggest that the DA input to the mPFC is not simply a result of the rewarding stimuli, but instead is activated mainly when conditions deviate from expected outcomes.[394]

An issue that is becoming increasingly important is the fact that the mPFC appears be asymmetrical (or lateralized).[58,74,344,458,459] In this regard, it has been demonstrated that the right mPFC may be critical in the mediation of responses to stressful stimuli. For example, lesions to the right or bilateral mPFC, but not the left mPFC blunted stressor-induced increase in corticosterone levels and ulcer development

(an index of autonomic activation).[459] However, to our knowledge, very few studies have assessed reward-related cortical asymmetries. Thus, one of the objectives of this study was to assess whether laterality differences would be evident within the mPFC in response to the anticipation and consumption of a palatable snack.

While the role of DA systems in behaviors motivated by reward has been firmly established, there is increasing evidence that other neurochemical processes may also be involved in the regulation/modulation of feeding/reward and its anticipation. For example, microdialysis studies revealed that, in response to both stressful and rewarding stimuli, increased release of 5-HT is elicited from neurons that are co-distributed with DA in certain brain regions, including the mPFC.[44,343] Likewise, ethanol administration has been shown to induce the release of both DA and 5-HT in the nucleus accumbens and alcohol-preferring rats have abnormal 5-HT and/or DA systems in various reward-related nuclei including the mPFC.[290]

Appetitive and aversive events (such as feeding and restraint stress, respectively) have been shown to activate similar neurochemical substrates.[311] Specifically, *in vivo* microdialysis revealed that both an acute exposure to a brief period of restraint stress and spontaneous ingestion of rat chow induced the release of CRH and BN-LP at the CeA.[311] However, not much is known about potential rewarding properties of these peptides. BN-LP's, like CRH, are known to stimulate the HPA axis and decrease food intake including operant responding for both food and water reward.[16,151,152,154,230,231,308,310]

However, to our knowledge, no studies have assessed the role of CRH or BN-LPs in the anticipation of a food reward.

In the current investigation, we assessed the potential role of DA, 5-HT, CRH and BN-LP in the "anticipation" and subsequent ingestion of a palatable snack. Rats were trained using auditory, visual and olfactory cues to "anticipate" the presentation of a rewarding palatable snack. The aims of the present investigation were to assess neurochemical changes within the right and left mPFC in response to the 1) "anticipation" and 2) consumption of a palatable "snack".

Materials and Methods

Subjects

Male Long Evans rats, weighing 275-300 g, were obtained from Charles River (St. Constant, Quebec). Two days after arrival in the vivarium, animals were individually housed in custom designed "anticipation" cages (clear Plexiglas boxes, 350 cm x 250 cm x 340 cm with a wire mesh floor and a "safe" cubicle of black Plexiglas 150 cm x 150 cm x 190 cm located in one corner of the cage). Cages were equipped with a speaker that delivered a 60-dB white noise. Animals were fed *ad libitum* (except during training and testing – see below) and housed in a quiet room, separated from other animals in the facility, in a controlled environment maintained at 21°C with 60% relative humidity. All experimental procedures followed the Canadian Council on Animal Care guidelines and were approved by the Research Ethics Committee of the University of Ottawa.

Training

Animals in the three groups were exposed to their respective training paradigms over a 4-week period (two weeks before surgery and two weeks following recovery). Briefly, animals were weighed each morning during which time their cages were cleaned and their food was removed. So that the presence of the experimenter did not become a conditioned stimulus, over the next 2-3 hours, the experimenter randomly entered and exited the room, mimicking sample collection. Two "anticipatory" cues (a 60-dB white noise and the sight/odor of the snack) were presented simultaneously each day for variable time periods (5-40 min) prior to snack delivery. Training took place between the hours of 1100-1300. Animals were randomly divided into three groups, one treatment group and two control groups. The treatment group (Cue Relevant group) was exposed to the cues and then given access to the palatable snack at which point the auditory cue was immediately silenced. The first control group (Extinction group) was similarly trained (exposed to the cues and given access to the snack), but snack delivery was discontinued at least 2 weeks prior to testing. The second control group (Cue Irrelevant group) was presented with the cues but the snack was never paired with the anticipatory cues.

Surgery

Following the initial two-week training period, rats were anaesthetized using pentobarbital (60 mg/kg, intraperitoneal) with halothane supplement (1.5 L) as required, and placed in a stereotaxic instrument with level skull. Blunted ear bars were used in order to protect the rats' eardrums. Two 20 gauge guide cannulae, each containing a

removable 24 gauge stainless steel obturator, were implanted at the right and left mPFC. The placement coordinates (Paxinos and Watson[362]) with level skull were anterioposterior = +3.2, lateral = ± 0.8, and dorsoventral = -3.5). The guide cannula (which protruded from a custom designed Delrin™ pedestal) was then secured to the skull with four stainless steel screws and acrylic dental cement.

In vivo microdialysis

One day prior to testing, animals were briefly anaesthetized using halothane, and the obturators (within the guide cannulae) were replaced with microdialysis probes. The concentric microdialysis probes (with 2 mm of active membrane; 250 μm outer diameter) of regenerated cellulose (6000 dalton molecular weight cutoff; Spectrum Medical Industries) were positioned above the target site such that they rested within the guide cannulae. Each probe was secured with a retaining screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel and a 2.5 ml infusion syringe (Hamilton) attached to a pump (model 22, Harvard). Microdialysis probes were perfused overnight at a rate of 0.5 μl/min with filtered KRB solution consisting of (in mM): K^+ 2.7, Na^+ 145, Ca^{2+} 1.35, Mg^{2+} 1.0, Cl^- 150, (pH 7.4) [327], and BSA (0.1%). On the following morning (first test day), animals were gently restrained and probes were gently lowered into the target position (within the mPFC) and the perfusion rate of the KRB was increased to 3 μl/minute. Sample collection was initiated 1.5 - 2 hours later. At the end of the first test day, the infusion rate was once again reduced to a stand-by rate of 0.5 μl/min overnight. The second test day was identical to the first day, except that the probes were already in place within the mPFC and, thus, did not have to be lowered. On

collection, each sample (50-60 μl) was immediately frozen on dry ice and stored -80°C until analysis by RIA and HPLC.

Experimental Design and Procedures

Twenty-eight rats with probes aimed at the mPFC participated in the study. The probes were continually perfused with KRB, and dialysates were pooled every 20 minutes throughout the experiment. After collecting five baseline samples, the rats were presented with the "anticipatory" cues. Specifically, a 60-dB tone was emitted through a speaker located in the cage top, and two small squares of Graham Wafers (Christie's Honeymaid®) (~1.8 g each) were placed on the top, front corner of the cage. Thus, animals could see and smell the graham wafer snack and could hear the tone. Dialysate samples continued to be collected every 20 min. At the end of the two "anticipatory" periods, the Cue Relevant group of rats were presented with and allowed to consume the snack. Meanwhile, for the two control groups, the snacks were removed from the top of the cage and discarded. Sample collection continued for an additional hour (i.e. three samples).

Radioimmunoassays

The detection and quantification of CRH was achieved through a solid-phase high sensitivity adaptation/modification[285] of the double antibody liquid phase RIA originally described by Vale and colleagues.[475] BN-LP(s) were detected using a similar solid-phase RIA.[378] Briefly, protein A/G (Calbiochem-Novabiochem Corp., La Jolla, CA) coated Immulon®-4 wells (Dynatech Laboratories, Inc., Chantilly, VA) were incubated with

anti-CRH serum (rC70 kindly provided by W. Vale) or anti-BN serum (α-BN2 kindly provided by Dr. T. Moody) for 2 hours at 20° C. Samples, standards (reconstituted in the KRB solution with BSA (0.1%)), ranging from 0.05 to 250 fmol/well or blanks were incubated for 24 hours at 4° C. Next, 25 µl assay buffer containing 5000 - 6000 CPM [^{125}I-Tyr^{0}]rCRF (Amersham Canada Ltd., Oakville, ON) or [^{125}I-Tyr^{4}]BN (iodinated in house, as per Salacinski et al. 1981,[409] was added to each well and incubated for an additional 24-hour period at 4° C. Finally, the wells were rinsed, separated and their residual radioactivity counted in a gamma-counter (Cobra® II Auto-gamma, Meriden, CT). A four parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically about 0.1 and 2 fmol/well for CRH and BN, respectively.

The specific anti-CRF serum used in the study recognized CRH_{1-41} and displayed negligible cross-reactivity with other related peptides[475] including urotensin 1 and Ucn (data not shown). The BN antibody used in the radioimmunoassays recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with amphibian BN (100%) and certain mammalian BN-LPs including GRP_{1-27} (110%), and GRP_{18-27} (neuromedin C or NMC (82%)), but only weakly with GRP_{1-16}, NMB-10, NMB-32 or substance P (≤0.1%).[332] We have shown in the past that the major source of BN-LP immunoreactivity from the hypothalamus is attributable to GRP.[307]

High Performance Liquid Chromatography

Levels of DA, DOPAC and 5-HIAA (5-HT was below detection limit) were determined using an HPLC system (Agilent Technologies, Waldbronn, Germany). Samples collected on the second test day (55-60 µl) were injected via autoinjection (Agilent 1100 series Autosampler, Waldbronn, Germany) (50 µl/sample for the detection of DA and 5 µl/sample for the detection of DOPAC and 5HIAA) into the HPLC system equipped with a single cell electrochemical detector (Antec Leyden Model Intro, Montreal, P.Q., Canada) with applied potential of 0.650 nA, filter of 1 s., and range of 0.1 nA/V. Separation of these analytes was achieved by their passage through an ESA, 4.6 x 150 mm, 5 Micron analytical column (Zorbax Eclipse XDB-C8, Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of 90 mM sodium dihydrogen phosphate (monobasic), 1.7 mM 1-octane sulfonic acid (sodium salt), 50 nM EDTA, 10% (200ml/2L) acetonitrile, 50 mM citric acid (monohydrate) and 5 mM KCL (final pH = 2.4)) was delivered at a flow rate of 1.0 ml/min. Quantification of the various analytes was accomplished by comparing their area under the curve to those of known external standards (calibrated at 2.5 pg/50 µl, 5.0 pg/50 µl and 50.0 pg/50µl) using computerized Agilent ChemStation chromatography data acquisition system (Agilent Technologies, Waldbronn, Germany).

Behavioral Monitoring

During testing, animals were videotaped and their behaviors were analyzed over a 120 min time period (during baseline, "anticipation", and "cookie" sampling periods). The frequency of sleeping, exploring, drinking, rearing, vigilance, and grooming were

scored every 10 seconds (see Table 1 for operational definitions of behaviors recorded). Cumulative scores over 20-minute sampling periods were subjected to statistical analysis.

Table 1

Behaviors measured during 120 min test period

Behavior	Definition
Exploring	The animal is moving around, actively examining an area of its testing compartment.
Rearing	The animal raises both forepaws off the floor of the cage and stands on hind legs with body extended.
Grooming (Scratching and/or Washing)	Scratching – Contact of the hindpaw with the side of the face, head or body/flank followed by a scratching action. Washing – Wiping of the face with circular movements of the forelimbs or active licking of the abdomen and thorax.
Vigilance	The animal is awake and alertly watchful.
Resting/sleeping	The animal is inactive, crouched or lying down with its head down

Histology

At the end of the experiment, animals were deeply anaesthetized using Halothane and decapitated. Their brains were extracted, sectioned, and stained for histological examination. Only animals with verified placement in the right and left mPFC were used in this study.

Statistical Analysis

All results are expressed as a mean ± S.E.M. All microdialysis data were analyzed by first averaging the first four baseline samples (denoted as 100%), with subsequent samples expressed as a percentage of that baseline. The changes of DA, DOPAC, CRH, GRP and 5-HIAA levels in the mPFC as well as the behavioral data were analyzed using mixed measures ANOVAs with Group (Cue Relevant, Extinction, Cue Irrelevant) as the between group factor and Time (final baseline sample, the two anticipatory samples and the first two snack or "cookie" samples) as the within-subjects factor. The Huynh-Feldt correction was applied to each analysis. Where there was a significant interaction, post hoc comparisons were conducted for the effect of Group using a one-way between-subject ANOVA (with Time as the within-subject factor and Group as the between group factor) followed by the Tukey HSD test. For the main effect of Time, post hoc comparisons were conducted using Paired Sample t-tests with Bonferroni correction, comparing the fifth baseline sample (B5) to each of the two anticipatory samples (A1 and A2), the sample during which the rats consumed (or not) the snack (C1), and the sample immediately following that one (C2). For the behavioral

analysis, each behavior (Exploring, Rearing, Grooming, Vigilance and Resting/Sleeping) was analyzed separately as above.

Results

Effect of exposure to the anticipatory cues and the subsequent consumption of a palatable snack on interstitial levels of CRH and GRP:

Figure 1 depicts the interstitial levels (presented as a percentage of baseline values) of CRH at the mPFC in response to the presentation of the "anticipatory" cues followed by the consumption (or not) of a palatable snack (Cue Relevant right and left, n = 8 and 10 respectively; Extinction right and left n = 8 and 6 respectively; Cue Irrelevant right and left, n = 6/group). The mixed measures ANOVA revealed, among animals with correctly positioned probes, a significant interaction (Sampling Period x Group) ($F_{19,145}$ = 2.35; $p<0.01$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t-tests of specific comparisons within the simple effect of Time revealed, in the Cue Relevant group, a significant rise in interstitial levels of CRH at the right and left mPFC in response to the "anticipatory" cues ($p<0.01$) when compared to baseline. Although levels began to increase immediately following the cue onset, the increase did not reach statistical significance until the second "anticipatory" sampling period. In the Cue Relevant group, the release of CRH during the second "anticipatory" sample (A2) in the right mPFC was significantly greater than in the left mPFC ($F_{1,16} = 4.23$; $p = 0.056$) (see Figure 1).

Figure 2 depicts the interstitial levels (presented as a percentage of baseline values) of GRP at the mPFC in response to the presentation of the "anticipatory" cues

followed by the consumption (or not) of a palatable snack (Cue Relevant right and left, n = 7 and 10 respectively; Extinction right and left n = 8 and 7 respectively; Cue Irrelevant right and left, n = 7/group). The mixed measures ANOVA revealed that, among animals with correctly positioned probes, there was also a significant Sampling Period x Group interaction ($F_{20,160} = 1.89$; $p<0.05$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t-test of the simple effect of Time revealed that, in the Extinction group, when compared to the baseline sample, there was a significant rise in interstitial levels of GRP in the left (but not in the right) mPFC in response to the presentation and consumption of the palatable snack ($p<0.05$). This effect was protracted, as the elevated GRP release was still significant at the sample following ingestion ($p<0.01$) (see Figure 2).

Effect of the anticipatory cues and the subsequent consumption of a palatable snack on interstitial levels of 5-HIAA, DA, and DOPAC:

Figure 3 depicts the interstitial levels of 5-HIAA at the mPFC (expressed as a percentage of baseline) in response to the presentation of the "anticipatory" cues followed by the presentation (or not) of a palatable snack (Cue Relevant right and left, n = 10/group; Extinction right and left n = 8 and 7 respectively; Cue Irrelevant right and left, n = 9 and 10 respectively). The mixed measures ANOVA revealed that, among animals with correctly positioned probes, 5-HIAA varied as a function of the Sampling Period x Group interaction ($F_{19,186} = 1.79$; $p<0.05$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t-tests of specific simple effects revealed that in the Cue Relevant group there was a significant rise (relative to the baseline sample) of interstitial 5-HIAA levels at the right mPFC in response to the presentation and consumption of the palatable snack (C1

sample) ($p<0.01$). As seen in Figure 3, there was also an increase in the interstitial levels of 5-HIAA at both the right and left mPFC in response to the anticipatory cues; however, this rise did not reach statistical significance. The one-way ANOVA comparison for the simple effect of group revealed a significant difference between the groups during the second "anticipatory" sampling period ($F_{5,48} = 4.02$; $p<0.01$) and the 20-min period immediately following the discontinuance of the cues (i.e. during and immediately following ingestion ($F_{5,48} = 3.41$; $p<0.05$). The post-hoc Tukey test revealed that, in the Cue Relevant group, the release of 5-HIAA during the second "anticipatory" sample (A2) in the right mPFC was significantly greater than that of the other two groups ($p<0.05$). Furthermore, the first consummatory sample in the left mPFC in the Cue Relevant group was significantly greater than in their Cue Irrelevant counterparts ($p<0.05$) (see Figure 3).

Although there was a trend towards increased interstitial levels of DA and DOPAC, these did not reach statistical significance (see Table 2).

Effect of the anticipatory cues and the subsequent consumption (or not) of a palatable snack on the animals' behavior:

Table 3 depicts the frequencies of the animals' behaviors (Cue Relevant, n = 11; Extinction, n = 10; Cue Irrelevant, n = 11) in response to the presentation of the "anticipatory" cues followed by the presentation (or not) of a palatable snack. During the 120-min test period rats spent a considerable amount of time sleeping. However, the mixed measures ANOVA revealed that there was a significant Sampling Period x Group interaction with respect to the number of occurrences of sleeping ($F_{6,89} = 6.15$; $p<0.01$).

The subsequent Bonferroni corrected ($\alpha = 0.013$) t-tests of specific comparisons within the simple effect of Time revealed that, in the Cue Relevant group, the animals had significantly less occurrences of resting/sleeping during the second "anticipatory" sampling period as well as during the following 20-min period relative to baseline ($p<0.01$). The one-way ANOVA comparison revealed a significant main effect of Group during the first and second "anticipatory" sampling periods ($F_{2,29} = 7.34$; $p<0.01$; and $F_{2,29} = 52.51$; $p<0.001$ respectively) as well as the 20 min period immediately following ($F_{2,29} = 172.42$; $p<0.001$). The post-hoc Tukey test revealed that the Cue Relevant group rested/slept significantly less than the other two groups during exposure to the "anticipatory" cues and the subsequent consumption of the palatable snack ($p<0.01$).

As seen in Table 3, analysis of vigilant behaviors revealed a significant Sampling Period x Group interaction ($F_{5,70} = 6.75$; $p<0.01$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t-tests confirmed that in the Cue Relevant group, the animals were more vigilant during the second "anticipatory" sampling period than at baseline ($p<0.5$). Moreover, the one-way ANOVA comparison revealed a significant main effect of Group during the first and second "anticipatory" sampling periods ($F_{2,29} = 12.52$; $p<0.001$; and $F_{2,29} = 22.92$; $p<0.001$ respectively) as well as the 20-min period immediately following ($F_{2,29} = 7.76$; $p<0.01$). The post-hoc Tukey test revealed that rats in the Cue Relevant group were significantly more vigilant ($p<0.01$) than those in the remaining two groups.

As seen in Table 3, animals infrequently engaged in exploring, although exploration varied as a function of the Sampling Period x Group interaction ($F_{8,109} = 4.31$;

$p<0.01$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t tests indicated that rats in the Cue Relevant group displayed significantly more exploratory behavior during the second "anticipatory" sampling period compared to the baseline period ($p<0.05$). The one-way ANOVA revealed a significant main effect of Group during the first and second "anticipatory" sampling periods ($F_{2,29} = 3.44$; $p<0.05$; and $F_{2,29} = 35.72$; $p<0.001$ respectively) as well as the 20-min period immediately following ($F_{2,29} = 23.64$; $p<0.001$). The post-hoc Tukey test revealed that relative to the other two groups, significantly greater exploration and rearing was evident in the Cue Relevant group ($p<0.01$).

Like exploration, rearing was not a frequent behavior, but was found to vary as a function of the Sampling Period x Group interaction ($F_{5,68} = 3.12$; $p<0.05$). The subsequent Bonferroni corrected ($\alpha = 0.013$) t-tests indicated that although there was a slight increase in the frequency of rearing during "anticipation" in the Cue Relevant group, this increase did not reach statistical significance. The significant interaction pertained to the differences between the groups. The one-way ANOVA comparison revealed a significant main effect of Group during the first and second "anticipatory" sampling periods ($F_{2,29} = 3.78$; $p<0.05$; and $F_{2,29} = 23.05$; $p<0.001$ respectively). This effect was protracted, being evident during both of the 20-min sampling periods following "anticipation" ($F_{2,29} = 17.27$; $p<0.001$; and $F_{2,29} = 5.82$; $p<0.01$ respectively). The post-hoc Tukey test demonstrated that this increase in the frequency of rearing was Group dependent. During the first 20 min of "anticipation" ($p<0.05$) and during the second 20-min sampling period following ($p<0.01$), the Cue Relevant rats displayed more

rearing compared to the Cue Irrelevant animals. During the second "anticipation" period and the 20-minute sampling period immediately thereafter, the Cue Relevant animals exhibited more rearing than both the Extinction and Cue Irrelevant rats ($p<0.001$).

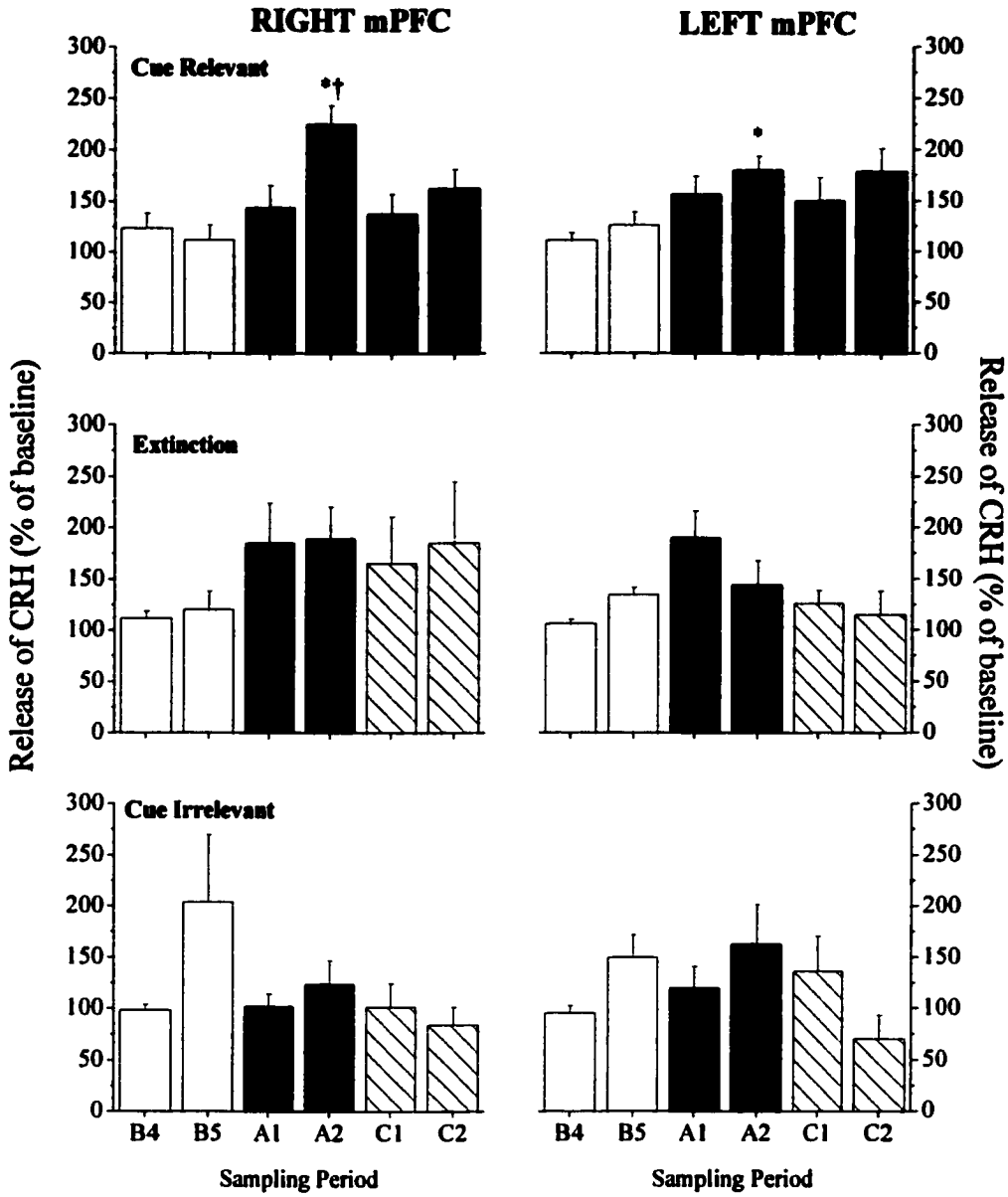


Figure 1: Effect of anticipatory cues and consumption of a palatable snack on the release of CRH in the right and left mPFC. Each bar represents the mean ± S.E.M. of interstitial levels of CRH during the 40 min prior to cue onset (open bars), the 40 min of cue presentation (solid bars), and the 40 min during and following the presentation of a palatable snack (gray and hatched bars). Groups represent animals that were trained to "anticipate" the presentation and consumption of palatable snack (Cue Relevant) (upper two panels), animals that were similarly trained but snack presentation was discontinued at least two weeks prior to testing (Extinction) (middle two panels), and rats that had never received the snack following cue presentation (Cue Irrelevant) (lower two panels). *Significantly greater than baseline; †Significantly greater than same group, left mPFC.

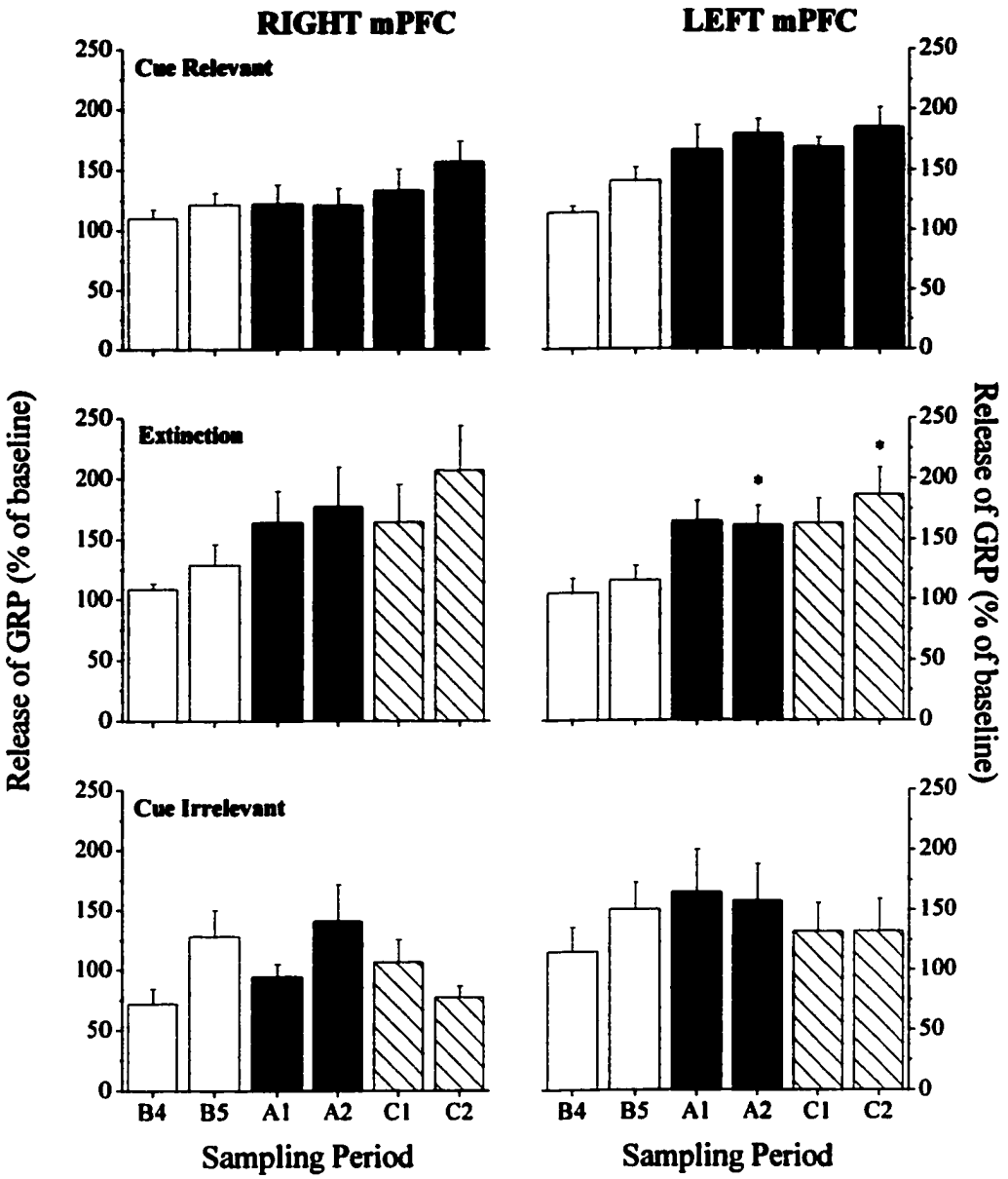


Figure 2: Effect of anticipatory cues and consumption of a palatable snack on the release of GRP in the right and left mPFC. Each bar represents the mean ± S.E.M. of interstitial levels of GRP during the 40 min prior to cue onset (open bars), the 40 min of cue presentation (solid bars), and the 40 min during and following the presentation of a palatable snack (gray and hatched bars). Groups represent animals that were trained to "anticipate" the presentation and consumption of palatable snack (Cue Relevant) (upper two panels), animals that were similarly trained but snack presentation was discontinued at least two weeks prior to testing (Extinction) (middle two panels), and rats that had never received the snack following cue presentation (Cue Irrelevant) (lower two panels). *Significantly greater than baseline.

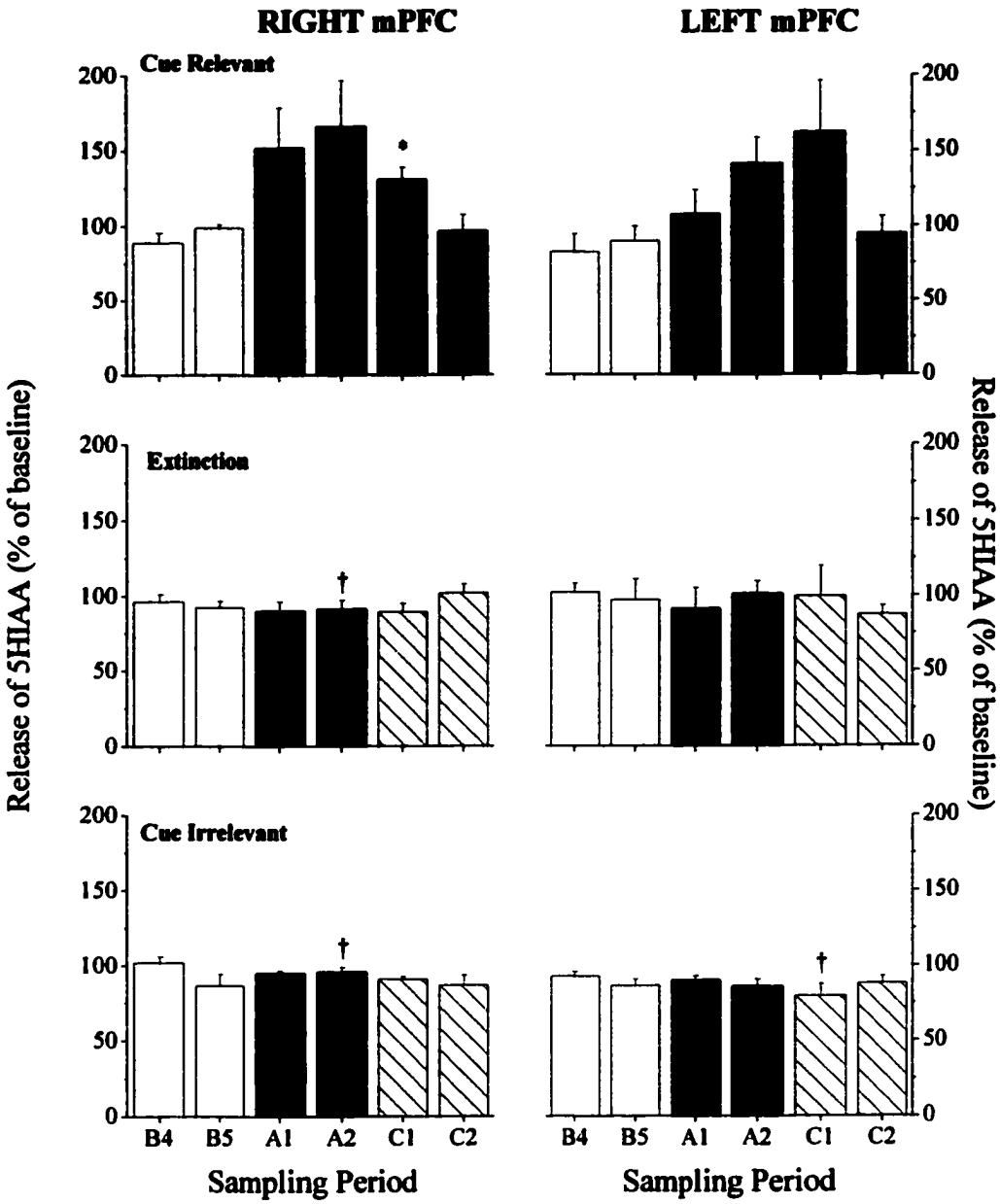


Figure 3: Effect of anticipatory cues and consumption of a palatable snack on the release of 5-HIAA in the right and left mPFC. Each bar represents the mean ± S.E.M. of interstitial levels of 5-HIAA during the 40 min prior to cue onset (open bars), the 40 min of cue presentation (solid bars), and the 40 min during and following the presentation of a palatable snack (gray and hatched bars). Groups represent animals that were trained to "anticipate" the presentation and consumption of palatable snack (Cue Relevant) (upper two panels), animals that were similarly trained but snack presentation was discontinued at least two weeks prior to testing (Extinction) (middle two panels), and rats that had never received the snack following cue presentation (Cue Irrelevant) (lower two panels). *Significantly greater than baseline. †Significantly less than Cue Relevant.

	B4	B5	A1	A2	C1	C2
DA-Right mPFC						
Cue Relevant	103.2 ± 9.2	114.6 ± 9.2	124.5 ± 14	153.7 ± 22	102.6 ± 9.4	109.4 ± 10
Extinction	103.4 ± 7.6	87.0 ± 5.6	95.6 ± 5.4	116.7 ± 19	125.8 ± 15	115.6 ± 13
Cue Irrelevant	94.1 ± 5.4	93.8 ± 5	107.9 ± 7.1	114.2 ± 9.6	122.2 ± 12	113.5 ± 10
DA-Left mPFC						
Cue Relevant	93.4 ± 9.9	94.8 ± 5.0	110.2 ± 6.1	120.4 ± 8.8	94.3 ± 8.4	101.7 ± 4.9
Extinction	110.3 ± 17	107.2 ± 14	117.9 ± 21	114.8 ± 33	121 ± 19.3	100.3 ± 13
Cue Irrelevant	93.5 ± 2.4	97.3 ±3.4	108.2 ± 11	104.2 ± 4.5	99.8 ± 7.4	95.1 ± 2.6
DOPAC-Right						
Cue Relevant	87.96 ± 8.8	83.2 ± 7.9	138.7 ± 15	115.4 ± 17	111.6 ± 13	95.98 ± 11
Extinction	92.8 ± 6	99.1 ± 13	105.9 ± 13	97.1 ± 14	102.2 ± 14	112.7 ± 28
Cue Irrelevant	98.2 ± 2.7	105.0 ± 3.9	104.3 ± 4	102.7 ± 3.3	107.1 ± 5.5	103.2 ± 3.3
DOPAC-Left						
Cue Relevant	107.7 ± 16	92.4 ± 6.5	126 ± 14	100.8 ± 6.1	116 ± 16	97.4 ± 8.9
Extinction	95.8 ± 2.3	97.0 ± 8.6	92.7 ± 2.6	92.1 ± 5.7	83.3 ± 6.9	76.2 ± 7.3
Cue Irrelevant	97.0 ± 3.8	91.5 ± 5.1	96.9 ± 2.0	93.9 ± 1.8	92.8 ± 3.3	88.6 ± 4.1

Table 2: Effect of anticipatory cues and consumption of palatable snack on the release of DA and DOPAC in the mPFC. Each cell represents the mean ± S.E.M. of interstitial levels of DA and DOPAC in the right and left mPFC during the 40 min prior to cue onset (B4 & 5), the 40 min of cue presentation (A1 & 2), and the 40 min during and following the presentation of a palatable snack (C1 & 2). Groups represent animals that were trained to "anticipate" the presentation and consumption of palatable snack (Cue Relevant), animals that were similarly trained but snack presentation was discontinued at least two weeks prior to testing (Extinction), and rats that had never received the snack following cue presentation (Cue Irrelevant).

Behavior/ Group	B4	B5	A1	A2	C1	C2
Exploring						
Cue Relevant	2.0 ± 1/3	2.5 ± 1.2	6.6 ± 2.1	9.5 ± 1.4**	8.6 ± 1.7**	1.5 ± 0.5
Extinction	1.1 ± 0.6	0.9 ± 0.4	2.0 ± 1.2	0.6 ± 0.5††	0.2 ± 0.2††	3.3 ± 2.7
Cue Irrelevant	0.1 ± 0.1	0.1 ± 0.8	1.5 ± 1.0†	0 ± 0††	1.5 ± 0.3††	1.5 ± 0.9
Rearing						
Cue Relevant	0.5 ± 0.5	1.5 ± 1.2	3.4 ± 0.9	6.5 ± 1.2*	3.2 ± 0.8	1.5 ± 0.5
Extinction	0 ± 0	0.9 ± 0.7	1.4 ± 0.8	0.7 ± 0.4††	0.1 ± 0.1††	0.4 ± 0.3
Cue Irrelevant	0 ± 0	2.1 ± 1.8	0.5 ± 0.5†	0 ± 0††	0 ± 0††	0 ± 0††
Grooming						
Cue Relevant	6.8 ± 4.8	12.5 ± 6.2	14.7 ± 4.8	21.1 ± 6.8	25.8 ± 4.7	17.7 ± 4.7
Extinction	7.1 ± 4.4	15.1 ± 8.3	12.4 ± 6.6	9.5 ± 4.9	1.8 ± 0.8††	10.1 ± 5.2
Cue Irrelevant	0.5 ± 0.3	7.6 ± 5.3	10.8 ± 5.2	1.1± 0.5†	4.0 ± 2.0††	11.4 ± 6.0
Vigilance						
Cue Relevant	4.7 ± 2.6	15.8 ± 7.4	43.4 ± 10.1	53.0 ± 9.7*	16.8 ± 3.8	7.5 ± 2.2
Extinction	2.9 ± 1.1	4.8 ± 2.2	5.3 ± 1.8††	7.5 ± 2.5††	3.9 ± 1.4	4.0 ± 2.2
Cue Irrelevant	0.6 ± 0.2	4.6 ± 2.4	5.0 ± 2.4††	1.1± 0.4††	4.5 ± 1.8	4.8 ± 2.2
Resting/Sleeping						
Cue Relevant	94.9 ± 10.4	76.6 ± 11.8	38.5 ± 11.7	18.5 ± 8.9**	7.6 ± 2.4**	78.6 ± 7.7
Extinction	96.0 ± 5.4	82.1 ± 11.4	78.9 ±12.1†	87.5 ± 8.3††	102 ± 6.7††	91.0 ± 12.0
Cue Irrelevant	116 ± 0.7	101 ±10.8	95.6 ± 9.2††	115 ± 0.8††	109 ± 3.3††	98.8 ± 9.0

Table 3: Effect of anticipatory cues and consumption of a palatable snack on behavior in rats. Each cell represents the mean ± S.E.M. of frequency of exploring, rearing, grooming, vigilance, and resting/sleeping during the 40 min prior to cue onset (B4 & 5), the 40 min of cue presentation (A1 & 2), and the 40 min during and following the presentation of a palatable snack (C1 & 2). Groups represent animals that were trained to "anticipate" the presentation and consumption of palatable snack (Cue Relevant), animals that were similarly trained but snack presentation was discontinued at least two weeks prior to testing (Extinction), and rats that had never received the snack following cue presentation (Cue Irrelevant). *,** significant difference from B5, †,†† significant difference from cue relevant group at $p<0.05$ and $p<0.01$.

Discussion

The reinforcing properties of naturally rewarding behavior, such as food intake, are known to involve the mesolimbic and mesocortical dopaminergic systems.[27,42,126,416,443,496] The current investigation highlights significant elevations in release or availability of CRH and 5-HIAA at the mPFC in response to "anticipatory" cues and the subsequent consumption of a palatable snack.

As indicated earlier, the dopaminergic system has been the focus of research into the reinforcing properties of natural rewards. On the basis of earlier studies it was hypothesized that animals exposed to cues associated with a reward stimulus (ingestion of a palatable snack) (i.e. the Cue Relevant group) would display a significant increase of DA release in response to the "anticipation" and presentation of the snack. However, although the Cue Relevant group displayed a trend toward an increase of DA at the right mPFC during the second "anticipatory" sampling period (see Table 2), no significant increase in levels of either DA or DOPAC occurred within either the right or left mPFC. Any number of factors could account for the lack of the predicted effect. For instance, our detection limits for DA required fairly long (20 min) sampling periods, which may have contributed to the masking of short-term increases. In addition, animals in the current study were exposed to prolonged period of habituation to the palatable snack. This contrasts with other studies investigating the role of DA where the training schedules were typically very short.[3,27,126] Bassareo & Di Chiara (1997) [27] demonstrated an increase of extracellular DA levels in the mPFC in response to the consumption of a novel food (Fonzies). Although this effect was still present after three successive

presentations of Fonzies, this habituation/training period is much shorter than the four weeks of training given to the animals in the current study prior to testing. It is also noteworthy that DA efflux in the mPFC appears to be sensitive to sensory-specific satiety.[3] In this context, rats offered a different palatable food during two consecutive meals ate significantly more during the second meal and displayed a significantly greater release of DA at the mPFC compared to rats that were offered the same food on both occasions.[3] Thus, it appears that repeated exposure to the same palatable food, i.e. the same stimulus, may induce a blunting in DA efflux over time. Interestingly, the blunting of stressor-induced DA efflux, particularly in the right mPFC, has been reported in response to repeated exposure to the same stimulus.[58]

It is of particular interest that the Cue Relevant group (i.e. those trained via auditory, olfactory and visual cues to "anticipate" the presentation and consumption of a palatable snack) displayed increases of CRH that were greater at the right (relative to the left) mPFC. The increased CRH release became progressively evident at the time proximal to the actual presentation of the snack. When the anticipatory cues had been extinguished (Extinction group) or when the cues were not associated with snack presentation (Cue Irrelevant group) an increase in the release of CRH was not evident. To our knowledge, this is the first demonstration of the release of CRH in response to the "*anticipation*" of an appetitive event.

Ample evidence exists regarding the role of CRH in the mediation of the multiple components of the stress response.[41,283] Central administration of CRH is known to

activate the sympathoadrenal system, generate arousal and anxiety-like behaviors, suppress immune functions and decrease food intake.[41] However, little is known regarding a role for CRH in the mediation of reward.[283] Previously, we demonstrated the release of CRH (and BN-LP) at the CeA in response to both an aversive (acute restraint stress) and appetitive (spontaneous food intake) event.[311] However, it is not clear whether the release of CRH in response to food intake is related to the rewarding properties of the food, the vigilance required or associated with the acquisition and consumption of food, or the influx of foreign material into the body. Recent evidence suggests that CRH may actually decrease the rewarding effects of intracranial self-stimulation from the lateral hypothalamus.[283] Specifically, ICV administration of CRH or Ucn (the recently discovered neuropeptide that shows a 43% sequence identity with CRH and binds with high affinity to CRF receptors) dose dependently increased intracranial self stimulation thresholds without altering performance measures.[283] CRH was somewhat more potent than Ucn and its effect was completely blocked by the CRH_1 and CRH_2 antagonist, D-Phe CRF_{12-41}. The fact that, in the current investigation, there was in increase in the release of CRH from the mPFC in response to an apparently rewarding/appetitive event, at first glance, appears contradictory. However, intracranial self-stimulation and consumption of a palatable snack are very different paradigms and potentially under very different neurochemical control. Furthermore, the effects of exogenous administration of pharmacological doses of CRH are potentially different from physiologically relevant endogenously released peptide. It is possible that, at pharmacological doses, exogenous CRH may induce a state of anxiety/stress in the animals, whereas endogenous release of CRH (at lower or physiological levels) induced a

state of arousal enabling the animals to remain vigilant for the presentation of the awaited snack. Behaviorally, the release of CRH into the mPFC did not appear to be associated with attenuation in the rewarding value of the snack presentation. In fact, during the "anticipatory" period, the Cue Relevant animals slept/rested significantly less, were more vigilant, and actively explored their environment more than the Extinction and Cue Irrelevant groups (see Table 3). Furthermore, they immediately consumed the entire snack when it was offered, suggesting that the palatable snack was indeed rewarding. Thus, the increased CRH release in advance of food presentation likely is related to anticipatory factors. Alternatively, it may actually be stressful for a rat to be able to see and smell a palatable snack and have to wait for it to be presented. Thus, while it is tempting to suggest that CRH is being released in response to the appetitive event of "anticipating" a reward, this "anticipation" period may, in fact, be stressful.

It is noteworthy that glucocorticoids, the end product of HPA axis activation in response to stressor exposure, display rewarding properties.[283,369] Specifically, glucocorticoids have been shown to enhance food-directed behaviors, be released in response to positive reinforcers such as food or exposure to a receptive sexual partner, facilitate the rewarding effects of drugs of abuse, be self-administered, and induce the release of DA in the nucleus accumbens.[369] In the current investigation, blood sampling was not done, thus we cannot report on plasma corticosterone levels. However, we have previously demonstrated palatable snack-induced increases of CRH release as well as plasma ACTH and corticosterone levels.[311] This poses an important question: why does the application of both a stressful and an appetitive or rewarding event appear to

stimulate the same neural systems? The suggestion has been made that the rewarding properties of glucocorticoids actually facilitate coping responses in stressful situations.[369] Given our findings that similar neurochemical systems are activated in response to both appetitive and aversive events,[311] it is tempting to speculate that glucocorticoids may enhance the rewarding properties of appetitive events.

There appears to be general agreement that the 5-HTergic system is involved in the regulation of food intake.[433] Microinjections of 5-HT or its agonists into the hypothalamus decreases food intake, and microdialysis sampling from the lateral and medial hypothalamus revealed increases in the release of 5-HT in response to not only food consumption, but also its anticipation (i.e. the smell of the food).[195,417] In regard to feeding, hyperactivity of the HPA axis has been associated with abdominal obesity, and treatment with a 5-HT-releasing agonist, dexfenfluramine, was able to reduce this hyperactivity of the HPA axis.[53] Taken together, the serotonergic system could be important in the emotional aspects of food intake. Consistent with this supposition, in the Cue Relevant group, the presentation of the "anticipatory" cues initiated an increase of 5-HT turnover (as evidenced by an increase in the level of its metabolite, 5-HIAA), an effect that became significant upon snack presentation. Neither the "anticipatory" cues nor snack presentation induced any changes in the levels of 5-HIAA in the Extinction and Cue Irrelevant groups. In addition, for the Cue Relevant group, during the "anticipatory" sampling period closest to the presentation of the snack (A2), the level of 5-HIAA (although not significantly greater than baseline) was greater than in the other two groups. Again, this effect was more pronounced in the right mPFC compared to the left

mPFC where, although there was a trend toward an increase of release, the effect was too variable to reach significance. The results of the present investigation concur with a previous report indicating that 5-HT was released from the lateral and medial hypothalamus during pre-ingestive events and during eating.[417] Furthermore, activation of 5-HT synthesis has been demonstrated in the mPFC, nucleus accumbens and striatum in response to intracranial self stimulation of the medial forebrain bundle.[343]

While the results of the current investigation suggest that the serotonergic system plays a role in the "anticipation" of a palatable snack and, potentially, its rewarding value, the mechanisms of 5-HT action require further elucidation. In this regard, its interaction with the dopaminergic system may be relevant. Interestingly, it has been demonstrated that 5-HT receptors modulate cortical DA function in the mPFC.[365] Specifically, when infused directly into the mPFC, the selective 5-HT_{2A} receptor antagonist, M100,907, blocked K^+-stimulated DA release as well as increases of DA induced by systemic administration of the 5-$HT_{2A/2C}$ agonist, DOI, indicating that cortical 5-HT_{2A} receptors potentiate the release of mesocortical DA.[365] Furthermore, 5-HT itself may contribute to the rewarding effects of drugs of abuse since the nucleus accumbens is innervated by 5-HT, and amphetamine treatment has been shown to deplete 5-HT in the nucleus accumbens core and dorsal striatum.[68] Correspondingly, it has been demonstrated that 5-HT is an important modulator of the excitatory drive to ventral tegmental area pathways following psychostimulant exposure.[212]

Although the satiety effects of BN-LPs have been well studied,[16,151,152,154,230,231,308,310] there is a paucity of information available on BN-LPs' effect on reward mechanisms. In the current investigation, the Cue Relevant and Extinction groups displayed a similar magnitude of increase of GRP, a mammalian form of BN-LP, in the left mPFC. However, in the Cue Relevant group, this increase was not statistically significant (probably due to a higher baseline value) while in the Extinction group, the increase was significantly greater than baseline in the "anticipatory" sampling period closest to the presentation of the snack than in the sample following the consumption of the snack. These results are interesting but difficult to explain. One study, involving the placement of electrodes in the lateral hypothalamus revealed that intraperitoneal administration of BN increased the thresholds for stimulation-induced feeding, but did not influence intracranial self stimulation, indicating that the effect of BN-LPs on stimulation-induced feeding were similar to that of normal feeding.[72] Taken together with the lack of effect in the Cue Relevant animals, these results could suggest that BN-LPs are unrelated to the rewarding properties of food. At first blush this would seem to contradict the results obtained in the Extinction group, as they showed an increase in the release of GRP. However, it has been shown that GRP is released, not only in response to food intake,[311] but also in response to stressor application.[230,308] It might be considered that frustration/stressor associated with the snack being withheld was responsible for the increased release of the peptide in the Extinction group. Behaviorally, however, these animals did not appear to be stressed (see Table 3).

Several studies now have highlighted left/right hemispheric functional asymmetries within the mPFC.[52,58,344,458-460] For example, animals with lesions to the right mPFC displayed less anxious behavior in the elevated plus maze than animals with left or sham operated animals.[460] Furthermore, similar lesions to the right mPFC decreased both pre-stress and peak stress-induced corticosterone response.[459] Also, *in vivo* voltammetry studies measuring DA function revealed that, depending on the type of stressor involved, the right mPFC appears to have a specialized role in the integration of emotional and physiological responses to stressful situations.[58,458] In addition, positron emission tomography studies in human subjects have revealed a right hemispheric activation of the dorsolateral prefrontal cortex with a deactivation in the left mPFC in response to cue-induced cocaine craving.[52] The present findings are consistent with these reports in that the increased trend toward the release of DA occurred only in the right mPFC and the increase in the release of CRH was greater in the right mPFC compared to the left. Collectively, these findings highlight the importance of laterality biased peptidergic responses (in favor of the right mPFC) in the regulation of an organism's responses to emotionally laden events. It also emphasizes the importance of sampling/testing from both the right and left mPFC.

It is noteworthy that, when animals are restricted to a scheduled feeding period each day, they will increase their activity approximately 1-3 hours prior to feeding.[326,353,366,428] For example, rats that are expecting their food at a particular time of day will display intense pre-feeding increases in locomotor activity including consolidated bouts of running or foraging-related activity and increase in wheel running.

This activity is generally referred to as "feeding anticipatory activity". The behavioral activity that we observed concurred with previous results. It is interesting that all rats experienced an episode of grooming at least once during the testing period. However, in the sampling period that included consumption of the snack, the Cue Relevant rats groomed significantly more than the other two control groups. Similarly, during the "anticipation" sampling period closest to the presentation of the snack as well as the consumptive sampling period, Cue Relevant rats explored more than the other two groups. Cue Relevant rats also reared more, were more vigilant and rested/slept less during "anticipation" and consumption sampling periods compared to both baseline samples and the other groups. Of course, it cannot be determined from these data whether the Cue Relevant animals were anticipating the snack, or whether they were frustrated and stressed by the delay in snack presentation. After all, heightened vigilance, grooming and active exploration of a familiar environment are all part of an animal's behavioral repertoire following stressor exposure.[118,188] This notwithstanding, these behaviors do indicate that the animals were aroused and vigilant to the "anticipatory" cues and that these cues represent incentive salience.

In conclusion, the present investigation demonstrates that CRH is released in the mPFC in response to the "anticipatory" cues signaling the availability of a palatable snack. Increased interstitial levels of 5-HIAA in the same area are also apparent in response to the ingestion of the snack. This effect was more prominent in the right, compared to the left mPFC. The present study did not show a significant release of DA in this area in response to either the "anticipation" or consumption of the snack, which

may relate to the fact that the animals were well habituated to the "anticipatory" cues as well as the snack. Previously, we have shown that CRH is released at the CeA in response to both spontaneous feeding and the application of a stressful event. It is interesting that similar neurochemical substrates appear to be involved in two apparently diverse behaviors: feeding and response to stressful stimuli. The present study further demonstrates that CRH release not only occurs in response to a physiological event (ingestion of food), but also to a psychological event (the "anticipation" of a palatable snack). In future, it would be interesting to assess and compare neurochemical responses to the "anticipation" of an aversive event. In any event, the current study should provide insights into emotional arousal and, potentially, into mechanisms associated with emotional and/or anxiety related eating disorders such as anorexia nervosa and bulimia.

Preface to Chapter IV

If the organism's response to both ingestion (an appetitive event) and stressor exposure (an aversive event) are being subserved by common neurochemical mechanisms, one would expect that stimuli that affect the stress response would impact on ingestive behavior or vise versa. Individual responses to stressors are dependent on a multitude of factors including the severity (e.g. weak or strong), duration (e.g. acute or chronic), or quality (e.g. physical or psychological) of the stressor, as well as on experiential (e.g. early life experiences or previous adult stressor exposure) and individual (or organismic) variables (e.g. species, genetics, age or gender). The following chapters in this thesis examine the impact of experiential or organismic variables (that are known to alter the stress response) on various elements of ingestive behavior.

Neonatal handling and maternal separation are known to induce permanent alterations in the HPA axis and behavioral responses to stressors. The next experiment examined the effect of these early life manipulations on the animals' physical development, anxiety level, ingestion of a palatable snack, and response to known satiety peptides during adulthood.

CHAPTER IV

Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender dependent effects.

Abstract

Environmental manipulations during early development can induce permanent alterations in HPA and behavioral responses to stressors. However, little is known about the impact of early life experiences on appetitive responses. The present investigation assessed the effects of brief handling/separation or protracted separation from the dams, on feeding and anxiety responses during development. During the first 3 weeks post-partum, Sprague-Dawley rat pups were exposed daily to either brief (15 min) handling/isolation, a more protracted (3 hour) period of maternal separation, or were not handled. When tested on the elevated plus maze (at 5-6 weeks) Handled groups displayed less anxiety than Non-Handled gender-matched controls. Surprisingly, so did the Maternal Separation females. At weaning (Day 22), the Maternal Separation rats weighed significantly less than both the Handled and Nom-Handled animals. The difference between the Handled and Maternal Separation was more robust and persisted throughout the experiment (Day 62). The Handled animals of both genders, and the females of the Maternal Separation group, consumed more of the palatable "snack" than their Non-Handled counterparts. The feeding suppressant response to the various satiety peptides (BN, CCK, and amylin) was not affected by the early life experience, with exception of CCK effects, which were more pronounced in Handled and Maternal Separation males. These results suggest that early life events may contribute to anxiety and/or ingestive disorders such as anorexia nervosa, bulimia and obesity.

Introduction

Animals separated from the dam during the neonatal period may exhibit profound and long lasting alterations of endocrine and behavioral responses to stressors.[138,169,235,270,300,301,490] It is important, however, to distinguish between the impact of brief periods of separation (handling) from those elicited by more protracted periods of maternal separation. Pups briefly handled (10-15 min separation from the dam) daily, for the first 2-3 weeks of life, show reduced HPA responsivity to stressors encountered in adulthood. Although basal corticosterone levels of the neonatally handled rats and their non-handled counterparts are remarkably similar at adulthood,[303] the Handled (H) rats display a smaller stressor-elicited rise in circulating ACTH and both total and free corticosterone.[269,302] As well, these hormonal increases return to basal levels more quickly in the H animals than their Non-Handled (NH) counterparts.[302] The effects of handling may be related to the increased glucocorticoid negative feedback sensitivity stemming from increased expression of hippocampal and frontal cortex glucocorticoid receptors.[112,301,441]

In addition to the endocrine variations, early neonatal manipulations also engender persistent behavioral changes. For instance, compared to NH rats, H animals exhibit less anxiety/emotionality on such tests as the elevated plus maze, hyponeophagia paradigm, and the emotionality rating test,[47,129,346] open field exploration,[271] two-way active avoidance test,[346] and the Porsolt swim test.[193] These animals also display less voluntary alcohol consumption[193] and generally more adaptive responses to stressful events.[89,96,128] Thus, brief early life separation/stimulation has robust effects on adult

behavior and neuroendocrine stressor response, which may vary according to the gender of the subjects.[253,347,461] It should be noted, however, that the reports of the differential interactions between early life experiences and gender are inconsistent. This variability may partly be due to the diversity of procedures employed (e.g., different strains, maternal separation paradigms, and/or duration of separation) across the various studies.

In contrast to brief handling, longer periods (>2 h) of daily maternal separation are reported to increase HPA responsivity to stressors.[15,254,272,301,457,481,487] In this respect, maternal separation is associated with increased CRH receptor binding at extra-hypothalamic sites, including the raphé and parabrachial nuclei.[254] Moreover, relative to NH or H rats, the Maternal Separation (MS) rats displayed greater stressor-elicited rise in plasma ACTH and corticosterone, decreased hippocampal and cortical glucocorticoid receptor binding,[481] and increased hypothalamic CRH mRNA expression.[380]

It has been suggested that changes in HPA reactivity associated with early life experiences have implications for individual differences in responses to subsequent environmental challenges and may contribute to psychiatric disorders.[254,301] In addition to the induction of anxiety-related disturbances, there is some clinical evidence suggesting that early life experiences may also influence ingestive behavior and may be an important etiological factor in the later development of eating disorders.[236,294]

Much of the pharmacological research on the treatment of eating disorders has focused on the potential serotonergic hypothesis of bulimia and anorexia nervosa.[1]

However, it may also be important to consider the possibility that altered responsiveness to putative satiety peptides may be clinically relevant in this respect. Indeed, some of these satiety peptides (e.g. CCK and BN-LPs), can not only suppress food intake,[151,156] but can also elicit anxiety.[55] It was thus of interest to establish whether early life experiences would impact on the ingestive responses elicited by a variety of putative satiety peptides. The suggestion that neonatal experience influences feeding behaviors is reinforced by anecdotal evidence suggesting that early life experiences and/or stress history may contribute to the various ingestive disorders such as anorexia nervosa[236,294] and obesity.[96] In brain regions fundamental to the regulation of both anxiety and feeding responses (e.g. the CeA[139]) variations of CRH and BN-LPs have been detected in response to both appetitive and stressful conditions.[311]

The aim of the present investigation was to characterize the effects of early manipulations of mother-pup interactions on ingestive responses among male and female rats. Specifically, we assessed the effects of neonatal handling and maternal separation on 1) the developmental changes in weight gain, 2) anxiety responses, 3) propensity to consume palatable "snack" food and 4) the feeding-suppressant effect of various satiety peptides (CCK, BN, and amylin).

Materials and Methods

Subjects

Male and female Sprague-Dawley rat pups were bred and reared in our laboratory colony from experienced Sprague-Dawley females (Charles River, St.

Constant, Quebec). On the day following birth (PD-1), litters were randomly assigned to NH, H and MS groups. Litters of the NH group were left undisturbed throughout the first 21 days of life. The remaining pups were removed from the home cage, pooled, their sex determined, and then randomly assigned to sex specific H and MS groups (n = 10 each). Dams had *ad libitum* access to food (Purina Lab Chow) and water. All cages were cleaned once per week by removing half of the soiled wood chip bedding and replacing it with clean wood chips without disturbing the pups. The colony room was maintained at 20°C with relative humidity of 60% on a 12 hour light-dark cycle (lights on at 0700 hours). All experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care, and subjected to review by the University Animal Care Committee.

Neonatal handling and maternal separation treatment

Pup manipulations (handling and maternal separation) were conducted once daily (between 0800 and 1100) from postpartum day 2 through postpartum day 21. *Handling* consisted of removing the dam from the nest, picking up each pup, weighing it, gently stroking it dorsally from head to tail for 15 seconds, and then placing it into a cup lined with wood chip bedding. This cup was placed within an incubator at 32-33°C for the first 2 weeks and 20°C during the 3rd week. Following a 15-min period, pups were again stroked for 15 seconds, and returned to the nest, after which the dam was reunited with her pups. *Maternal separation* involved removal of the dam from the nest, followed by individual pup removal and weighing. The pups were then individually placed in wood-chip lined cups (as above) for 180 min, after which time they were returned to the nest.

This was followed by the return of the dam. The NH group of animals was left undisturbed throughout the 21 days of treatment, and was not weighed in order to preclude any effects of tampering with the pups. On postpartum day 22 all animals were weaned and placed in same sex, same treatment groups of 3-4 animals per cage until they were tested on the elevated plus maze, after which they were housed individually.

Neonatal pup weights

As previously noted, H and MS pups were weighed daily from postpartum day 2 through postpartum day 21. All pups were weighed when weaned at postpartum day 22, as well as at postpartum day 29, 40, 50 and 62.

Elevated plus maze test

When pups were approximately 5 weeks old, they were tested on the elevated plus maze. The elevated plus maze consisted of two opposing open arms (50 cm x 10 cm with a 5 mm clear Plexiglas lip), an open 10 x 10 cm square in the center, and two opposing closed arms (50 cm x 10 cm with 40 cm high walls). The entire maze was elevated 60 cm from the floor. Initially, each pup was placed in an open field (90 cm x 90 cm with 15-cm high walls) for 5 minutes. Immediately thereafter, the pup was placed in the center of an elevated plus maze facing one of the open arms of the maze. The number of entries to and the time spent in each arm of the maze, as well as in the center square of the maze, were recorded during a 5-min test period.

Ingestion of palatable snack

Starting at 6 weeks of age (2-3 days after the plus-maze test), non-food deprived animals (n = 8/group) were offered a high carbohydrate, palatable "snack" of Graham Wafers (Christie®, 6.7 g/wafer, 71.6% CHO) in their home cage for 30 min, between 0900 and 1100 hours, for a period of 7 consecutive days.

Response to exogenous administration of BN

The same animals, now familiarized with Graham Wafers (age 7 weeks) were used to assess the effects of BN administration. Each animal was injected with 4 doses of BN (0 (Saline), 4, 6, and 8 μg/kg, intraperitoneal), over 10 days, in a randomized Latin Square design. BN (Bachem, CA) was freshly dissolved in saline immediately prior to administration (between 0900-1200). Approximately 10 min following the injection, animals were presented with the Graham Wafer snack for 0.5 hour, and the amount consumed during the 30-min period was recorded.

Response to CCK and amylin

Two days after completion of the BN tests (discussed above), animals were assessed for their response to CCK 8 sulphated (CCK-8s) and amylin. All peptides were freshly dissolved in saline. Animals first received an injection of CCK-8s (4 mg/kg, intraperitoneal), and offered the Graham Wafer snack as above. Two days later, rats were injected with amylin (4 mg/kg, intraperitoneal) and the Graham Wafer test was repeated.

Statistical Analysis

Weight changes in the pups during the pre-weaning period (postpartum Day 2-21) were compared by a mixed design analysis of variance with daily weights considered as a within measure, and group (handling condition) as a between factor. Post-weaning and adult weight differences at various time-points (postpartum 22, 29,40, 50 and 62) were analyzed individually (using ANOVA with group as a between factor) due to the large between-day variability. Graham Wafer intake across 7 days was analyzed as a repeated measures ANOVA with both early life handling/separation and sex considered as between subjects' variables, and daily consumption as a within subjects' variable. The anxiety measure (plus maze performance) was analyzed by 2-way ANOVA with sex and early-life experiences used as independent variables. The response to each dose of BN was analyzed using a repeated measure (Latin Square) ANOVA, with handling as the between group measure. The responses to single doses of CCK, amylin or BN (from previous study) were analyzed using a separate ANOVA. Finally, all the snack intake data (including BN dose response study as well as the response to other peptides) was re-analyzed after conversion or expression of values as a percentage of appropriate saline values (considered as 100%). Unless otherwise stated, all post hoc analyses were performed using the LSD test with Bonferroni's correction.

Results

Effect of neonatal manipulation on body weight gain

The H and MS groups were weighed daily, while the NH group was left undisturbed (by design) until after weaning. Body weight over the first three weeks of

life varied as a function of the Treatment (Handling and Maternal Separation) x Day (Age) interaction for both males and females ($F_{20,350} = 16.29$, $p<0.0001$ and $F_{20,377} = 4.24$, $p<0.0001$, respectively). As can be seen in Figure 1, all rats gained weight over the first 3 weeks of life, but the rate of weight gain in the MS pups was slower relative to their H counterparts. Initial (postpartum day 1) pup weights of the H and MS groups were similar (mean weights ± S.E.M. for each group: male Handled = 7.22 g. ± 0.21, male MS = 6.21 g. ± 0.23, female H = 6.83 g. ± 0.22, female MS = 7.17g. ± 0.19). However, starting on postpartum day 9 for females and postpartum day 11 for males, the pups from the MS condition weighed significantly less than their H counterparts (for both sexes, $p<0.05$). This difference in weight gain was robust and persisted up to weaning (see Figure 1) for both sexes, ($p<0.01$) and beyond (see Figure 2).

Following completion of the 3-week treatment regimen, the rats were weaned (on postpartum day 22), and weights of all 3 groups recorded on postpartum day 22, 29, 40, 50 and 62. At the end of the treatment period (postpartum day 22), analysis showed a significant Treatment effect (males: $F_{2,33} = 40.24$, $p<0.0001$; females: $F_{2,33} = 12.29$, $p<0.001$). Tukey post hoc analysis revealed that sex-matched pups of the H and NH groups weighed the same, whereas animals exposed to the MS condition weighed significantly less than both sex-matched H and NH cohorts (for both sexes, $p<0.01$). Thus, the pre-weaning weight difference noted between the NH and MS groups (see Figure 1) was attributable to a diminished weight gain in the MS condition rather than enhanced weight gain in the H condition.

Overall analyses of the post-weaning body weight changes revealed significant Treatment for males ($F_{2,128} = 29.79$, $p>0.0001$) and females ($F_{2,144} = 17.62$, $p>0.0001$). Individual analyses at specific days revealed that H and NH males gained weight almost at the same rate, whereas the MS males continued to weigh less than the H males throughout the experiment ($p<0.01$ - 0.05, see Figure 2 for details). Similar analyses on the female weight gain profile revealed that, like the males, there were no differences between the H and NH conditions, however, the MS animals weighed significantly less than the NH up to postpartum day 40 ($p<0.01$ - 0.05) and less than H group at postpartum day 22 ($p<0.01$), postpartum day 29 ($p<0.05$) and postpartum day 62 ($p<0.05$).

Effect of neonatal manipulation on elevated plus maze behavior

The time spent in the open arms of the elevated plus maze (expressed as percent of total time in the maze) revealed a significant Group effect ($F_{2,58} = 10.3$ $p<0.0001$). Post hoc analysis indicated that the male and female H as well as female MS animals spent significantly more time in the open arms of the maze in comparison to NH animals (male H: $p<0.05$; female H and MS: $p<0.01$). Female MS animals also spent significantly more time in the open arms of the maze compared to the male MS counterparts ($p<0.05$), (see Figure 3, upper panel). Thus, although the activity level (total number of entries into the open or closed arms of the maze) did not vary significantly across the treatment conditions, H animals (of either gender) or MS females spent significantly more time on the open arms of the maze, once there.

Effects of early experience on consumption of an unfamiliar palatable snack

Rats with differential neonatal histories were presented with a novel palatable snack for 7 consecutive days. The male rats consumed more of the snack than did the females. However, when intake was expressed as a percentage of body weight, the difference in the snack intake between the males and females was not significant ($F_{1,42}$ = 1.53, p = 0.22), and, as there were no gender-based differences, the data were analyzed separately. The analysis of snack intake (grams consumed) in males, revealed a significant Treatment ($F_{2,19}$ = 8.68, p = 0.002) and Days ($F_{6,114}$ = 105.99, p<0.0001) effect. As can be seen in Figure 4, all groups were equally neophobic to the novel snack upon the initial exposure (as revealed by small and similar amounts of new food consumed). However, all animals increased their intake of this snack over the course of 7 consecutive days. In the males, H animals ate consistently more than the NH ones on test day 2 (p<0.05) and also on days 4 to 7 (p<0.01), and more than the MS males from day 4 onwards (p<0.01). The MS and NH males ate similar amounts (see Figure 4). Among the females, consumption also varied as a function of the Treatment ($F_{2,19}$ = 4.16, p = 0.03) and Days ($F_{6,144}$ = 90.15, p<0.0001). Post hoc analysis revealed that the MS females consistently ate more of the snack than NH cohorts from test day 2 onward (p<0.05 - 0.01, see Figure 4). Like in the case of the males, the H females ate significantly more than the matched NH controls on day 2 (p<0.05) and from days 4 to 7 (p<0.01, see Figure 4).

Dose-related effect of BN on palatable snack consumption

In the males, BN administration affected snack intake in a dose related manner ($F_{3,60} = 15.8$, $p<0.0001$). However, the response to BN was not significantly influenced by the neonatal treatment they had received ($F_{2,20} = 3.41$, $p=0.053$). Post hoc analyses revealed that BN suppressed food intake in all animals in a dose-related manner, irrespective of their early treatment. Significant differences were detected starting at the 6 μg of BN ($p<0.05$) (see figure 5). At the 8 μg dose, whereas BN significantly suppressed intake in NH and H males ($p<0.05$), it failed to affect intake in the MS group.

In the females, there was a significant effect of BN Dose ($F_{3,63} = 6.75$, $p<0.001$) and neonatal Treatment ($F_{2,21} = 6.35$, $p<0.01$), but no Interaction effect. Although BN suppressed snack intake in all groups, the relationship between the BN dose and the extent of suppression was variable. Post hoc analyses revealed that BN-elicited suppression in the NH females was significant only at the 6 μg dose ($p<0.05$). Furthermore, as shown in Figure 5 (lower panel), the response to BN in the H females was significant only at the highest dose ($p<0.05$) whereas the MS females were significantly suppressed at the 4 and 6 μg doses ($p<0.05$) but not at the 8 μg dose (see Figure 5).

Comparison of the feeding-suppressant effects of amylin, CCK and BN

Data from animals treated with CCK or amylin were analyzed using ANOVA. For comparison purposes, BN data from the above experiment (for the 6 μg dose only) was included in this analysis. In the males, ANOVA revealed a significant Drug

(peptide) x Neonatal Treatment Interaction for the snack intake ($F_{6,60} = 3.59$, $p<0.01$). Post hoc multiple comparisons indicated that among males, BN (6 μg/kg), CCK (4 μg/kg) and amylin (4 μg/kg) each significantly reduced the snack intake, irrespective of the early-life manipulation ($p<0.05 - 0.01$; for details see Figure 6, upper panel). When the data were expressed in terms of percent suppression (with saline controls expressed as 100%; shown at the bottom of appropriate columns), ANOVA revealed a significant neonatal Treatment effect ($F_{2,21} = 4.79$, $p = 0.019$). Tukey post hoc analysis revealed that among the NH males, the feeding suppressant effects of the 3 peptides were roughly equivalent (suppressing intake by 23 – 37 %), whereas among the H and MS males, the suppression elicited by CCK (62% and 71%, respectively) was significantly greater than that elicited by BN ($p<0.05$).

In the females, there was a main Drug (peptide) effect on the snack intake ($F_{3,63} = 13.57$, $p<0.0001$), but no Treatment or Interaction effects. This was attributable to the fact that there was no statistical difference in the pattern of response to the 3 peptides, across the Neonatal Treatment conditions ($F_{2,21} = 0.74$, $p = 0.49$). Post hoc analyses revealed that BN suppressed snack intake in the NH ($p<0.05$) and MS ($p<0.01$), but not in the H females. CCK did not suppress intake in the NH group but markedly suppressed intake in the H ($p<0.05$) and MS ($p<0.01$) groups. Amylin was without effect on the NH and H groups but suppressed intake in the MS group ($p<0.01$) (see Figure 6).

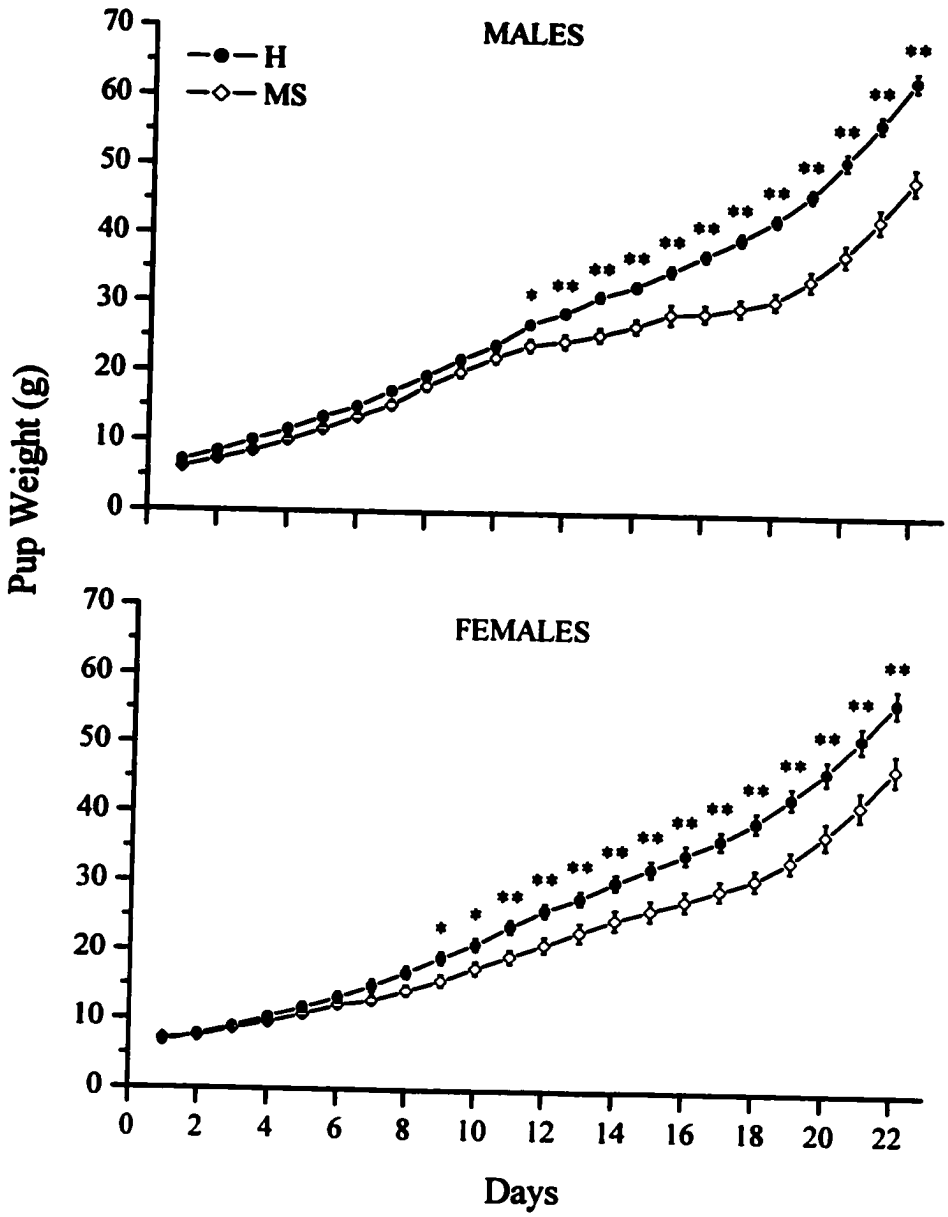


Figure 1: Effect of neonatal handling (H) and maternal separation (MS) on body weight gain, prior to weaning. Each value represents the mean ± S.E.M. of the body weight (g) of the males (upper panel) and females (lower panel), at various post-partum (PD) days. The H groups are depicted by filled circles and the MS groups by open diamond-shaped symbols. *, ** Significant age-matched group differences at $p<0.05$ and $p<0.01$, respectively.

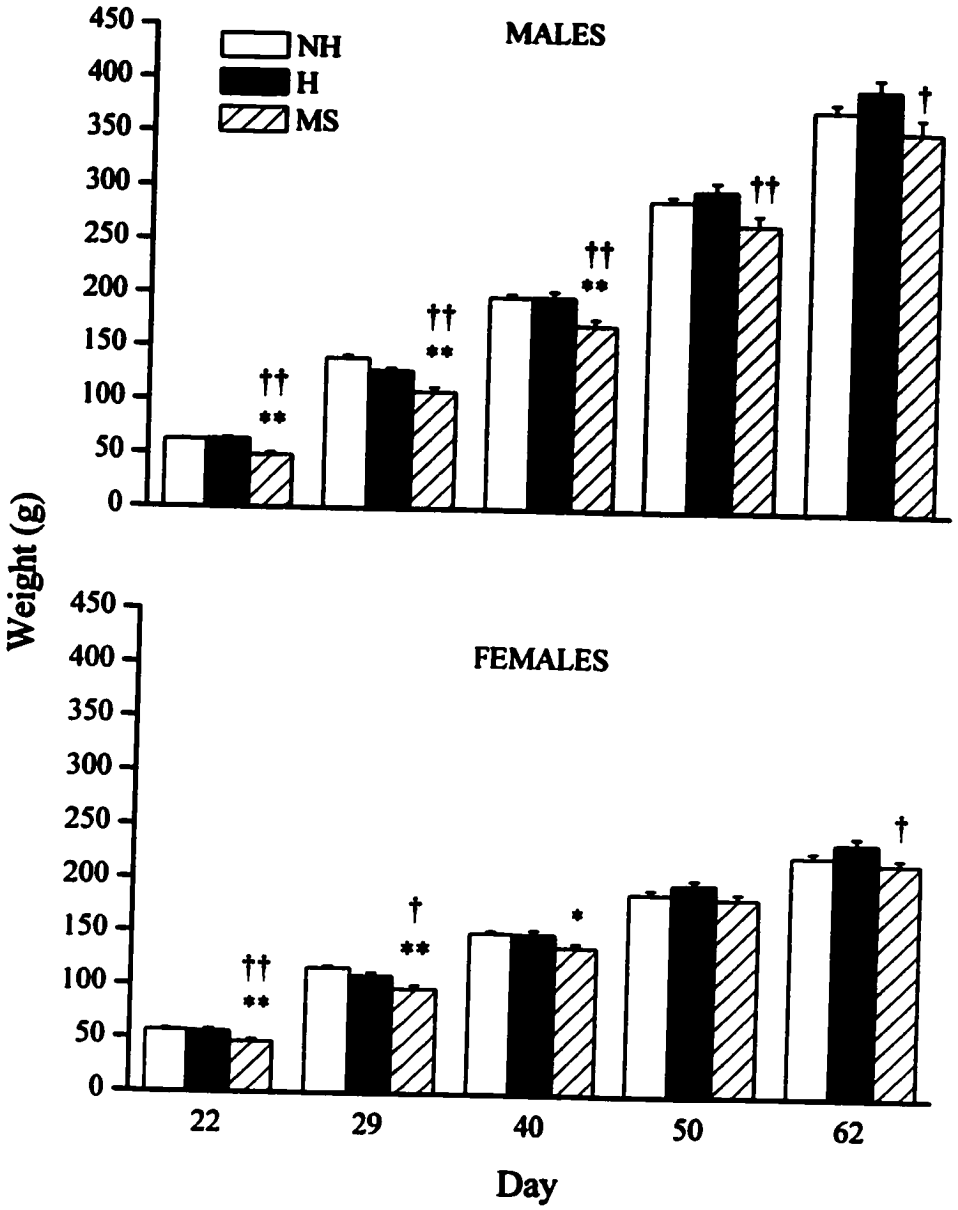


Figure 2: Effect of neonatal manipulation on weight post-weaning development. Each column represents the mean ± S.E.M. of the weight (g) over days, of male (upper panel) and female (lower panel) rats. These rats were previously subjected to handling (H: solid columns), maternal separation (MS: hatched columns) or non-handled control (MH: open columns) conditions. The abscissa depicts the days on which the rats were weighed. *, ** Significantly different from NH at $p<0.05$ and $p<0.01$ respectively. †, †† Significantly different from H at $p<0.05$ and $p<0.01$ respectively.

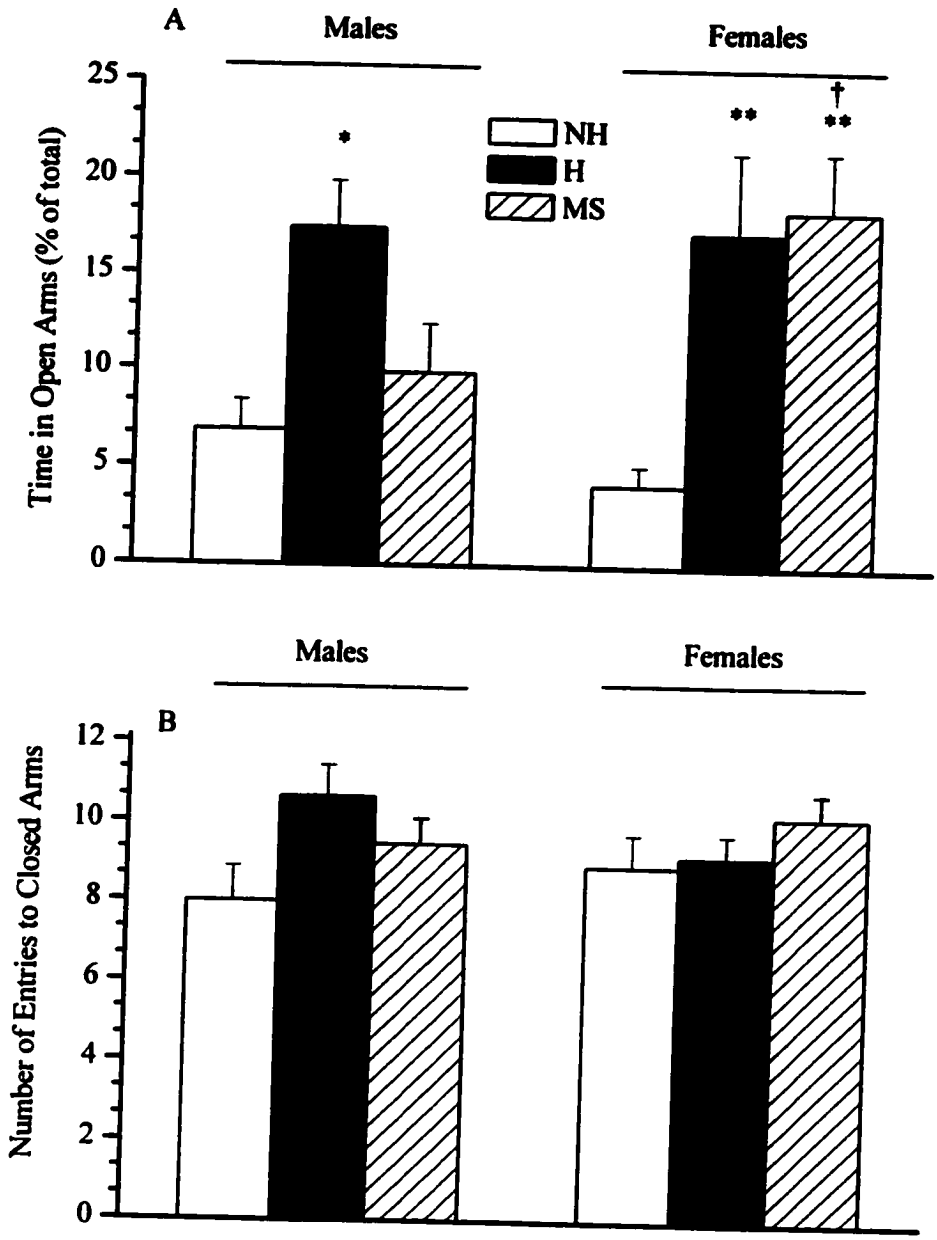


Figure 3: Effect of neonatal manipulation on elevated plus maze behavior. Each column represent the mean ± S.E.M. of time (expressed as % of total test time) spent in the open arms (upper panel) and number of entries made to the closed arms (lower panel) of the elevated plus maze by male (left panels) and female (right panels) rats. Rats neonatally exposed to NH condition are represented by open columns, those exposed to MS condition by hatched columns, and those exposed to H condition by solid columns. *, ** Significantly different from sex-matched NH group at $p<0.05$ and $p<0.01$, respectively. † Significantly different from male MS at $p<0.05$.

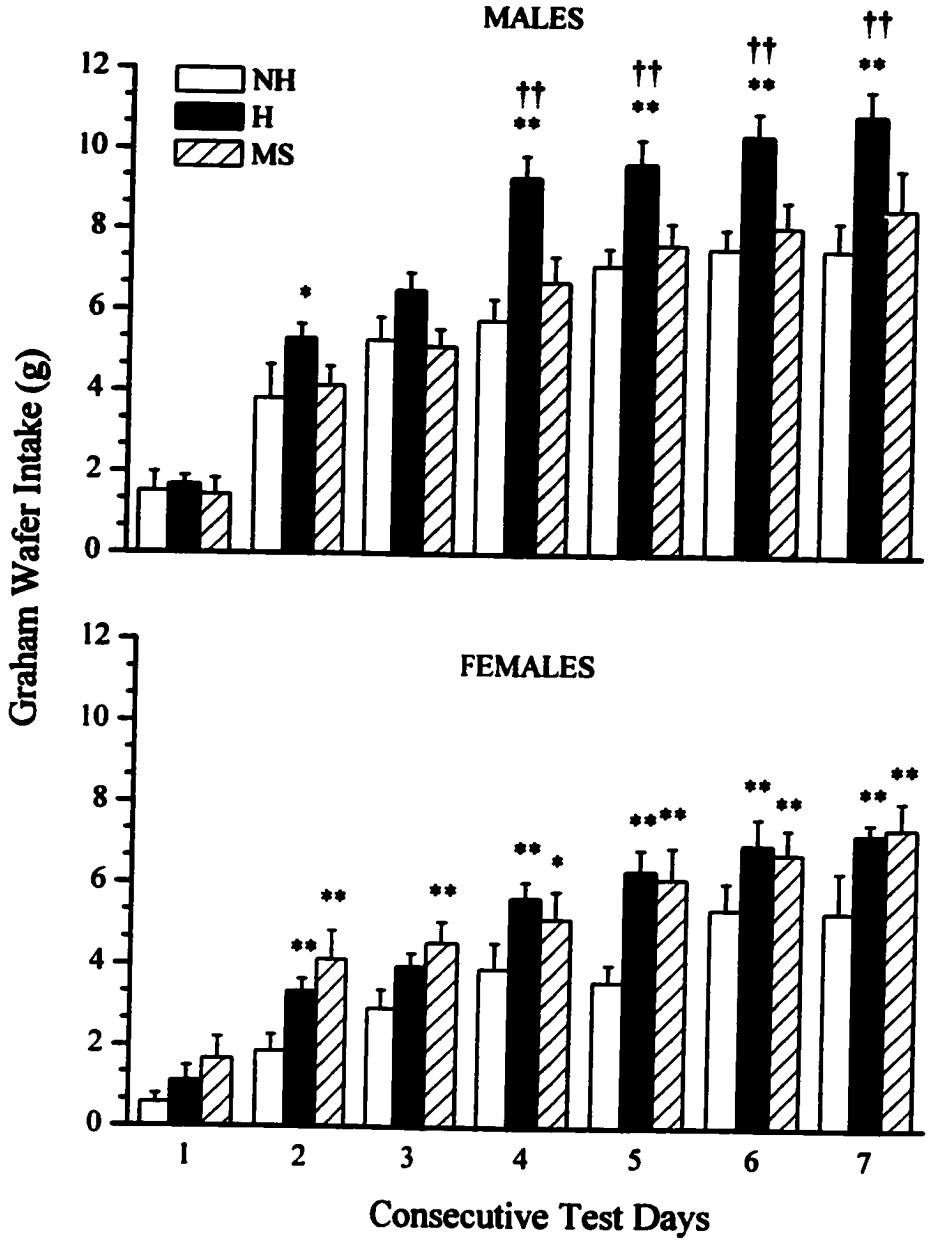


Figure 4: Effect of neonatal manipulations on consumption of an initially unfamiliar (novel) palatable snack (Graham Wafers). Each column represents the mean ± S.E.M. of the snack consumed (g) by males (upper panel) and females (lower panel), over 7 consecutive test days. Open columns represent the NH rats, hatched columns represent the MS group and solid columns represent the H group. *, ** Significantly different from time-matched NH group at $p<0.05$ and 0.01, respectively. †, †† Significantly different from time-matched MS group at $p<0.05$ and $p<0.01$, respectively.

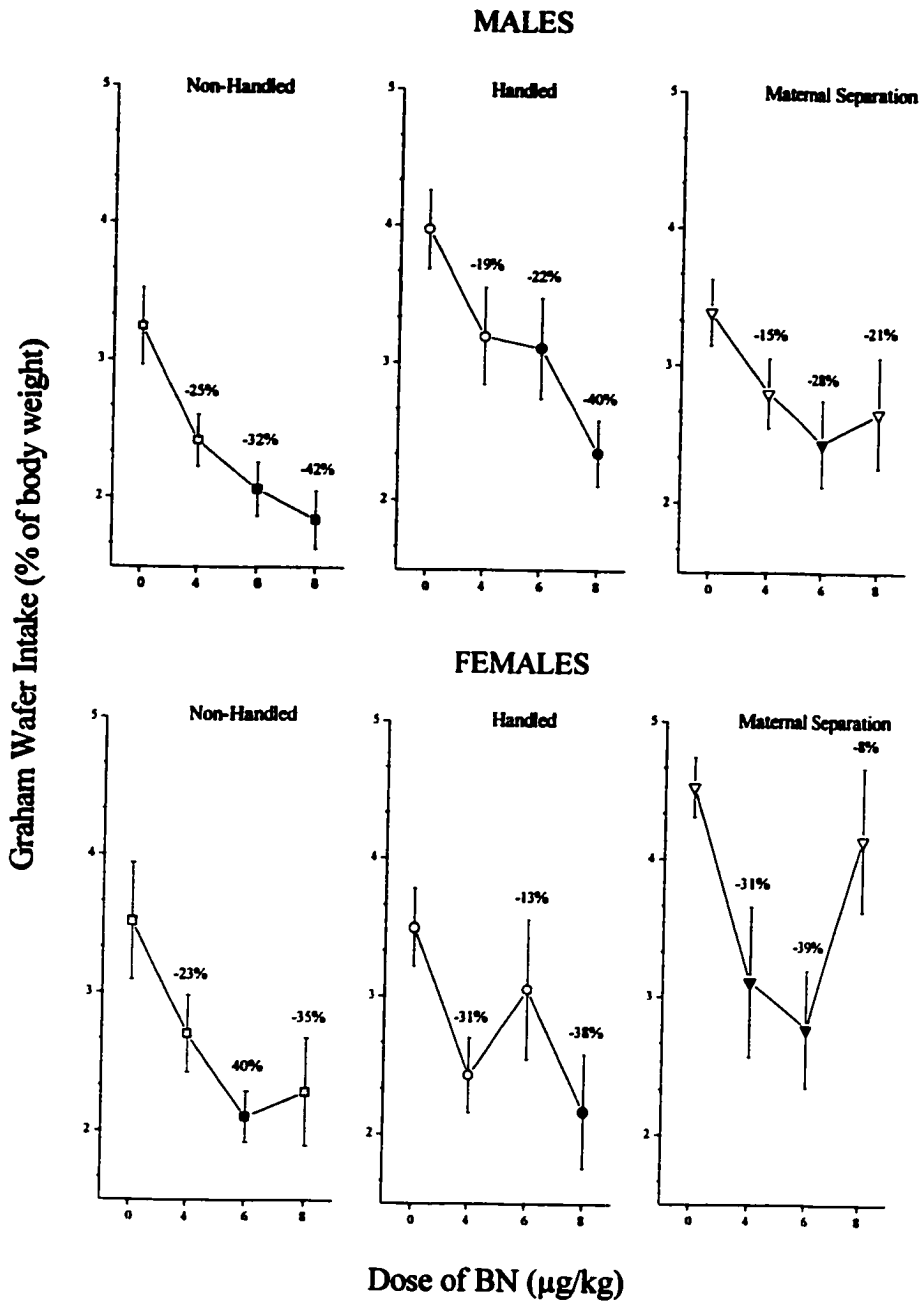


Figure 5: Effect of BN on snack consumption: Each point represents the amount of snack consumed (expressed as a percent of body weight) ± S.E.M, as a function of BN dose (depicted on the abscissa). The left, center and right panels depict the dose-response function of the NH, H and MS males (upper panels) and females (lower panels), respectively. Closed symbols represent a significant difference from the group- and sex-matched control condition (BN 0 µg/kg.), at $p<0.05$.

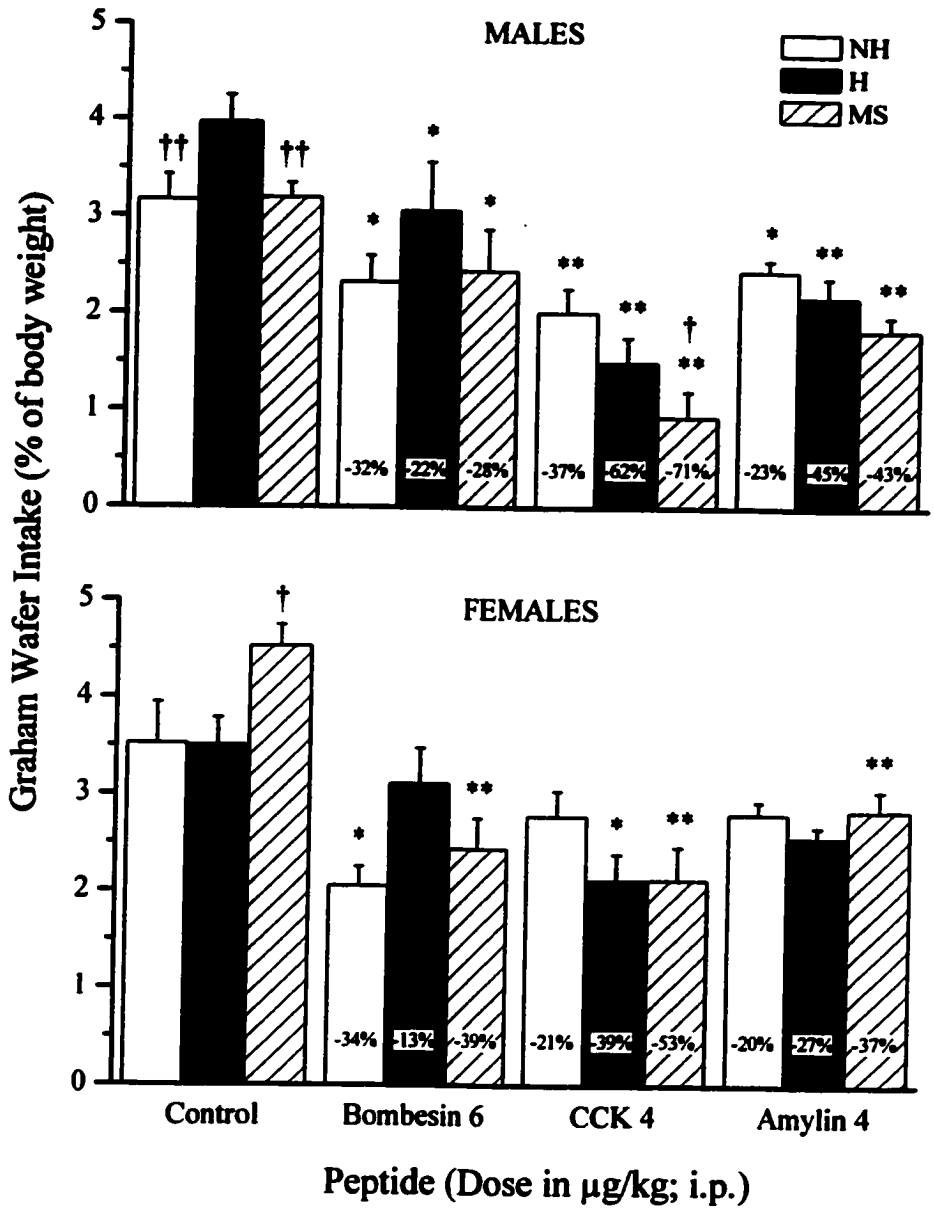


Figure 6: Effect of BN, CCK and amylin on snack consumption. Each column represents the mean ± S.E.M. of Graham Wafer intake (expressed as percent of body weight), following administration of Saline (control), BN (6 μg/kg; intraperitoneal), CCK-8s (4 μg/kg; intraperitoneal), or amylin (4 μg/kg; intraperitoneal). *, ** Significantly different from sex and group matched control (saline injection) condition, at $p<0.05$ and $p<0.01$ respectively. †, †† Significantly different from specific peptide-matched H condition at $p<0.05$ and $p<0.01$, respectively.

Discussion

It has been shown that rat pups exposed to brief periods of handling/separation from their dams and cohorts during the first 3 weeks of life exhibit less fear and/or anxiety in novel environments, later on in life.[128,129,193,271,301,346] This has been interpreted to reflect enhanced ability to cope with stressful events and, thus, reduced vulnerability to stressor-induced behavioral impairments or pathologies.[89,301] The results of the present investigation demonstrate that H animals (both male and female) displayed less anxious behavior than their NH controls on the elevated plus-maze, which is concordant with earlier reports using a similar paradigm.[47,129,346] Although it had been hypothesized that the MS animals would be more anxious than their NH or H cohorts,[11,304] this was found not to be the case. In fact, the female MS animals displayed significantly less anxious behavior than the sex-matched NH control group.

Given the known endocrine profile of the MS rats, the finding of reduced anxiety in this group was unexpected. It will be recalled that, in comparison with NH rats, animals subjected to prolonged periods of maternal separation exhibited increased stressor-elicited rise in the circulating ACTH and corticosterone levels.[15,254,301,457,481] In terms of gender specificity of early stressor effects, it is of interest to note that maternal separation (24-hour in duration at postpartum day 3) induced a down-regulation of the glucocorticoid receptors in the males but a glucocorticoid receptor up-regulation in the females.[461] Since the glucocorticoid receptors are critical in determining HPA reactivity,[301] a more efficient HPA system in the female MS animals may account for the less inhibited behavior on the elevated plus maze. In the absence of the appropriate

endocrine measurements, however, this contention remains speculative. It is also likely that other factors specific to our paradigm, such as some stimulation (associated with pup pick-up and weighing) and incubator-associated body temperature maintenance may have favored a reduction in anxiety.

In the present study, we found that not only was anxiety affected by the early-life manipulations, but so were the consummatory behaviors and gains in body weight. During the first 21 days of life, both male and female MS pups failed to gain weight at the same rate as their H counterparts. This reflected the failure of the MS animals to thrive, rather than accelerated growth and development of the H animals, as the NH controls (which were first weighed only at postpartum day 22) weighed the same as the gender-matched H groups. During the course of post-weaning development (from postpartum day 22 to postpartum day 62), the MS gained enough weight to catch-up with the NH controls, but not with the H rats (which tended to weigh more). As seen in Figure 2, this pattern was more robust in the males as compared to the females. There have been conflicting reports concerning the effect of neonatal handling on body weight.[476] One hypothesis forwarded to account for the reduced weight gain in the MS animals, suggests that mild hypothermia (resulting from the separation from the dam and cohorts) enhances levels of thyroid hormones (thyroxine and triiodothyroxine), which enhance fat metabolism.[96] However, as the environmental temperature was regulated in the present study, this explanation appears untenable.

An alternative hypothesis accounting for the weight gain differences is that separation (in excess of 2 hours) may suppress circulating growth hormone levels, whereas an opposite effect is elicited by handling.[304] Thus, lower levels of growth hormone could potentially affect development of the MS pups during the critical growth period. It also remains possible that early life manipulations may have affected dam-pup interactions and/or availability of maternal milk.[476] In fact, a recent study has confirmed that dams briefly separated from their pups subsequently spent more time licking and grooming the pups, and engaged in longer periods of arched-back nursing.[276,411]

The early-life manipulations also influenced the consumption of a palatable snack during adulthood. Although all groups were equally neophobic on the initial encounter with the novel snack (Graham Wafers), they markedly increased consumption of this snack over the 7 consecutive test days (30 minutes availability/day). The most striking observation was that, once habituated, the H animals (of both genders) consumed significantly more of the snack than the gender-matched NH controls. Although the effects of H were qualitatively similar in the males and females, the effects of MS were gender specific. Whereas MS condition failed to influence snack consumption (compared to NH controls) in the males, it markedly increased snack consumption in the females. Indeed, the MS females consumed as much of the snack food as the H females.

The mechanism(s) contributing to the early-life stimulation (or handling) induced enhancement of snack consumption in adulthood remain unknown. However, the effects of early-life manipulations on the CRH system(s) may be important in this context.

Meaney and colleagues[301,380] have found that CRH expression is reduced in adult animals subjected to early-life stimulation or handling, which they attributed to enhanced glucocorticoid negative feedback system. Since CRH is released during feeding[311] and CRH also suppresses food intake,[392] it remains possible that the potentially blunted CRH release in H animals may account for the enhanced snack consumption (or attenuated suppression of snack intake). Such a mechanism, however, may not account for enhanced snack consumption noted in the female animals that were deprived of maternal/cohort contact for 3 hours/day during first 3 weeks of life. That is because animals so treated, exhibited increased (rather than decreased) CRH mRNA expression in the hypothalamus, compared to NH rats.[380] Yet, as will be recalled, the snack consumption in the MS and NH rats was similar in the males, whereas in the females the consumption of MS was enhanced and resembled that of the H females. However, as increased mRNA expression does not necessarily translate to increased peptide release or utilization, the potential involvement of the CRH system can not be ruled out as yet. It would be of interest to test this contention using more direct analyses, such as *in vivo* microdialysis combined with RIA. Furthermore, other neuronal systems may also be important in this phenomenon. For instance, alterations in the 5-HT turnover have also been observed following early life manipulations,[301] which may be significant as serotonergic mechanisms are important in the regulation of food intake.[501] Thus, the serotonergic and other neurotransmitter systems may also be involved.

Termination of food intake is believed to be initiated and/or coordinated by so-called "satiety" peptides, which are released from the gut in response to food. These

peptides are thought to eventually invoke brain mechanisms, which limit further ingestion.[155] There are several of these putative satiety peptides, including amylin, CCK and BN-LPs. In the current study, we were interested in assessing whether changes in ingestive behavior elicited by early-life manipulations may somehow be related to altered responsiveness to these satiety-eliciting peptidergic signals. We report that exogenous administration of BN suppressed food intake in a dose-related manner in all male groups, and the pattern of suppression was similar between the groups. Furthermore, MS females were extremely sensitive to the lower doses of BN, but insensitive to the highest dose, resulting in an inverted-U dose-response. The response of the H females was erratic, showing suppression at the highest dose, but not the middle two doses.

The mechanism(s) responsible for the differential effects seen in females is not immediately evident. However, it is known that feeding in females varies as a function of their estrous cycle.[145] Since the estrous status was not monitored in the present study, the potential contribution of the gonadal hormone fluctuation to the females' snack consumption can not be determined. Further, it is possible that differences in glucose metabolism that occur between H and NH[476] may also have contributed to the gender differences, and need fuller assessment.

Our data indicate that early life experience does indeed influence subsequent feeding-suppressant responses to CCK. However, these changes were not in the anticipated direction. Because the H animals consumed more of the palatable snack than the NH controls, and tended to weigh more at maturity, it was expected that the CCK

effects would be blunted in the H animals. However, an opposite effect was found, suggesting that the differences between H and NH animals are not attributable to altered CCK sensitivity. While amylin effects were considered important, in light of its suggested role as a mediator of the satiety effects of BN and CCK,[279] the present data suggest that sensitivity to amylin may not contribute to the weight gain differences in H and MS animals.

Taken together, it appears that early life experiences differentially affect the potency and/or efficacy of various satiety peptides, and these effects may be gender-dependent. Of the three peptides assessed in the present investigation, only the trends in sensitivity to exogenous BN could be considered congruent with the weight changes associated with the different handling manipulations. However, as we did not measure 24-hour chow intake, it is not possible to distinguish between weight gain differences due to differential food intake versus that due to metabolic differences.

In summary, it appears that early life experience can produce long lasting effects not only on anxiety-related behaviors, as previously reported,[301] but also on physical growth, adult snack consumption and response to satiety peptides. It appeared as well, that the effects of early life manipulations on both weight gain and response to satiety peptides were gender-dependent. Some of these behavioral changes may be attributable to various neuroendocrine and/or neurochemical changes resulting from early-life manipulations. It is tempting to suggest that early-life experiences may also contribute to individual differences and/or eating disorders noted in humans; however, such

implications remain speculative. In this context, concurrent behavioral and neurochemical studies exploring the mechanism(s) surrounding the handling/maternal separation paradigm may shed new light on the etiology of feeding (patho)physiology and altered stress reactions associated with anorexia nervosa, bulimia, obesity and anxiety related disorders.

Preface to Chapter V

In addition to experiential variables addressed above, organismic factors can also impact upon behavioral responses. Research over the past few decades has highlighted the fact that, amongst other things, males and females may vary in their neurochemical responses to stressor exposure. In the next set of experiments, we compared the gender based differences in their propensity to consume food (Chow or a palatable snack). Furthermore, since food intake in females is influenced by their reproductive cycle, our experiments factored in the effects of estrus cycle.

Chapter V

Is "the Sweet Tooth" more of a female phenomenon?

Abstract

Typically, the bulk of behavioral and biological studies in rodents have been focused on the male gender, mainly because the female physiology and responses fluctuate according their estrus cycle. As a consequence, the potential gender differences are not well characterized, particularly as they pertain to the ingestive responses. Female rats have a 4-5 day estrus cycle characterized by four distinct phases, diestrus, proestrus, estrus and metestrus that can be differentiated according to their vaginal cytology. In the present investigation, we monitored the 24-hour consummatory behavior of male and female rats over a period of approximately 6 weeks. Non food-deprived rats were given a daily 1-hour access to a palatable, high carbohydrate "snack" (Graham Wafers) and rat chow pellets, concurrently. During this time the females' estrus cycle was monitored. At the end of one month, the snack was discontinued and 24-hr Chow intake was measured for an additional week. When food intake was expressed as a percent of body weight, females consumed almost twice as much of the Graham Wafers than the males. Furthermore, the females' 24-hour intake exceeded that of the males whilst regular access to the snack was available. However, when only Chow (without snack access) was available, this gender difference disappeared. Graham Wafer (snack) intake of the females decreased during the estrus phase of their cycle; however this effect only reached statistical significance during the first of two cycles. Taken together, these results suggest that female rats display a greater propensity to consume a palatable snack, compared to the males. This observation is of interest when considering ingestive disorders, such as obesity, anorexia nervosa and bulimia nervosa, which are more prevalent in females.

Introduction

Research over the past few decades has highlighted the fact that males and females can vary significantly in their behavioral and physiological responses to various challenges. For example, males and females have different patterns of food intake.[79] Whereas male rats consume approximately 65% of their total intake during the dark cycle, females eat 80% during that period.[260] Also, males maintain a constant intake by increasing meal size and decreasing meal number over time while females display fluctuations in both meal number and meal size that vary across their estrus cycle.[79,260]

The palatability of the food available might be an important determinant of how much will be consumed. It has been shown that, when given a choice, female rats generally prefer both sweet (sucrose) and salty (sodium, calcium) solutions to a greater extent than males[135] and consume more sweet solution than their male counterparts.[79,421,507] Thus females tend to prefer foods with higher carbohydrate content, as they tend to be more palatable.[507,508] In fact, one human study demonstrated that, although women perceived sweet snacks as unhealthy, compared to men, they rated them as more pleasant.[170,258] Also, when questioned about why they terminated a meal, women reported that they stopped eating "when the food stopped tasting good" (indicating the importance of hedonic factors) while men reported that they stopped "when the food was gone" (indicating the importance of external factors).[508]

These differences in food intake and taste responsivity could potentially be linked to the gender-based endocrine differences, particularly the sex hormones. The females'

cyclic fluctuations in their ovarian hormone levels in the rat (referred to as the estrus cycle),[79] are associated with fairly dramatic behavioral changes. For instance, during the estrus phase of the cycle (which occurs immediately following the release of the ovum), the female is sexually receptive to the male, is behaviorally more active,[143] and eats less food.[120,143,147,148,201,454] It has been suggested that estrodiol (the principal estrogen produced by the ovaries) may modify the taste and/or palatability of the food.[79] As such, the taste reactivity test demonstrated that female rats with high levels of estradiol displayed an increase in ingestive taste responses compared to male rats and females with low levels of estradiol.[79]

Most of the studies examining gender differences in palatable food consumption have used sweet tasting solutions such as sucrose and/or sweet carbohydrate mixtures such as polycose. In the current investigation we used a palatable Graham Wafer (Christie®) for which rats have shown a preference over other palatable snack such as cheese nips and chocolate chip cookies.[507] Thus, the purpose of the present investigation, was to 1) examine the consumption of both "bland" rat Chow and a "highly palatable" high carbohydrate "snack" over the course of the females' estrus cycle, 2) assess potential gender differences in both rat Chow and palatable "snack" intake and 3) compare the total food intake of males and females with the absence and presence of a palatable snack access.

Materials and Methods

Subjects

Male (n = 10) and female (n = 10) Long Evans rats, weighing 275-300 g and 150-175 g respectively, were obtained from Charles River (St. Constant, Quebec). Rats were housed individually in clear Plexiglas cages located in a sound attenuated feeding module maintained at 20° C with relative humidity of 60% on a 12-hour reversed light-dark cycle (lights off at 1200 hours). Rats had *ad libitum* access to water and to a pre-weighed (70-75 g) supply of food (Purina Lab Chow) between 1200 and 0800 hours (i.e. 20 hours per day). Animals were habituated to their test environment for 7 days, during which time they were individually handled (for about 5 min) each day. All experimental procedures followed the Canadian Council on Animal Care guidelines and were approved by the Research Ethics Committee of the University of Ottawa.

Tracking the females' estrus cycle

In order to monitor the estrus cycle of the female rats, vaginal swabs were taken each morning immediately prior to the "snack" period (between 1115 and 1145 hours). Animals were removed one at a time from their home cage and gently wrapped in a towel such that their heads and body were covered while exposing the vaginal opening. The saline soaked warm (approximately 37° C) cotton swab was inserted approximately 1.5 cm into the vaginal opening and gently rotated, to collect cells from the vaginal wall. The specimen was then transferred to an untreated slide, quickly air dried, and temporarily fixed in Cytoprep (Fisher Scientific).

Cells were subsequently stained using a modified Wright Stain technique, using the Diff-Quik® Stain Set (Dade). Specifically, the Cytoprep fixative was removed from the specimen by immersing the slides in 70% alcohol for 10 seconds. Cells were then re-fixed by dipping the slides 5 times for one second each time in Diff-Quik® Fixative Solution (1.8 mg/liter Triarylmethane Dye, 100% pure dye content (PDC) in methyl alcohol). The nucleoli and cytoplasm of the cells were stained by dipping the slides 5 times for one second each time first in Diff-Quick® Solution I (1 g/liter Xanthene Dye, 100% PDC, buffer and sodium azide (0.01%) as preservative) and Diff-Quik® Solution II (1.25 g/liter Thiazine Dye Mixture, 100% PDC & 0.625 g/L Azure A and 0.625 g/L Methylene blue in buffer). Slides were then cover-slipped using Permount (Fisher Scientific) as the mounting medium.

Slides were visually inspected each day using bright field microscopy in order to determine the phase of each female's estrus cycle for a particular day and the rhythm of the estrus cycle over the entire period of the study (40 days). During diestrus, cells are irregularly shaped and have large distinct nuclei. Often this stage is marked by the presence of leukocytes. During proestrus cells are spherical, have full round nuclei and are so abundant that they tend to cluster together. Estrus is marked by the proliferation of cornified, irregularly shaped epithelial cells frequently clumped together in thick, large sheets. Following estrus the animal enters metestrus, characterized by a proliferation of leukocytes (neutrophils) and large, irregularly shaped squamous epithelial cells.

Feeding protocol

At 0800 hours each morning, animals were weighed and the unconsumed Chow (from the previous 20 hours) was weighed to determine the intake over the preceding 20 hours. At the onset of the dark cycle (1200 hours), rats were given a 1 hour access to Purina Lab chow pellets (4-5 g each) and Graham Wafer biscuits (Christie®, 6.7 g/wafer, 71.6% CHO; 2.5 g). At the end of the one hour "snack" period, all leftover Graham Wafers and Lab Chow pellets were removed from the cages (under red light, thus not interrupting the animals' dark cycle) and measured. Rats then received their pre-weighed overnight (19-hour) supply of Lab Chow.

In order to assess the gender-based differences in the intake of just Lab Chow (i.e. in the absence of choice), the access to the "snack" was discontinued, and Chow intake was monitored for a 7-day period. Uneaten Lab Chow was removed each morning at 0800 and replaced with a pre-weighed (70-75 g) portion of Lab Chow. The females' estrus cycle was not monitored during this time.

Statistical Analysis

All results are expressed as a mean ± S.E.M. In order to compare the food intake of the male and female rats, all feeding data are expressed as a percentage of body weight. Male and female food intake was then analyzed using mixed measures ANOVAs with Gender as the between group factor and Day as the within-subjects factor. The Huynh-Feldt correction was applied to each analysis. Where there was a significant interaction, post hoc comparisons were conducted for the effect of Group using a one-

way between-subjects ANOVA (with Day as the within-subject factor and Gender as the between group factor) followed by the Tukey HSD test.

In order to analyze the "snack" intake of the females across the estrus cycle, the Graham Wafer and Lab Chow intake were averaged separately over each phase of the cycle. Food intake was then analyzed across two cycles using a mixed measures ANOVA with Phase as the between group factor and Day as the within-subjects factor. The Huynh-Feldt correction was applied to each analysis. Where there was a significant interaction, post hoc comparisons were conducted for the effect of Phase using a one-way between-subjectsANOVA (with Day as the within-subject factor and Phase as the between group factor) followed by the Tukey HSD test.

Results

Graham Wafer intake of male and female animals during the 1-hour snack period:

Figure 1 depicts the Graham Wafer (circles) and Lab Chow (squares) intake of male (filled symbols) and female (open symbols) rats during the daily 1-hour snack period. Although the Mixed measures ANOVA of the quantity of Graham Wafers consumed by the males and females during the 1-hour "snack" period did not reveal a statistically significant Gender x Day interaction ($F_{10,169} = 1.83$; $p = 0.06$), post hoc tests were conducted on the *a priori* predictions that had been made. These comparisons indicated that there was a robust main effect for Group ($F_{1,18} = 29.45$; $p<0.001$). The one-way ANOVA comparison for the simple effect of group revealed a significant difference between the males and females at each time point. As seen in Figure 1, when

the quantity of food consumed was expressed as a percentage of their body weight, the female rats consumed more of the Graham Wafer snack than the males.

Chow intake of male and female animals during the 1-hour snack period:

Also depicted in Figure 1 (open and closed squares) is the amount of Lab Chow consumed during the daily 1-hour snack period. Although the interaction was not significant, once again, post hoc tests were conducted on the *a priori* predictions that had been made. These comparisons indicated that there was a main effect for Group ($F_{1,18}$ = 12.98; $p<0.01$). The one-way ANOVA comparison for the simple effect of group revealed that males consumed more Lab Chow than females on Days 1, 4, 8, 11, 12, and 15 (see Figure 1).

Male and female animals' 24-hour intake with and without a snack period:

Figure 2 depicts the intake of male (closed circle) and female (open circle) rats during the 24-hour period in the presence (upper panel) and absence (lower panel) of a palatable snack. Mixed measures analysis of the rat chow consumed revealed a significant Days x Group interaction ($F_{7,124} = 2.48$; $p<0.05$). The one-way ANOVA comparison for the simple effect of group revealed that, after the first day of snack consumption, and with the exception of day 3, the females had a significantly greater total intake compared to the males (see Figure 2, upper panel). At the end of the experiment, the snack period was discontinued and the 24-hour Lab Chow intake was monitored for 7 days. The difference between the males and females disappeared once the snack period was discontinued (see Figure 2, lower panel).

Determination of estrus cycle:

Visual examination of vaginal smears under bright field microscope revealed that all females displayed a regular 4-5 day estrus cycle (with the exception of one female that exhibited a 6 day cycle). The cycle was clearly characterized by four major phases: diestrus, proestrus, estrus and metestrus.

Influence of estrus on Graham Wafer and Lab Chow intake of females during snack period:

Figure 3 depicts the intake of the females during the 1-hour snack period across the estrus cycle. Graham Wafer intake was reported according to the day of the estrus cycle. Although the interaction was not significant, post hoc tests were conducted on the *a priori* predictions that had been made. Mixed measures ANOVA of the quantity of Graham Wafers consumed during the snack period revealed a significant effect for Phase of cycle ($F_{3,54} = 6.53$ $p<0.01$). The Bonferroni-corrected Paired T-Test ($\alpha = 0.017$) for the first estrus cycle revealed that, compared to Estrus, females consumed more Graham Wafers during Proestrus ($p<0.017$). While Graham Wafer intake decreased during Estrus during Cycle 2, this decrease did not reach statistical significance.

The amount of Lab Chow consumed during the 1-hour snack period was similarly recorded. Again, although there was no significant interaction, there was a main effect of Phase of the cycle ($F_{3,50} = 3.44$; $p<0.05$). However, the Bonferroni-corrected Paired T-Test for the first phase of the Estrus cycle revealed that, compared to Estrus, there was a trend for the females to consume more Chow during Metestrus ($p<0.05$) ($\alpha = 0.017$).

There was no significant difference between days of the cycle during the second phase of the estrus cycle. Finally, mixed measures ANOVA revealed no significant effect of 24-hour intake across the estrus cycle (see Figure 3).

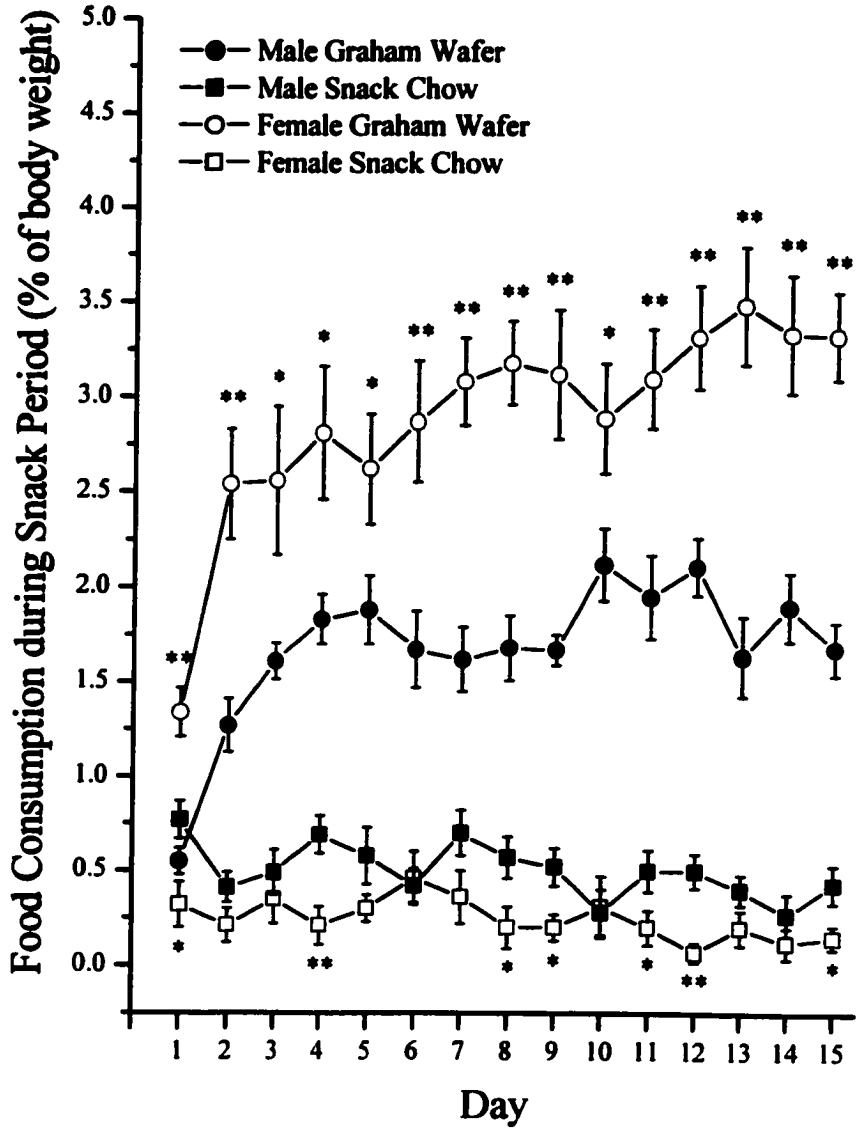


Figure 1: Effect of gender on the ingestion of a palatable snack (Graham Wafers) and Lab Chow during a 1-hour daily snack period. Each symbol represents the mean (± S.E.M) of Graham Wafer (circles) and Lab Chow (squares) intake of males (closed symbols) and females (open symbols) during a daily one-hour "snack" period over 15 test days. Intake is presented as a percentage of the rats' body weight. *,**Significantly different from male intake at $p<0.05$ and $p<0.01$ respectively.

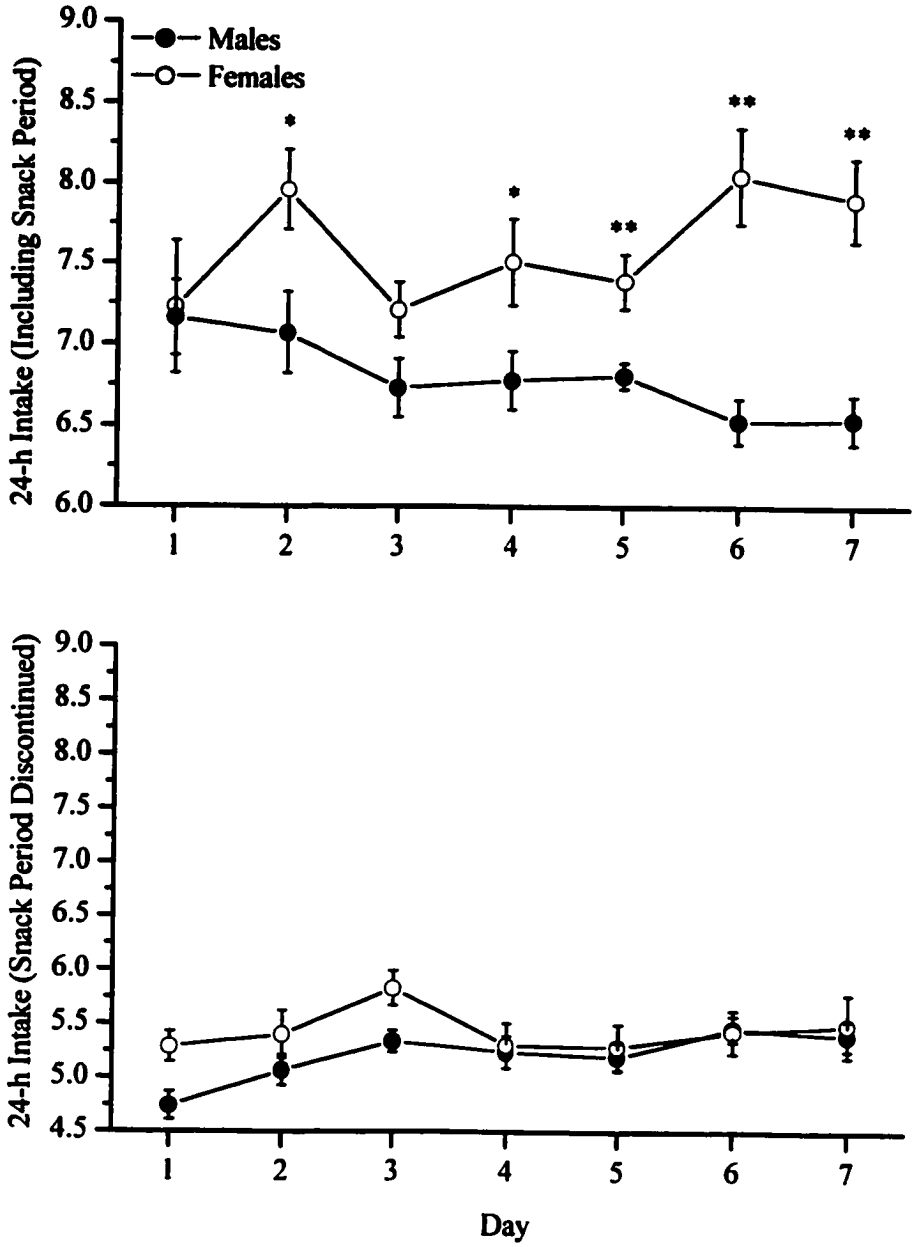


Figure 2: Effect of gender on total 24 hour intake of male and female rats during a 7-day period that included a palatable snack (top panel) and a 7-day period immediately following the discontinuation of the snack period (lower panel). Each symbol represents the mean (± S.E.M) of the total intake of male (closed symbols) and female (open symbols) rats during the 7-day period when the rats were receiving a daily 1-hour exposure to a palatable snack (top panel) and the 7-day period following discontinuation of the palatable snack (bottom panel). Intake is presented as a percentage of the rats' body weight. *,**Significantly different from male intake at $p<0.05$ and $p<0.01$ respectively.

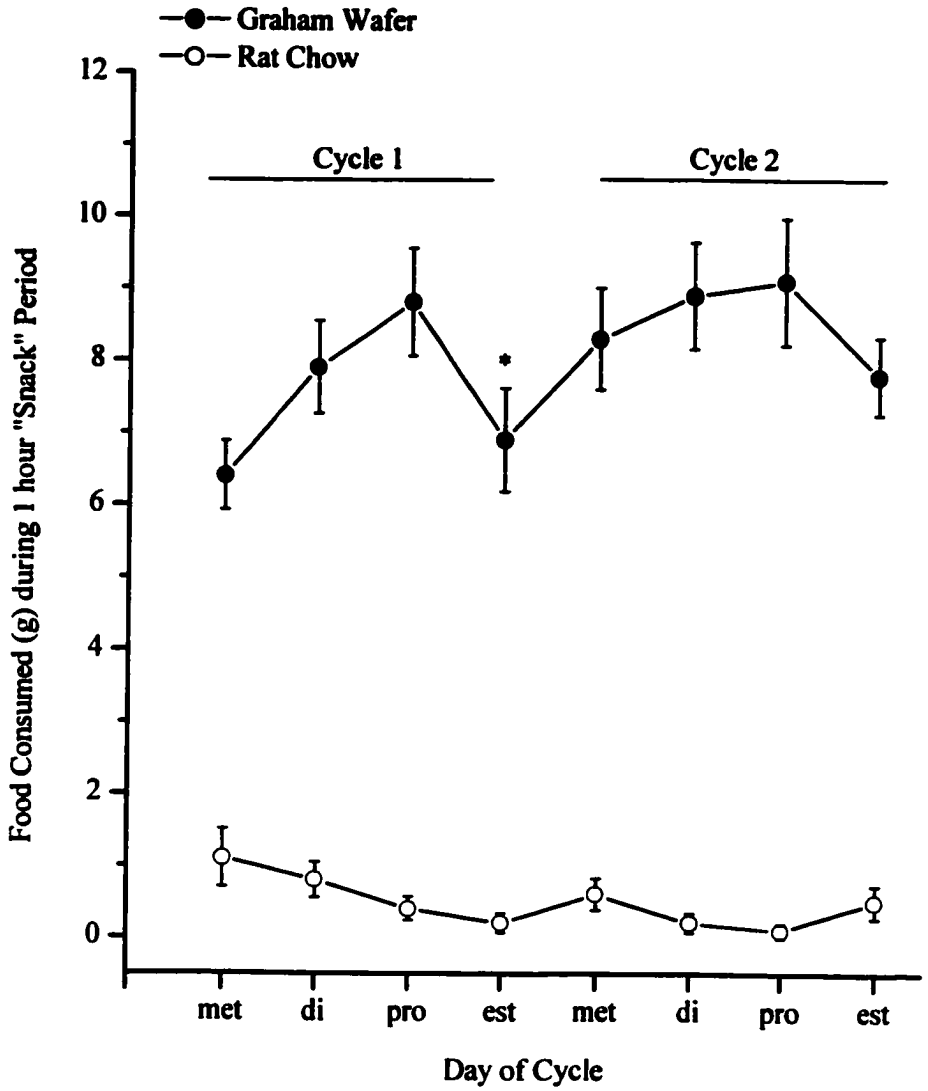


Figure 3: Effect of estrus cycle phase over two cycles on the consumption of a palatable snack (Graham Wafers) and rat chow. Each symbol represents the mean (± S.E.M) of the Graham Wafer (closed circle) and Rat Chow (open circle) intake of female rats during 2 estrus cycles. Intake is presented in grams. *,**Significantly different from proestrus at $p<0.05$ and $p<0.01$ respectively.

Discussion

The finding most worthy of note in the present investigation is that, when body weight is taken into consideration, the female rats consumed almost twice the amount of a palatable snack (Graham Wafers) than the males (in calories). Furthermore, when the continual Lab Chow availability is punctuated by palatable snack access, the 24-hour intake by females exceeded that of their male counterparts. Finally, consistent with previous reports,[144] the snack food intake of the female rats decreased during the estrus phase of their cycle. These data are interesting in light of the fact that females appear to be more susceptible to eating disorders such as obesity, binge eating disorder, anorexia nervosa and bulimia nervosa.[144]

It is tempting to speculate on the possibility that female rats are more responsive to the hedonic value of the palatable snack than the male rats. It is noteworthy that taste and palatability represent factors that are known to contribute to overeating and obesity.[422] When female rats were given a choice between a sweet (2% Polycose with 0.2% saccharin) and a bitter tasting solution (0.03% sucrose octaacetate), paired with the same postingestive consequence, the Polycose + saccharin group consumed 34% more total energy and gained more weight than the sucrose octaacetate group.[422] Moreover, it has previously been shown that when rats are given 24-hour access to 32% sucrose and 32% Polycose solutions, female rats increased their total caloric intake more and consumed more carbohydrate than did the males.[421]

It is generally agreed that there is a close association between feeding and gonadal hormones, particularly plasma estrogens.[144] Estradiol (the principal estrogen produced by the ovaries) appears to play a critical role in regulating the gender differences in ingestive behavior in the rat, and one line of thought is that it may do so through regulating the taste/palatability of the food.[79] Clarke et al (1998)[79] used taste reactivity tests to demonstrate that diestrus and proestrus (associated with high levels of estradiol) female rats exhibit a greater preference for infused tastants such as sucrose than males or estrus and metestrus females (in which estradiol levels were low). However, there appears to be some dispute regarding the effect of estrogen on palatability. Hrupka et al (1997)[201] examined the microstructure of licking behavior in ovariectomized female rats with and without B-estradiol 3-benzioate replacement. Both treatments failed to alter licking during the first minute of the meal and, thus, the authors concluded that estrogen did not appear to alter the palatability of the diet.[201]

Estrodiol has been shown to cyclically (during estrus) or tonically (during continuous estradiol treatment) reduce food intake by reducing meal size.[143,144,147,148,201] The present investigation examined whether the food palatability interacted with the estrus cycle. We found that females' intake of Graham Wafers peaked during proestrus during both of the two cycles examined, and decreased during estrus during both cycles, but only in the first cycle did that decrease reach statistical significance. The fact that Graham Wafer intake was lowest during estrus is interesting because that phase follows the surge in plasma estradiol.[79] Although this may appear to be contradictory given estrogen's known ability to reduce food intake,[121,141,144,146,174,201] recent studies have

demonstrated that estradiol cyclically increases the satiety signal of CCK, thereby decreasing meal size and food intake during the ovulatory or estrus phase.[144] As well, it has been shown that both food intake and CCK-8 injection increase c-Fos expression in the NTS, the PVN and the CeA, an effect increased in estradiol treated rats, suggesting that these brain sites may be important in the inhibitory action of estradiol on food intake.[144]

Sexual dimorphism of the rat HPA axis may be relevant to male/female differences in feeding behavior as CRH, the principal regulator of the HPA axis, is known to decrease food intake.[12,81] Furthermore, corticosterone, the end product of HPA activation is known to increase food intake.[12,81] Indeed, male rats have higher levels of CRH and lower levels of glucocorticoid receptor mRNA than diestrus females.[361] Moreover, in both sexes, exogenous administration of estrogen increases basal levels of corticosterone as well as stressor induced release of ACTH and corticosterone.[179] Comparatively, the female rats tend to have higher levels of corticosterone,[284] although it has recently been demonstrated that the corticosterone levels in females are dependent upon the phase of the estrus cycle.[12,179] Interestingly, there are no gender differences in basal corticosterone levels when females are in the estrus phase of their cycle, while at proestrus, the females' corticosterone levels peak and are significantly greater than those of males.[12] Given the ability of corticosterone to stimulate food intake, it is of interest that, in the current investigation, proestrus females also show peak levels of Graham Wafer intake during this phase.

Estradiol may be interacting not only with neuronal satiety signals, but may also be involved in the regulation of neuronal orexigenic mechanisms. NPY, a 36-amino acid residue peptide, is well known for its potency in stimulating feeding in sated animals.[429] The expression of NPY mRNA is increased at the arcuate nucleus of ovariectomized rats, and this is reversed by estrogen supplement.[429] Furthermore, the levels of NPY and release in the hypothalamus (PVN) is suppressed by estrogen treatment.[50] Since NPY is such a potent stimulator of food intake, it is possible that the anorectic effects of estrogen may be mediated, at least in part, by decreasing NPY release at the PVN.[50]

In summary, this study clearly demonstrates that food intake in general and palatable food intake in particular is sexually dimorphic. In the current investigation, the female rats appeared to display a preference for the daily palatable snack of Graham wafers compared to the males. Female rats consumed more of the snack (when body weight was considered) than their male counterparts, and exhibited a greater overall intake when a palatable snack was part of their diet. Moreover, palatable snack intake varied across the estrus cycle, implicating the involvement of gonadal hormones, particularly estrogen, in this effect. These results highlight the importance of including female animals in studies of feeding behavior, particularly given the prevalence of disordered feeding patterns among women.

Preface to Chapter VI

Genetic makeup is a fundamental factor that impacts on the organism's response to stressor exposure. We have characterized the stress responses of two lines of rats selectively bred for differences in amygdala kindling (Fast and Slow), and found that these rat lines differed in their endocrine responses to stressor exposure. As indicated in Chapter I,[311] there is ample reason to believe that common neural circuitry may be associated with stress/anxiety and with feeding, perhaps accounting for the fact that stress/anxiety is typically associated with altered food intake. In this respect, the Fast and Slow rats were compared as to their propensity to consume a palatable snack as well as their feeding-related neurochemical and endocrine profile. Their response to the known anxiolytic/orexigenic compound, diazepam, was also examined both in the home cage and the novel cage (a putative anxiogenic environment). Finally, micropunch survey of feeding-related peptidergic changes was undertaken in these two rat lines.

Chapter VI

Differences in carbohydrate snack consumption by the Fast and Slow kindling rats: effects of novelty-stress and diazepam

Abstract

Feeding behavior and anxiety may share several common neurochemical substrates. Using rats selectively bred for seizure susceptibility (Slow and Fast seizing) but also differing in anxiety, we assessed potential neuropeptidergic mechanisms that may subserve these processes. When offered a palatable snack (Graham Wafers or sucrose), the Slow rats consumed more than their Fast counterparts. Furthermore, Slow rats were more sensitive to diazepam, requiring relatively low doses of the drug to enhance their sucrose intake. The Slow rats were also more sensitive to the hypophagic effect (anxiety) of a novel environment, an effect that was reversed with diazepam. It is suggested that innate differences in GABA receptor sensitivity may contribute to their individual variability in snack intake. Given the involvement of CRH and BN-LP in both the regulation of feeding behavior and stress/anxiety responses, and the differences in stressor-related reactivity in the Fast and Slow rats, it was of interest to characterize the feeding-induced alterations in CRH and BN-LP tissue content as well as plasma levels of ACTH and corticosterone of the Fast and Slow rats. Thus, subsequent to behavioral testing, brain tissue analysis for CRH and BN-LP as well as trunk blood analysis for ACTH and corticosterone were conducted using *ex vivo* RIA. We found that, though there were no rat line differences in basal tissue levels of BN-LP, in comparison with Fast animals the Slow rats displayed a higher basal level of CRH peptide content at the PVN. Also, in the Slow rats' (compared to the Fast animals) feeding-induced tissue levels of CRH were significantly higher in the VMH and medial amygdala, while BN-LP tissue levels were higher in the VMH only. While both animals displayed a significant elevation of ACTH and corticosterone following consumption of the palatable snack, the

elevation in ACTH noted in the Slow rats was significantly greater than in the Fast animals. Interestingly, this did not translate into a significant elevation in plasma levels of corticosterone in the Slow rats, while the Fast animals did display a feeding-induced increase in corticosterone.

Introduction

Although critical to our understanding of normal as well as pathological ingestive processes, the neurochemical mechanisms governing individual differences in feeding behavior, and more specifically palatable food intake, remain largely unknown.[431] In addition to peptides, such as BN, CCK and NPY, it has been suggested that the widely distributed inhibitory amino acid transmitter, GABA may play a role in ingestive behaviors,[167,216,451,452] including that of palatable food intake, an effect thought to be mediated via $GABA_A$ receptors.[28,35,36,192,289,363,442,451-453] Indeed, GABA-induced changes of food intake are highly conserved across various species including rats,[85,86] hamsters,[40] pigs,[21] turkeys,[110] sheep[158] and rabbits.[287] Moreover, GABAergic mechanisms in various feeding- and taste-relevant brain regions, such as forebrain, hypothalamic and hindbrain nuclei, may influence the regulation of food intake.[35,37,80,191,217,228,239,240,277,324,386]

Increasing evidence has accumulated suggesting that anxiogenic responses and feeding may be linked to one another. Ordinarily, fear- or anxiety-provoking situations promote a reduction of food intake[425] and increase the latency to initiate feeding.[48,85] GABA agonists, such as benzodiazepine compounds reverse these effects.[391] While this reversal may be due simply to the anxiolytic properties of benzodiazepines,[167] it is also thought that benzodiazepines enhance the hedonic value of food, as evidenced not only by increased intake of palatable substances, but also by increased hedonic behaviors in taste reactivity tests.[36,167,192,363]

As in the case of variations of food intake, the behavioral changes elicited by situational stressors and/or pharmacological manipulations are also complicated by marked individual differences.[359] One approach to determining the mechanisms governing these changes involves the assessment of different strains (or the selectively bred lines) of animals with innate differences in appetite for certain foods.[83,359,431] To this end, we investigated feeding behaviors in two lines of rats ("Fast" and "Slow" kindling) that had been selectively bred for differences in amygdala excitability, as reflected by either fast or slow development of amygdala kindling epileptogenesis.[297,388] In addition to the differences in seizure susceptibility, various behavioral tests suggested that the Slow kindling rats are more anxious and fearful than their Fast kindling counterparts,[298,328] although the behavioral and neurochemical manifestations of their anxiety appear to be situation-specific.[312] The differences in emotionality and kindling rates in these animals suggest a potential GABAergic involvement. Indeed, pharmacological manipulations indicated that, relative to Slow rats, the Fast animals were more sensitive to the induction of seizures by GABA antagonists, whereas Slow rats were more sensitive to anaesthetic induction by sodium pentobarbital (an agonist for the barbiturate site on the $GABA_A$ receptor complex).[297] *In situ* hybridization histochemistry revealed $GABA_A$ receptor differences between the rat lines in selected limbic structures. Fast rats expressed only ~50% of the adult GABAα_1 subunit mRNA, while Slow rats had GABAα_1 levels that were ~70% greater than control animals.[383]

In contrast to diazepam, an orexigenic agent with anxyiolytic properties, the so called satiety peptide, BN,[310] as well as the "stress" peptide, CRH,[114,374,437] are known to

suppress food intake, and also possess anxiogenic properties.[308] For example, central administration of CRH, has been shown to induce anxiety-like behaviors and suppress food intake in normal rodents, including NPY- and tail-pinch induced feeding, indicating that the CRH system may modulate stressor-induced reduction in food intake.[187,198,199,246,437,445,465] Furthermore, in a novel (presumably stressful) environment, CRH exerts anxiogenic-like properties such as decreases in rearing and exploration in several paradigms, including the open field, the elevated plus-maze, the light-dark box, social interaction, and contextual fear conditioning.[100,206,437]

In addition to CRH, there are numerous reports that support the conclusion BN-LPs contribute to satiety processes.[310] For example, both peripheral and central administration of BN has been shown to dose-dependently decrease food intake, including stressor-induced feeding, and induce a repertoire of behaviors reminiscent of satiety.[151,152,155,292,314,334,340] Moreover, a role for BN-LPs in the regulation of an animal's response to stress/anxiety is also emerging.[230,231,308] Although data are not available profiling the effects of BN-LPs in standard animal models used to assess anxiety, such as the elevated plus maze, light-dark box, or fear potentiated startle, several other observations note the similarity of BN-induced and stressor- and/or anxiety-like behaviors.[308] For example, central administration of BN increases exploratory and grooming in a manner reminiscent of the behavioral response associated with exposure to a stressor.[306] Interestingly, BN-induced grooming behavior can be reversed by the administration of the anti-anxiety agents, diazepam and chlordiazepoxide.[331] Furthermore, when rats are offered a highly palatable snack in a familiar environment,

they readily consume the snack; however, when offered the same snack in a novel environment, rats will display a marked latency to initiate consumption and a reduction in food intake, an effect that can be reversed with anxiolytic treatment.[308] Thus, it was of interest to note that the anxiogenic effect of the novel environment can be attenuated with systemic administration of the selective non-peptide BN BB_1/BB_2 receptor antagonist, PD 176252, suggesting that BN-LPs might play a role in the mediation of anxiety.[308]

A previous study conducted in our lab attempted to characterize the responses of the Fast and Slow rats to stressor exposure.[309] It was found that Fast and Slow rats displayed differential neurochemical (CRH and BN-LP) and endocrine (ACTH and corticosterone) responses to stressor exposure (although this effect appeared to be dependent on the situation or type of stressor employed). Given involvement of CRH and BN-LPs in both the regulation of feeding behavior and stress/anxiety responses, and the differences in stressor-related reactivity in the Fast and Slow rats, it was of interest to characterize the feeding-induced alterations in CRH and BN-LP tissue content and plasma levels of ACTH and corticosterone of the Fast and Slow rats.

Given the likely involvement of GABA, CRH and BN-LPs in subserving emotionality differences between Fast and Slow rats, and the established involvement of these systems in palatable food intake, we investigated potential differences between Fast and Slow rats with respect to (1) their appetite for palatable snacks (Graham Wafer and sucrose), (2) stressor- (novel environment) induced suppression of snack intake, in the

absence and presence of the anxiolytic diazepam, and (3) regional variations in the endogenous CRH and BN-LPs levels across various brain regions.

Materials and Methods

Subjects

Age-matched Fast and Slow male (350-600 g) and female (130-200 g) rats were used in these experiments. These rats were selectively bred for differences in amygdala excitability, as realized by either their *Fast* or *Slow* kindled seizure development, without brother x sister matings.[297,388] The original parent line comprised Wistar and Long-Evans Hooded rats and were bred at McMaster University, Hamilton, Ontario, Canada until the 11th generation. Within six generations of selection, there was no overlap in the distribution of kindling rates between the two lines. After the 11th generation rats were transferred to Carleton University, Ottawa, Ontario, Canada, where selection was relaxed, and random breeding within each line has continued since that time. The rats used in the present investigation were from the 43rd and 44th generations. Rats were individually housed in standard rat cages. The colony room was maintained on a 12-hour light/dark cycle (lights on at 0800) at 23°C with 60% relative humidity. Animals had *ad libitum* access to food and water. All experiments were conducted in accordance with the guidelines set out by the Canadian Council on Animal Care and approved by the local university Research Ethics Committee.

Rat line (Fast/Slow) differences in palatable Graham Wafer consumption:

Free-fed male Fast and Slow rats (n = 10/group) were offered a palatable Graham Wafer (Christie HoneyMaid®) snack in their home cages for 30 min daily over a consecutive 7-day period. Consumption of the snack (30-min) was measured in grams and then expressed as a ratio of body weight (due to differences in the body weights between the two rat lines).

Effect of diazepam on sucrose consumption:

Following testing with the Graham Wafers, male (n = 10) and female (n = 10) rats were offered a palatable sucrose (granulated sugar) snack in their home cages for 20 min daily over a consecutive 8-day period. Consumption was measured in grams, and then expressed as a percentage of body weight. As the sucrose intake of the males was relatively low (data not shown) only the females were used for the next part of the study.

To assess the effect of diazepam, the female rats (n = 10/group) were habituated to the sucrose snack over an 8-day period as described in the preceding experiment. Following sucrose habituation, each rat was injected with various doses of diazepam (0 (vehicle), 0.5, 1.0, or 2.0 mg/kg; subcutaneous) over a five-day period. An additional higher dose of diazepam (3.0 mg/kg. subcutaneous) was used in the Fast line. This dose was not employed in the Slow line, as it was clear from preliminary results that it rendered Slow rats somnolent. Diazepam (Roche) was freshly diluted in vehicle (50% distilled water, 40% propylene glycol, and 10% alcohol). Thirty minutes following

injection, the rats were presented with the sucrose snack for 20 minutes and the amount consumed was recorded.

The effect of novel environment on sucrose consumption:

It has been shown that when animals are placed in a novel environment, the latency to consume food is increased[48,85] and consumption is reduced,[425] an effect reversible by anxiolytics. It was of interest to determine whether the two rat lines would exhibit differential sucrose consumption in a novel environment, and whether diminished consumption in an unfamiliar environment in the two rat lines would be differentially attenuated by diazepam. Accordingly, the female rats from the preceding study were used to assess palatable snack intake in a novel environment. The rats were handled briefly, and then returned to their home cages. Thirty minutes later, they were placed in a novel cage (clear Plexiglas rat cage with wire lid and no bedding) where they were given 20-min access to a sucrose snack. The amount of sucrose ingested by each rat was recorded. The following day, all of the Slow rats and five of the Fast rats were given diazepam (0.5 mg/kg; subcutaneous). The remaining five Fast rats received the higher dose (3.0 mg/kg diazepam). Thirty minutes following the injection, the rats were removed from their home cages, placed in the novel cages and offered access (20 min) to the sucrose snack, and the amount of sucrose consumed was recorded.

At the end of each experiment (one day to one week following the last day of testing), animals were decapitated, and brains were removed, immediately frozen in a stainless steel brain matrix resting on dry ice, then subsequently stored at –80°C. Using a

cryostat, brains were sliced into serial 600-μm coronal sections and a total of 9 discrete nuclei were micropunched from these sections.[357] Selected hypothalamic nuclei included the PVN, DM, VMH, LH, AH and the arcuate nucleus. Extra-hypothalamic nuclei included the medial and central nucleus of the amygdala and NTS. Punched tissue was immediately placed in 200 μl of 0.2 M acetic acid solution and heated to 80°C for 1 hour. Tissue was then sonicated and 40μl of the homogenate removed for assessment of the protein content. The remaining homogenate was centrifuged, and the supernatant lyopholized and stored at -80°C. Lyopholized samples were later reconstituted with RIA buffer and analyzed for immunoreactive CRH and BN-LPs using highly sensitive solid phase RIA.

Radioimmunoassay for CRH, BN-LP, ACTH and corticosterone:

The detection and quantification of CRH and BN-LP(s) was achieved through a modification of a high-sensitivity solid-phase RIA procedure originally described by Maidment and Evans, 1991.[285] Briefly, Immulon-4 well plates (Dynatech, Chantilly, VA) were coated with protein A/G (a genetically engineered protein (Calbiochem, La Jolla, CA) that combines immunoglobulin G binding profiles of both protein A and protein G; 0.1 μg/100μl/well) at least 24 hours prior to use. After incubation, the plates were rinsed with wash buffer (0.15 M phosphate-buffered saline, pH 7.4), and subsequently incubated with either the anti-CRH serum (rC70 kindly provided by W. Vale) or anti-BN serum (α-BN2 kindly provided by Dr. T. Moody) for 2 hours at 20°C. Following this incubation, the plates were rinsed with wash buffer. Either CRH or BN standards and lyophilized brain samples were reconstituted in phosphate buffered saline

(pH 7.4) and pipetted into designated wells, then incubated for 24 hours at 4°C. Reference and blank samples were also used to determine total and non-specific binding of the radioligand, respectively. Following incubation, 25 µl assay buffer containing a fixed concentration of either ~5000 - 6000 CPM [^{125}I-Tyr^{0}]rCRF (Amersham Canada Ltd., Oakville, ON) or [^{125}I-Tyr^{4}]BN (iodinated in house, as per Salacinski et al. 1981[409] was added to each well and incubated for an additional 24 hours period at 4°C. Finally, the wells were rinsed with wash buffer, blotted dry, and separated into 12 x 75 glass tubes. Residual radioactivity was determined using a gamma counter (Packard Cobra II Autogamma model D5002). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 and 2 fmol/well for CRH and BN, respectively.

Corticosterone and ACTH levels were measured using commercial RIA kits (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

Statistical Aanalysis

The body weights of the Fast/Slow male and female rats (measured at one time point for each group) were compared separately using one-way ANOVAs. Graham Wafer and sucrose intake for the rats was expressed as a percentage of body weight. Consumption of each food was analyzed separately using a mixed measures ANOVA with Consumption over days as the within measure and Rat Line (Fast/Slow) as the between factor. The females' response to diazepam was analyzed using a mixed measures ANOVA, with Dose as the within group and Rat Line (Fast/Slow) as the

between group measure. The effect of exposure to a novel environment on snack intake and response to diazepam in the novel environment was analyzed using a mixed measures ANOVA with Condition (home cage, novel cage, and novel cage with diazepam) as the within group factor and Rat Line as the between group factor. Post hoc analyses for differences in consumption over days were performed using Paired t-tests (with Bonferroni correction) comparing each day to Day 1, while independent t-tests were used to compare the Rat lines. Rat line differences in CRH and BN-LP content within various discrete brain nuclei and plasma levels ACTH and corticosterone were analyzed using two factor ANOVA (Rat line x Treatment). Where significant, ANOVAs were followed by Tukey's HSD multiple comparisons of the means or in the case of interactions, comparisons between the means comprising the simple effects.

Results

Rat line (Fast/Slow) differences in palatable Graham Wafer consumption:

Slow rats weighed significantly more than their Fast counterparts ($F_{1,19} = 25.43$, $p<0.001$). Thus, Graham Wafer intake was measured in grams and then expressed as a percentage of body weight. Graham Wafer intake varied as a function of the Rat Line (Fast and Slow) x Days interaction ($F_{6,108} = 7.80$; $p<0.001$). Fast and Slow rats displayed a significant and sustained increase of Graham Wafer intake, such that consumption on Days 2-7 exceeded that seen upon initial presentation of the Graham Wafers. The effect of Rat Line was evident in that the Slow rats consumed more Graham Wafers than their Fast counterparts from Day 3 onwards (see Figure 1).

Dose-related effect of diazepam on palatable sucrose consumption:

As no Rat Line differences in sucrose consumption were evident for the male animals (data not shown), only the females were used to assess the effects of diazepam on consumption. In order to replicate the Rat Line-related differences in the consumption of palatable snack, new groups of female rats (n = 10 Fast and 10 Slow) were habituated to the sucrose snack under the same conditions as Experiment 1 (data not shown). In this case, Fast females weighed significantly more than their Slow counterparts ($F_{1,18} = 41.99$; $p<0.001$). Snack intake varied as a function of Rat line (Fast/Slow) x Days interaction ($F_{7,126} = 5.19$; $p<0.001$). The intake of the Slow females increased during the 8 test days ($F_{7,63} = 6.76$; $p<0.001$), an effect which became statistically significant on Day 4 and onwards. The sucrose intake by the Fast females remained constant throughout testing. Over the entire test period, the Slow females consumed more sucrose than did the Fast females ($p<0.001$) (data not shown).

Following the habituation/stabilization period, the impact of diazepam on sucrose intake was assessed. Consumption varied as a function of Rat Line x Dose interaction ($F_{3,54} = 17.17$; $p<0.001$). The multiple comparisons of the simple effects indicated that, in the Slow females, even the lowest dose of diazepam (0.5 mg/kg) increased snack intake. The stimulation of snack intake began to taper off at the 1.0 mg/kg dose and was significantly reduced (from baseline) at the 2.0 mg/kg dose, a mildly sedating dose. In contrast, the Fast females were resistant to these doses (0.05-1.0 mg/kg) of diazepam. Indeed, an even higher dose of diazepam (3.0 mg/kg) was required to stimulate feeding in the Fast line ($p<0.05$) (see Figure 2).

The effect of novel environment on palatable sucrose consumption:

Following the re-establishment of stable baseline consumption in their home cages, the effects of stress associated with the transfer to a novel environment was assessed with or without diazepam treatment (0.5 mg/kg for the Slow and five of the Fast rats; 3.0 mg/kg for five Fast animals). Snack intake was found to vary as a function of the Rat Line x Condition (novel environment exposure, with or without diazepam) interaction ($F_{2,26} = 3.76$; $p<0.05$). As shown in Figure 3, exposure to the novel environment alone significantly reduced the sucrose intake of the Slow females ($F_{2,18} = 10.83$; $p<0.001$), an effect which was reversed by the administration of diazepam. The snack intake of the Fast animals remained unchanged by the novel environment and remained unaltered by diazepam treatment (see Figure 3). This was the case even with the higher dose of diazepam (3.0 mg/kg, data not shown).

Effect of rat line on CRH and BN-LP (GRP) content within various brain nuclei:

ANOVA revealed no differences in the basal levels of CRH between the two lines of rats with the exception of the PVN, ($F_{1,18} = 7.31$, $p<0.05$). Post hoc analysis showed that Slow animals displayed higher levels of CRH content compared to the Fast rats (see Figure 4, upper panel). Upon consumption of the palatable snack, Slow rats (compared to their Fast counterparts) displayed significantly greater levels of CRH at the VMH ($F_{1,15} = 4.75$, $p<0.05$), and medial amygdala ($F_{1,15} = 4.95$, $p<0.05$) (see Figure 4, lower panel).

There were no basal differences in the levels of immunoreactive BN-LP between the two lines of rats (see Figure 5, upper panel). However, upon consumption of the Graham Wafer snack (lower panel), immunoreactive BN-LP levels were significantly greater in the Slow rats, compared to the Fast animals, at the VMH ($F_{1,15} = 7.18$, $p<0.05$) (see Figure 5, lower panel).

Rat Line differences in the endocrine response (trunk blood) to snacking:

Although the Rat Line x Feeding condition interaction was not statistically significant, based on *a priori* predictions, independent analyses were conducted for each rat line. This comparison revealed a main effect of Group for both ACTH ($F_{1,47} = 14.3$, $p<0.01$) and corticosterone ($F_{1,48} = 5.8$ $p<0.01$). Post hoc analysis revealed that basal levels of plasma ACTH and corticosterone were similar in both lines of rats and, upon eating, ACTH levels increased significantly in both the Fast ($F_{1,19} = 18.0$, $p<0.001$) and Slow rats ($F_{1,25} = 18.6$m $p<0.001$). In contrast, corticosterone levels increased upon consumption only in the Fast animals ($F_{1,22} = 4.9$, $p<0.05$). However, despite baseline similarities, the feeding-elicited rise in the corticosterone levels of the Slow rats was significantly higher than that seen in the Fast animals (see Figure 6).

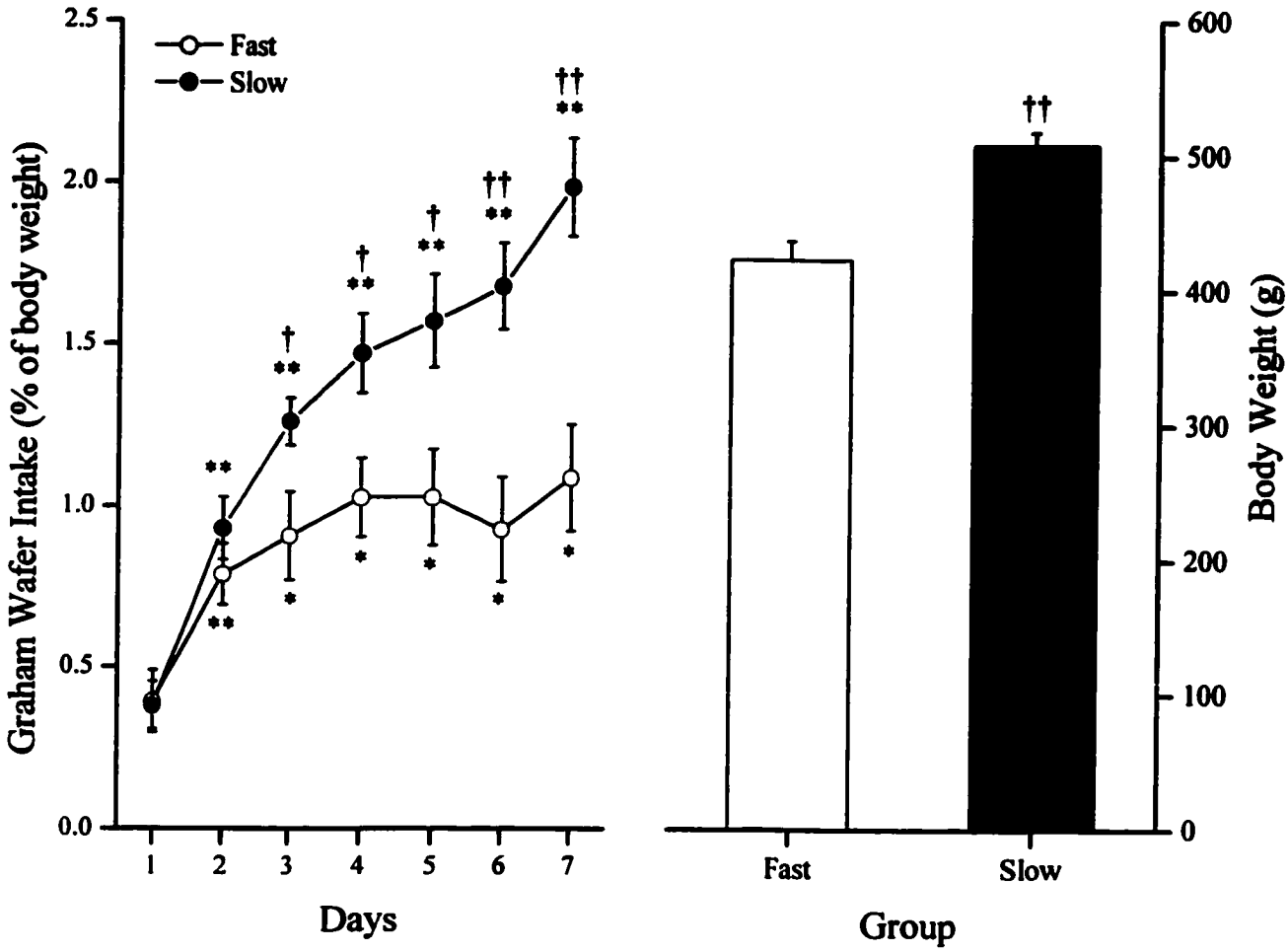


Figure 1: Effect of rat line on Graham Wafer intake and body weights. Values (left panel) indicate means ± SEM of Graham Wafer intake (expressed as a percentage of body weight) of Fast (open circles) and Slow (closed circles) rats. Bars (right panel) represent mean (± SEM) of body weight of Fast (open column) and Slow (solid column) animals. (*,** Significantly different from Day 1; †,†† Significantly different from Fast rats; $p<0.05$ and 0.01 respectively).

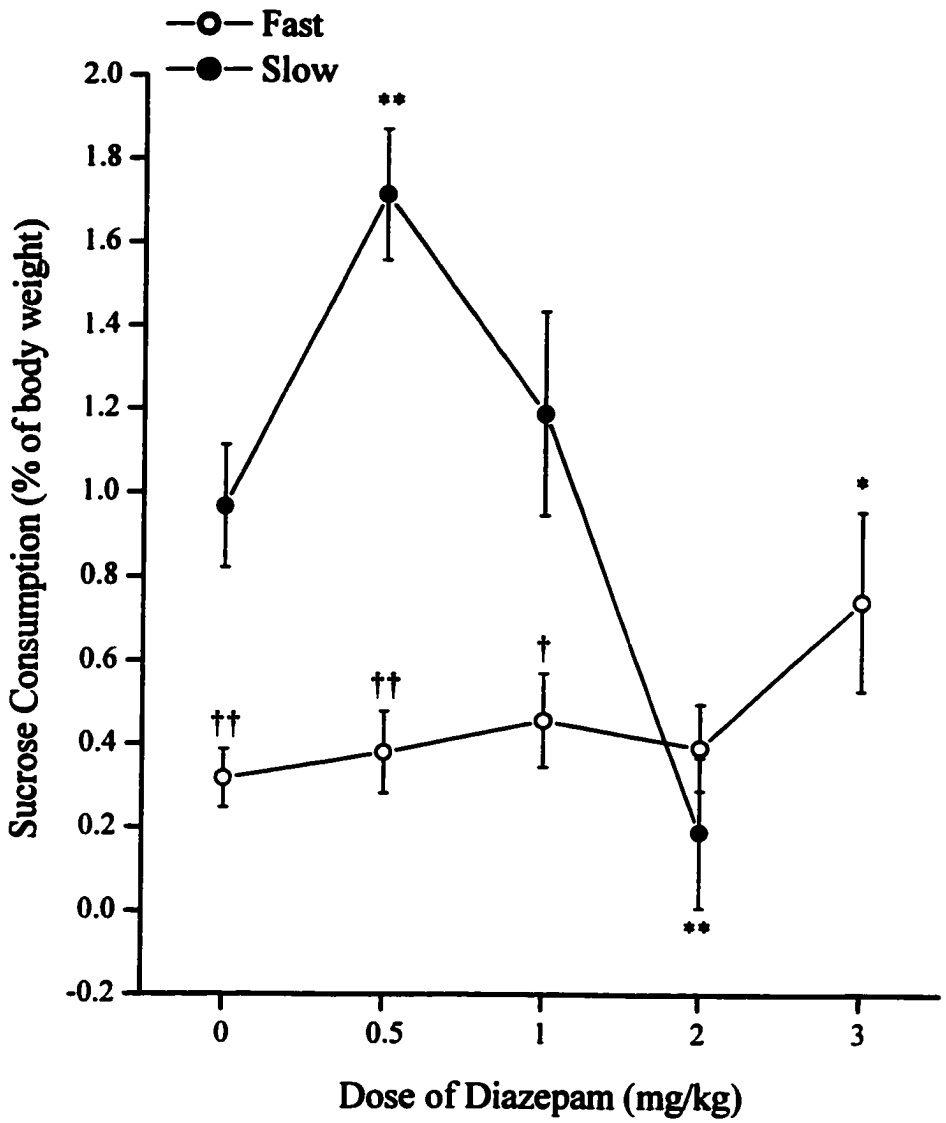


Figure 2: Effect of diazepam on sucrose consumption in Fast and Slow female rats. Fast and Slow female rats were administered diazepam (0, 0.5, 1.0, 2.0 mg/kg subcutaneous). Fast animals received an additional dose of 3.0 mg/kg subcutaneous. Each point represents the mean ± S.E.M. of sucrose intake, expressed as a percentage of the body weight. Open circles represent Fast rats and closed circles represent Slow animals. *,** Significantly different from baseline at $p<0.05$ and $p<0.01$ respectively. †,†† Significantly lower than Slow rats at $p<0.05$ and $p<0.01$ respectively.

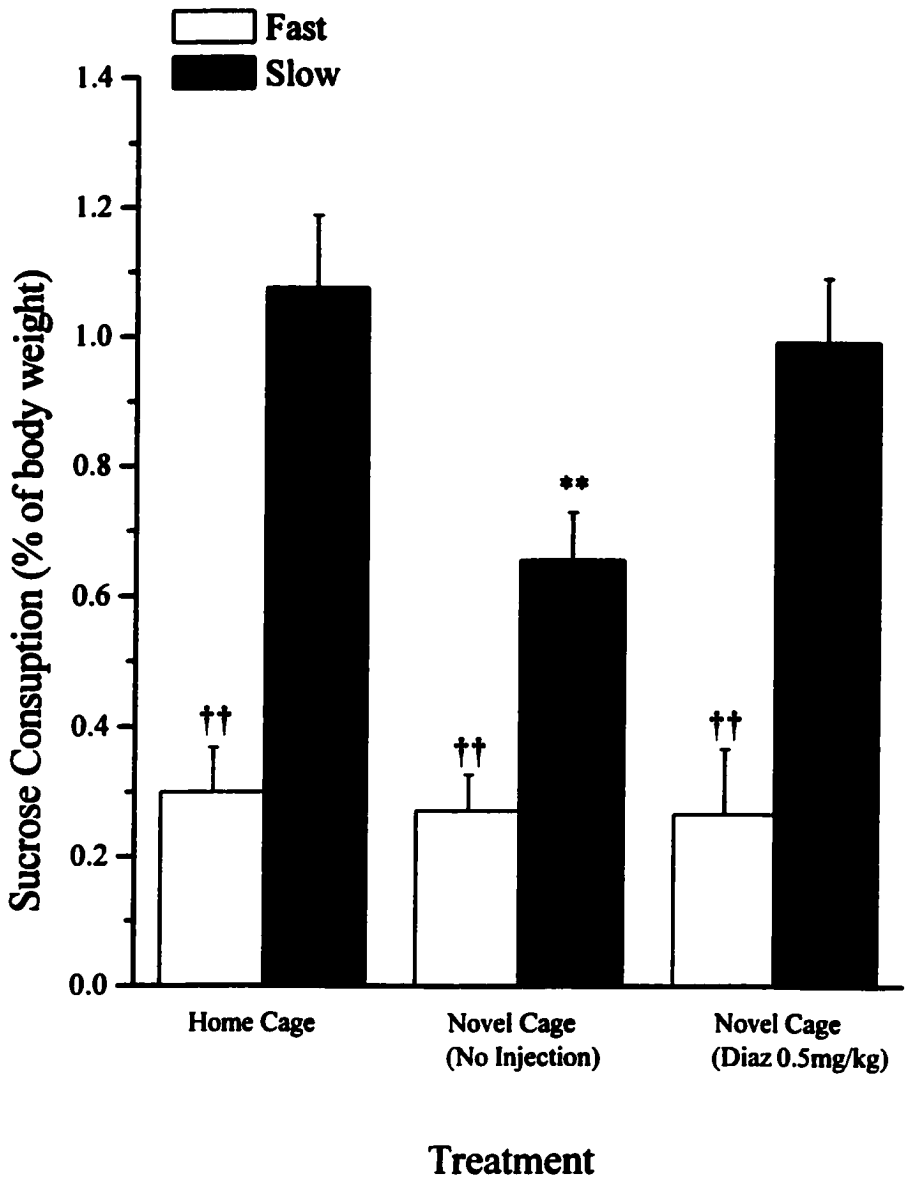


Figure 3: Effect of novel environment with and without diazepam administration on sucrose consumption of Fast and Slow Female rats. Fast and Slow animals were presented with granulated sucrose for 20 min in their home cage to establish a baseline comparison (set of bars at left). On the following day, rats were placed in a novel environment and presented with the same sucrose snack (middle set of bars). The next day, all of the Slow rats and five of the Fast rats were administered 0.5 mg/kg diazepam (set of bars on right), while the remaining Fast females received 3.0 mg/kg (data not shown). Bars represent sucrose consumption $\pm$ S.E.M., measured in grams and converted to a percentage of body weight. Fast rats are represented by open bars and Slow rats by closed bars. ** Significantly different from baseline at $p<0.01$. †† Significant Line differences at $p<0.01$.

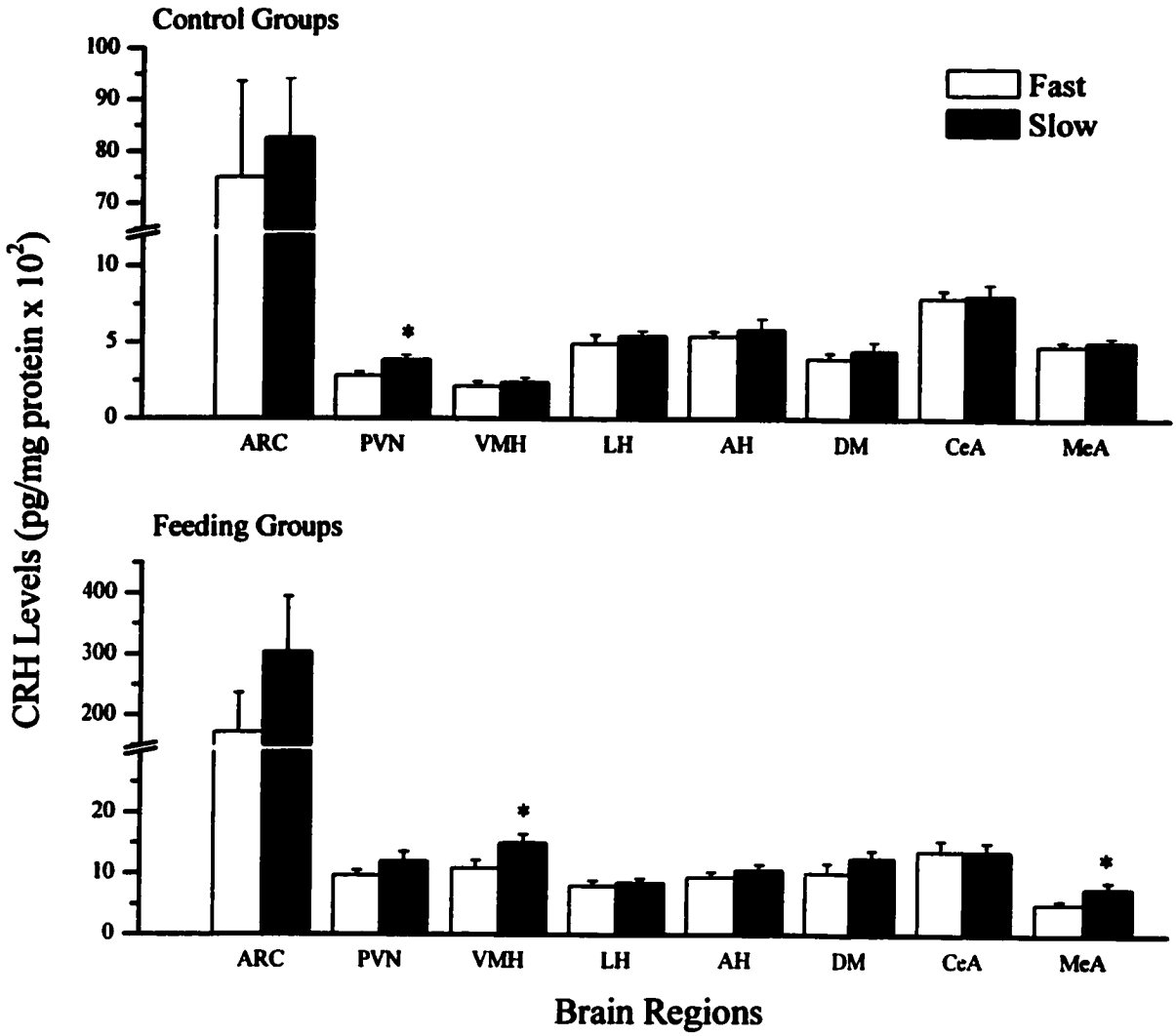


Figure 4: Regional differences in CRH content of brain regions following consumption of a palatable Graham Wafer snack. Bars represent the mean (± SEM) of CRH content of Fast (open bars) and Slow (solid bars) rats following the presentation of an empty dish without food intake (Controls, upper panel) and following consumption of a submaximal quantity of Graham Wafer (Feeders, lower panel). * Significantly greater than Fast, $p<0.05$.

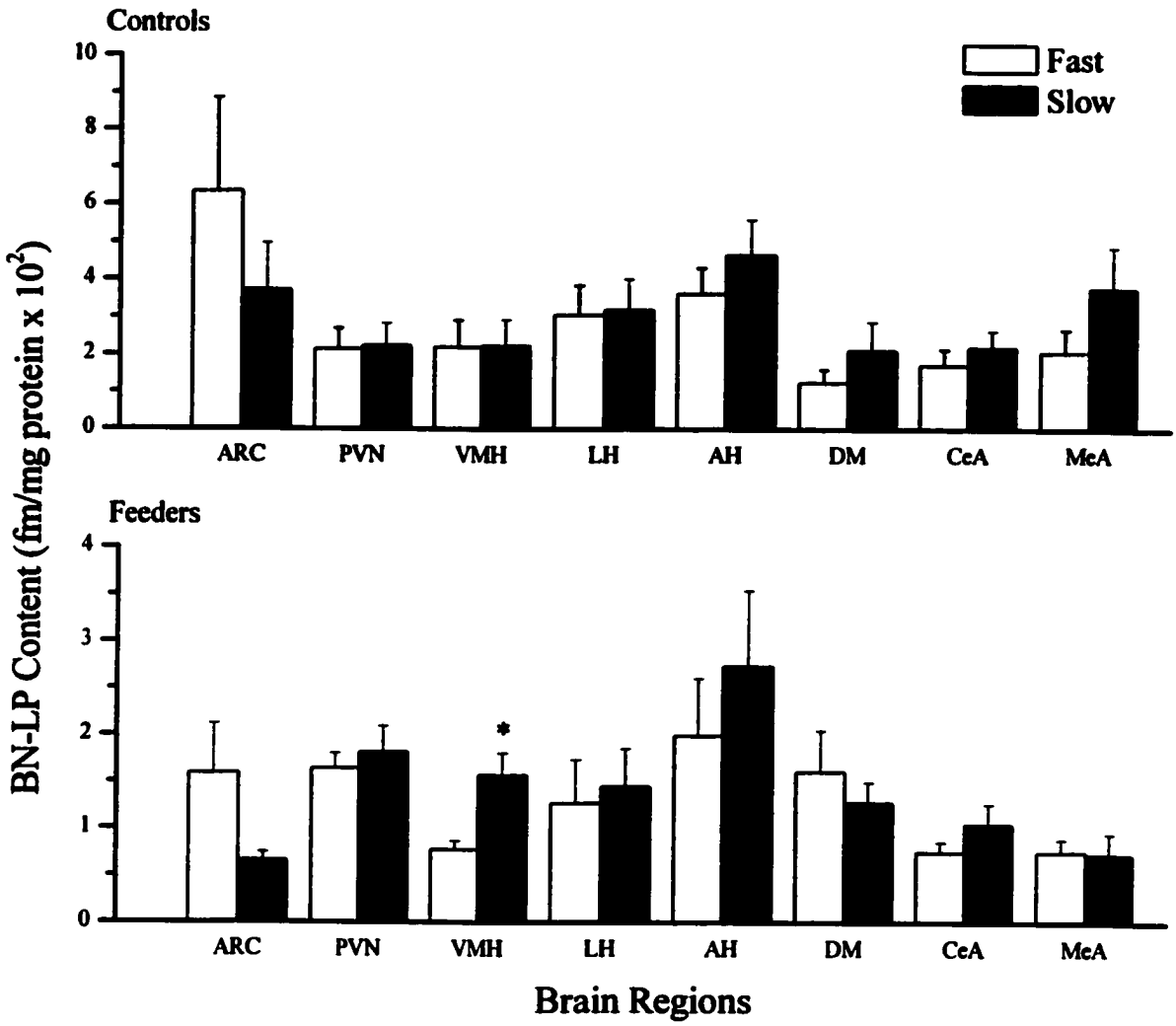


Figure 5: Regional differences in BN-LP content in brain regions following consumption of a Graham Wafer snack. Bars represent the mean (± SEM) of BN-LP content of Fast (open bars) and Slow (solid bars) rats following the presentation of an empty dish without food intake (Controls, upper panel) and following consumption of a submaximal quantity of Graham Wafer (Feeders, lower panel). * Significantly greater than Fast, $p<0.05$.

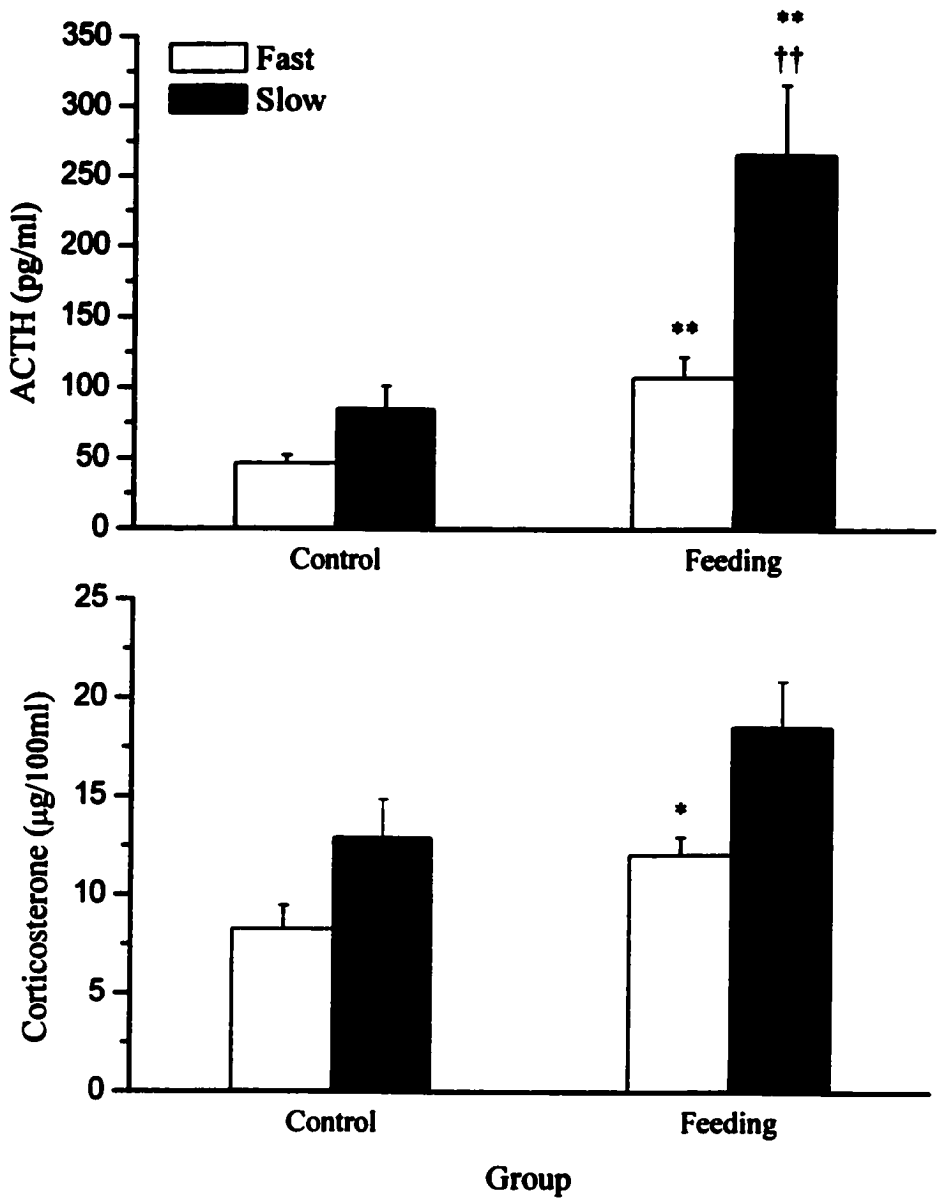


Figure 6: Rat line differences in endocrine response to the ingestion of a palatable snack. Bars represent mean (+ SEM) plasma levels of ACTH (upper panel) and corticosterone (lower panel) in the trunk blood following exposure to an empty food dish (control) (open bars) or consumption of a submaximal quantity of Graham Wafers (solid bars). *,** Significantly different from control, $p<0.05$ and 0.01 respectively; †† Significant line difference, $p<0.01$.

Discussion

It will be recalled that naïve Slow kindling rats exhibit more pronounced signs of anxiety than do Fast rats,[328] and they are more sensitive to positive GABA modulators (e.g., pentobarbital).[297] Moreover, Slow rats express an abundance of the $GABA_A \alpha_1$ receptor subunit (the adult form of the $GABA_A$ receptor complex), while Fast rats overexpress the embryonic $GABA_A$ α2,3&5 subunits, suggesting arrested development of the $GABA_A$ system of Fast rats.[383]

Despite the fact that Slow rats were found to exhibit greater signs of anxiety than Fast rats, behavioral and neurochemical consequences of stressors in these animals appeared to depend on the situation and type of stressor to which they are exposed.[9,298,312] For instance, in response to a purely psychogenic stressor (such as predator exposure), Fast rats displayed more immobility than did Slow animals, whereas in response to restraint (a mixed psychogenic and neurogenic stressor), the Fast animals struggled while the Slow rats displayed more immobility.[298] While basal levels of ACTH and corticosterone levels did not differ between the rat line, these neuroendocrine substrates were increased in response to the stressors in a manner that paralleled the behavioral effects.[9,298] Thus, although the more anxious behavioral profile of Slow rats seems to be contradictory given their more active GABAergic system, this profile may depend on situational factors and the type of stressor encountered.

As the Fast and Slow kindling rats exhibited clear differences with respect to GABA functioning, and in light of the possibility that GABAergic mechanisms may

contribute to food intake, the possibility was considered that palatable snack consumption may also differ in these rat lines. Snack consumption was assessed in a familiar (home cage) environment and in a novel environment that ordinarily results in suppressed food intake. Over the course of habituation to the palatable snack, the Fast and Slow male rats exhibited increased Graham Wafer intake; however, the increase in the Fast animals reached a plateau earlier than in Slow rats. Furthermore, when offered the opportunity, Slow rats consumed more sucrose than did Fast rats, and Slow rats were significantly more sensitive (relative to the Fast line) to the diazepam-induced enhancement of snack intake, as reflected by their increased sucrose intake at the low dose (0.5 mg/kg) of the drug. In fact, this dose was six-times lower than that required to stimulate feeding in Fast animals. This finding is consistent with other pharmacological observations in these rat lines with regard to Slow animals' sensitivity to anesthesia,[297,383,426] as well as their native overexpression of GABAα_1 receptors in limbic structures compared to Fast rats.[383]

It has been reported that, in rodents, anxiety may be correlated with the propensity to consume sucrose.[111] Given that Slow rats consumed more sucrose, it might be expected that these rats would exhibit less anxious behavior in animal models of anxiety. In the present investigation sucrose consumption in a novel environment was used to assess potential differences of anxiety. Ordinarily, placement of a rat into a novel environment increases their latency to initiate feeding and reduces the amount of food consumed.[48] This feeding-suppressant effect is marked and persists over repeated test sessions.[233] Administration of diazepam and the GABA agonist, abecarnil, has been shown to reduce the anxiogenic effect of a novel environment.[233,391] As well,

chlordiazepoxide reduces the latency to initiate eating, prolongs the total time spent eating, and abolishes food neophobia in a novel environment.[130] In the present investigation, placement of the rats into a novel cage significantly reduced the sucrose snack intake of the Slow rats, but had little effect in Fast rats, a finding consistent with the view that Slow rats are more anxious. Interestingly, the reduced consumption in the Slow rats was completely reversed by administration of 0.5 mg/kg diazepam, suggesting a possible anxiolytic action. In contrast, the novel environment did not influence sucrose intake in Fast rats, and diazepam did not affect that consumption.

It has been reported that 50-65% of CRH expressing neurons of the PVN co-express α_1 and β_2 subunits of the $GABA_A$ complex.[94] This raises the possibility that the activity of the HPA axis may be modulated, at least in part, by the GABAergic system. Since CRH is known to reduce food intake,[168] and the GABA system has an inhibitory effect on the HPA axis,[375] it is possible that the Slow animals (with the up-regulated $GABA_A$ receptor system) may not release as much of the anorexant peptide and hence be prone to consume greater quantities of sucrose. However, this would depend upon the triggered release of GABA, and it is not known whether the Slow rats release greater or lesser amounts of GABA during stress/feeding that Fast rats. One might expect that Slow rats release less GABA than Fast rats as they appear more anxious,[328] which is evidenced here by their reduced snack intake in a novel environment.

Previous research in our lab has shown that these two lines of rats differentiate on the basis of behavioral, endocrine and neurochemical responses to stressor exposure,

although this varies depending on the situation.[309] Behaviorally, in response to immobilization stress, the Fast rats struggle whilst the Slow rats adopt an immobility posture. Meanwhile, in response to predator exposure, the Fast rats freeze while the Slow rats emit active avoidance responses. Furthermore, the Fast and Slow rats' endocrine responses mirrored their behavioral responses.[309] Ferret exposure induced a greater increase in plasma ACTH and corticosterone concentrations in the Slow than in the Fast rats,[309] while in contrast, immobilization provoked a greater rise of plasma ACTH levels in the Fast rats.[309]

The results of the current investigation support our previous findings in that the Fast and Slow rats can be differentiated on the basis of their food intake-induced neurochemical and endocrine responses. CRH and BN-LP were abundantly distributed among all nuclei examined. Similar to previous reports,[464] levels of CRH were high in hypothalamic nuclei such as the arcuate nucleus, PVN, VMH, LH, AH, and DM, limbic areas such as the CeA, medial amygdala, and in the brain stem (NTS), all regions known to play a role in mediating autonomic and neuroendocrine responses. Similarly, BN-LP distribution was consistent with previous reports in that high levels were present in the PVN,[90] and other hypothalamic areas including the arcuate nucleus, VMH, LH AH,[159,292,358] and limbic structures such as CeA.[78,323,332,358,504] It is particularly significant that, although there were no rat line differences in basal tissue levels of BN-LP, in comparison with Fast animals, the Slow rats displayed a higher basal level of CRH peptide content at the PVN, which is an important area in the regulation of the HPA axis and autonomic homeostasis.[5,34] This suggests the possibility that the generally more

anxious behavioral profile of the Slow rats [328] may be due, at least in part, to an up-regulated CRH system (compared to the Fast rats) within the HPA axis. Interestingly, in response to the consumption of a palatable snack, the difference between the Fast and Slow rats in CRH levels at the PVN disappeared. Meanwhile, in the Slow rats' (compared to the Fast animals) feeding-induced tissue levels of CRH were significantly higher in the VMH and medial amygdala, while BN-LP tissue levels were higher in the VMH only. The feeding-related changes in CRH and BN-LP tissue levels appear to parallel the generally more anxious profile of the Slow rats. However, they are a little difficult to explain in regard to feeding. In the first part of this experiment, we demonstrated that the Slow animals appear to have a greater propensity to consume a palatable snack than the Fast rats. As CRH and BN-LPs are known to suppress food intake [267], one might expect that the basal and feeding-induced levels of these peptides would be lower in the Slow rats compared to the Fast animals. However, it is important to keep in mind that analysis of neuropeptides in post mortem tissue at a single time point does not necessarily reflect the dynamic changes that may be occurring in response to a particular stimuli such as food intake. For example, such analyses cannot distinguish between alterations in synthesis, release or degradation of the peptide being examined. Perhaps the higher tissue levels of CRH and BN-LP observed at the VMH and, for CRH, at the medial amygdala means that the release of these peptides is being held back in the Slow rats, permitting greater consumption of the food in this rat line. Alternatively, it is possible that the higher CRH levels seen in the Slow animals (compared to the Fast) represent a degree of frustration at not being permitted to eat as much as they wanted as, in this particular phase of the experiment, rats were given a submaximal portion of

Graham Wafers. It is also important to mention that it is difficult to say whether the differences in peptidergic levels seen in the Fast and Slow rats represent any sort of deviation from normal. Since these rats were only compared to each other and not an outbred strain of animals, it is not known if the Slow rats are more reactive than the normal population or if the Fast rats are hyporesponsive compared to the normal population. In future, it would be interesting to compare the neurochemical responses of the Fast and Slow rats to an outbred strain of rats such as the Sprague Dawley or to a strain of rats that has been previously characterized.

It is interesting that in the VMH, the post ingestion tissue levels of both peptides tested (CRH and BN-LP) were significantly higher in the Slow rats compared to the Fast animals. The VMH was once regarded as the "satiety" center of the brain.[19,371] Although that notion has changed, the VMH is still recognized as being involved in energy balance and subsequent eating behavior.[162,371,387] Interestingly, it has been suggested that CRH provides a link between food intake and the environment and, thus, interest in the VMH has increased in part due to the characterization of CRF type 2 receptors within this nucleus.[30] Work in our lab, using the micropunch brain survey approach, indicates that BN-LPs may contribute to the regulation of the CRH hypothalamic system in that ICV administration of BN (0.25 and 0.5 μg) significantly decreased levels of immunoreactive CRH at various hypothalamic and extrahypothalamic brain sites, including many stress- (and feeding)-relevant regions such as the VMH, AH, PVN, NTS and CeA.[231] It is tempting to speculate that BN-LP and CRH may be interacting to increase the general arousal/vigilance in response to the biologically relevant stimulus of the palatable snack.

In terms of the feeding-related endocrine response, while both animals displayed a significant elevation of ACTH and corticosterone following consumption of the palatable snack, the elevation in ACTH noted in the Slow rats was significantly greater than in the Fast animals. Interestingly, this did not translate into a significant elevation in plasma levels of corticosterone in the Slow rats, while the Fast animals did display a feeding-induced increase in corticosterone. The elevation in ACTH noted following snack consumption is consistent with previous work done in our lab demonstrating a comparable rise in both ACTH and corticosterone following a similar snack intake.[311] The fact that the snack-induced rise in ACTH was greater in the Slow rats (compared to the Fast animals) is interesting in light of the fact that the Slow rats generally tend to exhibit greater signs of anxiety as compared to Fast rats.[328] Furthermore, the feeding-induced rise in ACTH also consistent with the higher basal levels of immunoreactive CRH at the PVN and the higher snack-induced immunoreactive CRH levels at the VMH and medial amygdala. Although highly speculative, these results may point to an upregulated CRH system(s) in the Slow rats perhaps leading to a "sensitization" of the HPA axis in this rat line. Consistent with this contention is the fact that Slow rats displayed increased stressor-induced levels of CRH at the PVN, median eminence/arcuate nucleus and the pituitary.[309] The fact that the dramatic increase in the level of circulating ACTH was not followed by a significant increase in corticosterone is interesting but difficult to explain. Alternatively, the discrepancy between ACTH and corticosterone release observed in the Slow rats may represent altered adrenal sensitivity, the possible influence of other secretagogues on adrenal responses and/or a ceiling effect that made differences between the strains undetectable.[309]

In summary, the Fast and Slow rats, selectively bred for differences in amygdalar excitability, displayed different propensities to consume a palatable snack. As anxiogenic behaviors and consumption of palatable substances may involve common GABAergic mechanisms, we exploited the opportunity to test animals with known GABAergic system differences, namely differences of amygdalar $GABA_{\alpha 1}$ receptor binding. It was observed that the Fast and Slow rats were readily distinguishable from each other not only with respect to the amount of palatable snack consumed, but also in endogenous CRH and BN-LP levels and endocrine levels, snack consumption in a novel (or stressful) environment, and their response to diazepam. Taken together, the findings indicate that these rat lines potentially represent a novel model for the study of genetic differences in palatable snack consumption. Furthermore, this model may be pertinent to the analyses of eating disorders associated with anxiety, including anorexia nervosa, bulimia, and obesity.

General Discussion

Any event (real or implied) that serves to shift an organism away from homeostasis (i.e., a threatening stimulus) can be referred to as a stressor, and requires a physiological response to restore equilibrium.[188,207,243,295,449] Such a response typically requires the expenditure of energy, which at some point needs to be replenished through nutrient ingestion.[392] However, if eating occurred tightly associated with energy store replenishment, obesity and other eating disorders, such as anorexia nervosa and bulimia, would not be as prevalent.[371,488,493] Clearly, feeding is a complex behavior governed by a variety of internal (organismic) and external (environmental, psychosocial) factors.[310,336] Evidence gathered over the last decade suggests that some of the internal signals that regulate ingestion arise from different levels of the gastrointestinal tract and may involve several different peptides, including the BN-LPs.[310] Interestingly, some of the brain peptidergic systems activated during ingestion are also triggered by external forces such as stressor exposure. One example of a peptide with such a "dual role" is CRH. While CRH is a primary mediator of an organism's characteristic physiological (endocrine, autonomic and immune) response to stressors and the maintenance of homeostasis,[114,374,437] there is also considerable evidence that this peptidergic system plays a physiological role the modulation of feeding behavior.[88,267] Likewise, while BN-LPs are well recognized for their role in satiety systems,[90,151,152,155,292,307,310,314,334,340] emerging evidence supports the notion that they may also play an important role in the modulation of the stress response.[308] It appears likely, then, that these peptidergic systems may be serving additional roles or be playing a much broader role than previously envisioned. Thus, the overall objective of this thesis was to elucidate the role

of BN-LPs and CRH in feeding and its anticipation, and to examine the intricate relationship between feeding and stress response.

CRH and BN-LPs are released in response to both aversive and appetitive events: Neurochemical approach

While stress is generally thought of in a negative light (distress), the definition cited earlier could include almost any external or cognitive stimulus that induces a shift away from equilibrium. In this context, a stressor may not necessarily be aversive, but could encompass appetitive events as well. As such, it follows that both aversive and appetitive events would share some neurochemical responses. Indeed, earlier experiments in this laboratory had revealed feeding status-related fluctuations in tissue levels of BN-LPs and CRH at feeding-relevant brain sites, including hypothalamic nuclei and the CeA.[378] However, much of the evidence collected had been indirect and relatively static in nature. In other words, altered regional peptide tissue concentrations only suggested potential involvement and failed to reveal if the utilization or release of these peptides was associated with various ingestive phases. Furthermore, these reflected a "snap shot" view that did not reveal the *in vivo* dynamics of peptidergic involvement. Accordingly, the initial set of experiments served to validate the use of *in vivo* microdialysis and push-pull perfusion to dynamically monitor peptide release in behaving animals. This method was then used to directly and conclusively establish whether CRH and BN-LPs were involved in the responses mounted by the body to deal with both aversive and/or appetitive events. In chapter I, using *in vivo* microdialysis, we demonstrated that both CRH and BN-LP(s) are released from the CeA (classically

thought to contribute to fear responses) in response to both restraint stressor exposure and spontaneous food ingestion. Since CRH-positive neurons are also known to project from the CeA to hypothalamic structures,[185] it was expected that changes in CRH release at the CeA might be reflected in stimulation of the HPA axis. In fact, significant increases in plasma ACTH and corticosterone accompanied the ingestion of a palatable snack as well as stressor exposure (chapter I). Next, in chapter II, we assessed whether the release of CRH and BN-LPs observed at the CeA, was reflected as increased availability of these peptides downstream at the anterior pituitary (an integral target for CRH action). For technical reasons, *in vivo* microdialysis could not be used at the anterior pituitary and, thus, *in vivo* push-pull perfusion was utilized. Results revealed that interstitial levels of BN-LPs (GRP and NMB) and CRH (but not AVP) were significantly elevated at the anterior pituitary following consumption of a palatable snack (Graham Wafers). It is noteworthy that, in a concurrent study, it was demonstrated that interstitial levels of CRH, AVP, NMB, GRP and corticosterone increased at the anterior pituitary in response to an air puff stressor. It was then of interest to determine whether the release of these peptides reflected a purely physiological process or whether it involved a psychological component. For example, was it simply the ingestion and processing of nutrients into the system that accounted for peptidergic utilization, or were psychological components of feeding behavior (such as the anticipation of the meal) contributing to this response? Again *in vivo* microdialysis was employed and revealed that CRH, BN-LP (GRP) and 5-HIAA were released at the mPFC (a site recognized for its role in higher cognitive functions such as attention, memory and learning, personality, intellect and decision

making processes[206,479]) in response to the "anticipation" of a palatable snack (chapter III).

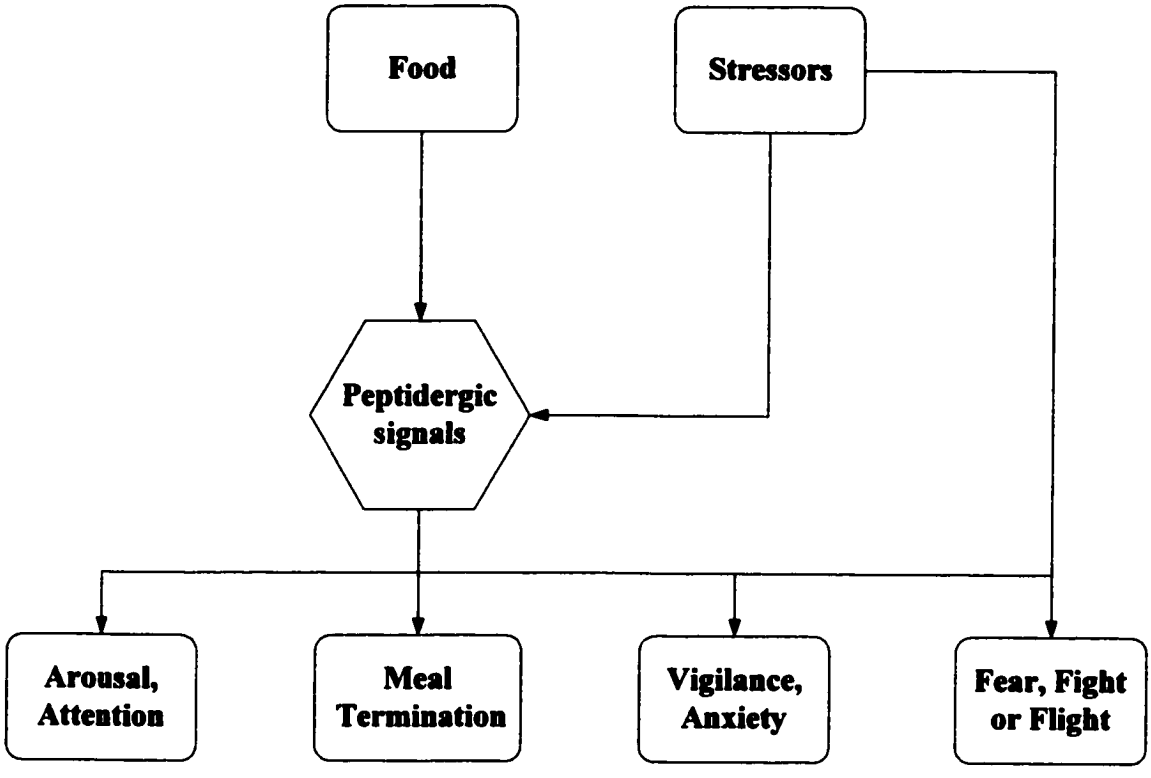
The intricate relationship between feeding and stress

The results of these experiments raised the intriguing possibility that some aspects of both the feeding behavior and the organism's response to stressor exposure are being subserved by a common neurochemical mechanism(s). At first blush, this appears to be counterintuitive. Why would such apparently dissimilar behaviors be subserved by a common neurochemical substrate? One possibility is that there may be an intricate relationship between stress and feeding. Exposure to a stressor induces activation of two separate but interrelated systems, the sympathetic division of the autonomic nervous system and the HPA axis.[449] The principal regulators of these two systems are NE and CRH respectively.[114,374,437,449] Although CRH promotes several behavioral effects, including reduction of food intake, it is primarily reputed as being a stress peptide, preparing an organism for fight, fright or flight responses.[114,374,437] Interestingly, it has been recently shown that the release of CRH can also be provoked by BN-LPs,[231] which are known chiefly for their role in mediating satiety.[310] An end result of the activation of these two systems (amongst many) is activation of the HPA axis, sympathetic nervous system and the mobilization of energy stores (glucose), required for mounting a stress response.[97,207] In the short run, stressor exposure diverts the organism away from such activities as feeding (having to deal with a charging bull is no time to grab a snack). However, in the long run, reacting to a stressor depletes energy stores, which then must be replenished.

Given the similarities in the neuroendocrine response to feeding and to stressors, one wonders whether, in fact, eating could be regarded as a stressor. There are indeed several attributes of food intake that could be interpreted as stressful for various species. For example, animals (as well as our early ancestors) must journey out into hostile territory to forage for food leaving them vulnerable to predatory attack. In such a situation, there is a need for extreme vigilance. Aggression, arousal and vigilance may also be necessary when hunting for food and protecting the catch from others. These issues may seem irrelevant to people in western society where food supplies are plentiful, and social governance makes meals enjoyable and safe experiences. It is possible, however, that relevant neurochemical signaling mechanisms may have remained intact through evolution, despite the socialization of this behavior (which potentially could involve overlying neocortical circuits). Indeed, it has been shown in humans that cortisol levels are increased in response to food intake.[220] Further to the same issue, the ingestion of food represents an influx of nutrients and foreign matter (which could potentially be toxic) into the body, which could perturb homeostasis. It would seem that CRH and BN-LPs might be serving a much broader role than had previously been envisioned. It is logical that, for functions as critical to the survival as feeding and response to stressor exposure, there would be overlapping and redundant systems. Thus, if one system became flawed through genetic mutation or disease, another system would be in place to act in a compensatory fashion. Finally, it can be hypothesized that stressors, to facilitate the flight or fight response, may deploy those physiological mechanisms normally used by the body for termination of a meal, to suppress the desire to eat while coping with an emergency situation. Regardless, as reflected in Figure 1, it has been our contention that,

rather than simply evoking feelings of fear and anxiety, these systems may serve to draw attention to events or cues that are biologically salient to the organism, such as those associated with food availability or those posing a threat to survival.

Figure 1: Why would stressors and appetitive events activate some of the same peptidergic systems?



Both ingestion and the response to stressor exposure may be utilizing similar neurochemical mechanism(s)/systems to draw attention to events or cues that are biologically salient to the organism. As such, these systems serve to induce arousal, meal termination, vigilance as well as fear and anxiety.

Impact of "anticipation"

When assessing the neurochemical concomitants of food ingestion, it may be important not only to assess the effects of ingestion itself, but also the anticipation, or incentive motivation, associated with a meal. Indeed, it was observed in the present investigation that, when non-food-deprived rats were offered a cue-paired palatable snack, the mere "anticipation" of the presentation of the snack (i.e., upon later presentation of these cues) was sufficient to induce the release of CRH at the mPFC (chapter III). This suggests that the release of CRH is relevant to the rewarding properties of food intake, and particularly the anticipation of food (incentive motivation). Importantly, in this particular experiment the animals had not been food-deprived, and hence the anticipation/ingestion of the snack likely represented hedonic responses, distinct from the processes associated with food intake and anticipation in a food-deprived organism (i.e., the reflection of hunger or hunger abatement).

Research concerning neurochemistry of reward processes has predominantly focused on the meso-limbic dopaminergic systems.[27,42,126,443] For example, it is generally accepted that the release of DA at certain central nervous system structures, such as the nucleus accumbens, plays a role in reward-related processes.[369] Furthermore, it has frequently been demonstrated that, in response to rewarding stimuli as well as conditioned predictors of reward, increased DA activity occurs within the mPFC.[126,416,443,496] Contrary to these previous findings, however, we found that, although there was a trend toward an increase of interstitial levels of DA at the right mPFC during the "anticipation" of the palatable snack, it did not reach statistical significance. As

indicated in chapter III, there could be many reasons for this lack of effect, including technical considerations and the animal's state of hunger. Ultimately, by assessing anticipatory processes it may be possible to discriminate between the contribution of such factors to the provocation of DA variations.

In contrast to DA, scant attention has been devoted to the role of peptidergic systems in the mediation of reward.[283] It was reported that CRH may actually decrease the rewarding effects of intracranial self-stimulation from the lateral hypothalamus.[283] However, the fact that a significant increase of interstitial levels of CRH was observed in response to cues associated with a palatable snack, it appears that this peptide is involved in reward processes or acts to draw attention to an event of biological significance. Yet it should be underscored that different neurochemicals do not serve in isolation, but rather work in concert in subserving different motivational states. In this respect, it is important to consider that, in response to the "anticipation" of the palatable reward, 5-HT turnover increased in the mPFC.

Chapters I, II and III highlight the importance of the CRH and BN-LP systems in the regulation of ingestive processes. One of the end results of the activation of the CRH system is the release of glucocorticoids. Glucocorticoids are generally thought to be released in response to stressor exposure, so much so that their release has been taken as evidence of events being appraised as being stressful.[206] Of course, adrenal glucocorticoid release is influenced by central neuronal processes and, thus, may reflect central states. For instance, DA (among other transmitters) appears to play a significant

role in the functioning of the HPA axis.[197] In this respect, intravenous cocaine administration, a dopaminergic agonist, increases plasma cortisol levels in humans,[29] and there is reason to believe that glucocorticoids may play a role in the mediation of reward process.[369] While the primary role of glucocorticoids in the periphery is to increase energy metabolism by stimulating glycogenolysis and gluconeogenesis,[369,412] glucocorticoids have many other functions.[412] In fact, as suggested in the present investigation and in a recent review,[369] glucocorticoids appear to fulfill the characteristics that ordinarily are assumed to reflect the biological substrates of reward. Specifically, glucocorticoids are secreted in response to rewarding stimuli such as food intake, a receptive sexual partner, or drugs of abuse.[369] Also, glucocorticoids increase reward-related behaviors such as food intake (but only when this behavior is naturally activated such as at the beginning of the activity period). Finally, animals will self-administer glucocorticoids indicating that they have intrinsic positive reinforcing effects.[369] On the basis of such findings it was argued that glucocorticoids may have a modulatory action, and their role may be to amplify the rewarding effects of positive reinforcers.[369] Thus, once again we see two apparently divergent states (related to stressor exposure and reward process) being modulated by a common neurochemical substrate (glucocorticoids). In effect, glucocorticoids may be important in helping an organism cope with and respond to a stressor-induced (either aversive or appetitive) shift from homeostasis.[369]

Factors that influence an organism's response to stressor exposure also impact feeding behavior: Neurochemical and Behavioral approaches

It appears that seemingly diverse stimuli and behaviors such as food intake, incentive reward, and stressor exposure activate common neurochemical substrates. If the stress response and feeding behavior are, at some level, subserved by common neurochemical mechanisms, it would be expected that variables that affect an organism's stress response would also have an impact on feeding behavior and related neurochemical processes. As previously mentioned, several variables are known to alter an organism's neurochemical stress response, including stressor characteristics (type of stressor, its controllability, predictability and chronicity), experiential (previous stressor history or early life experiences) and biological/organismic (age, genetics, gender) variables.[10] Chapters II, IV, V and VI of this thesis dealt with how some of the variables known to influence the stress response do, indeed, appear to influence feeding behavior and/or neurochemical responses to feeding. For example, in chapter II it was observed that prior exposure to a processive stressor (restraint) influenced the feeding-induced peptidergic availability of CRH, GRP and NMB at the anterior pituitary. In chapter IV, it was shown that early life experiences influenced neonatal growth and development, as well as the adult animal's propensity to consume a palatable snack and respond to known satiety peptides. Chapter V demonstrated that gender influenced the animals' penchant for a sweet snack, an effect also influenced by the phase of the females' estrus cycles. Finally, in chapter VI, using two lines of rats selectively bred for differences in amygdala excitability (reflected by either fast or slow development of amygdala kindling

epileptogenesis ("Fast" and "Slow" kindlers), it was demonstrated that the organism's genetic makeup influenced feeding.

Impact of previous stressor experiences.

In chapter II, we noted that basal interstitial levels of CRH, AVP, GRP, NMB and corticosterone at the anterior pituitary were significantly altered in animals previously exposed to repeated stressor episodes. Likewise, such effects were evident with the passage of time following a single episode of restraint stress. Furthermore, it was observed that the response to ingestion of a palatable snack was altered by prior exposure to a stressor. In fact there was a blunting of the rise in interstitial peptidergic levels ordinarily observed following stressor exposure. Several lines of investigation have revealed that the chronicity of stressor exposure influenced the organism's neurochemical response to subsequent exposure to a heterotypic (distinct) stressor.[472] Likewise, it was reported that, with time, following a single stressor experience, the neurochemical organization of the HPA axis was affected, in that many of the CRH neurons terminating at the median eminence began co-expression of AVP.[26,102,414,472] Since the HPA axis serves as an important arm of the hormonal system(s) regulating energy balance,[97] we hypothesized that this shift in neurochemical composition may have implications for energy balance and, consequently, feeding behavior and/or the neurochemical response to ingestion. Indeed, it was demonstrated that repeated stressor episodes (14 sessions) or a single episode of restraint followed by the passage of time (14 days) altered the basal levels of CRH, GRP and NMB. Furthermore, in cases where basal levels were high, animals displayed a blunted response to the ingestion of a palatable snack (chapter II).

As feeding was not measured directly, any suggestion that prior stressor history affected the animals' propensity to consume a palatable snack would remain speculative. However, it is commonly observed that food intake is reduced following stressor exposure.[17,123,404,432]

Summarizing, prior stressor exposure influenced the availability of both stress- and feeding-related peptides in a brain region that is pivotal in the regulation of the HPA axis, namely the anterior pituitary. The increased basal levels of these peptides along with the blunted peptidergic response to food intake could be relevant to eating disorders, particularly because dysregulation of the HPA axis has been associated with both obesity and anorexia nervosa.[49,318,364] It has been reported that hypercortisolism, present in underweight anorectic patients (along with a blunted ACTH response to CRH), persists after short-term weight restoration, but disappears once weight gain has stabilized.[318] It is tempting to speculate that systems using BN-LPs might similarly be involved in the disturbed feeding patterns associated with eating disorders, and could potentially provide new avenues for therapeutic strategies.

Impact of early life experiences.

As already discussed, early life experiences may profoundly influence behaviors in adulthood. In rodents, extended periods of separation from the dam (3 hours/day over 7-14 days) increase adult HPA reactivity to stressors, whereas brief periods of postnatal stimulation (handling) increase adult stress resilience, an effect that may be mediated in part by differences in maternal care of infants in the different handling

conditions.[15,254,291,299,303,304,424] In the present investigation we demonstrated that early life events also affected appetitive responses. Specifically, in chapter IV, we reported that, at weaning, rats that had been exposed to protracted periods of maternal separation weighed significantly less than both the briefly handled or the non-handled animals. Male rat pups that were exposed daily to brief handling ingested significantly more palatable snack compared to rats exposed to a more protracted (3-hour) period of maternal separation, or rats that were not handled. Interestingly, female rats in both treatment groups ate significantly more than non-handled female animals. Also, handled rats, particularly the females, showed a blunted response to systemically administered BN or amylin (a pancreatic polypeptide known to suppress food intake.[59,60] This is interesting in light of the fact that pups that are exposed to daily brief periods of handling (10-15 minutes of separation from the dam) for the first 2-3 weeks of life show reduced CRH mRNA.[301,380] While it is uncertain whether mRNA for BN-LPs or amylin are reduced in handled animals, the available evidence continues to support the contention that feeding and stress share overlapping neurochemical processes. Indeed, it was demonstrated in the elevated plus maze test that handled male rats (and females from both treatment groups) displayed less anxious behavior than their non-handled cohorts. Once more the mechanism(s) governing this effect remain to be elucidated. However, here, again, the HPA axis appears to be involved.[304]

Meaney and his colleagues have shown that CRH expression is reduced in adult animals subjected to brief periods of neonatal handling, an effect attributed to enhanced glucocorticoid negative feedback.[301,380] Since CRH is known to reduce food intake,[392] a

blunted CRH release during feeding in the neonatal handled animals may account for enhanced snack consumption (or attenuated suppression of snack intake). This would not account for the increased snack consumption and decreased anxiety observed in the female rats that were exposed to more protracted, 3-hour periods of maternal separation, since such maternal separation is typically characterized by enhanced CRH mRNA expression. However, increased mRNA expression does not necessarily translate into peptide release or utilization. A more direct analysis through *in vivo* microdialysis may provide further clues to the dynamics involved. Future studies could examine peptidergic release in response to both stressor exposure and palatable food intake in animals with varied early life experience, across various stress- and feeding-relevant nuclei. Overall, it is tempting to speculate that early life experience, which influences the stress response, may also affect the animal's sensitivity to satiety signals. This could potentially account for some of the individual variability seen in feeding behaviors and may contribute to disordered eating patterns observed in anorexia nervosa, bulimia and obesity.

Impact of gender.

Organismic variables including gender are known to influence the stress response.[308] For instance, male and female mice display opposite reactions to social stressors on anxiety behavior,[356] and it has been demonstrated that female mice secrete significantly more ACTH than males in response to footshock, a difference abolished by ovariectomy.[395] In chapter V of this thesis, we explored the relationship between gender and feeding behavior. It was observed that female Long Evans rats consumed significantly more of a palatable snack compared to their male counterparts. In fact,

when the short snack access was a part of their daily diet, the females exhibited significantly greater total intake compared to the males. This may be significant given that eating disorders such as anorexia nervosa are found predominantly in young females.[488] A gender difference was noted again in chapter VI where Slow female rats consumed more sucrose compared to their male counterparts. Interestingly, in the neonatal handling study (chapter IV) gender differences were attributable to handling conditions, and were not present in control (or non-handled) animals. Since the Fast and Slow animals were derived from Long Evans rats and the animals in the neonatal experiment were Sprague-Dawleys, genetic factors may also have contributed to the observed outcomes. Nonetheless, it has been shown that males and females display different patterns of food intake.[79] Males tend to eat more in the morning while females have smaller meals in the morning but increase their intake toward evening.[480] Similarly, male rats consume approximately 65% of their total intake during the dark cycle whereas females eat 80% during the dark and significantly less than males during the light cycle.[260] Also, male rats maintain a constant intake by increasing meal size and decreasing meal number over time, while females display fluctuations in both meal number and meal size and these effects vary over their estrus cycle.[79,260] The mechanisms underlying gender differences have still not been fully elucidated, but hormonal fluctuations have been the main target of investigation. It is generally agreed that there is a close association between feeding and gonadal hormones, particularly plasma estrogens, which have a regulatory role in locomotor activity and metabolism as well as food intake.[144]

Impact of genetic differences.

There is ample evidence that genetic factors contribute to the individual variations in response to stressors.[18,227] For example, transgenic mice that exhibit elevated plasma levels of ACTH and glucocorticoids, also display an increase in anxiogenic behavior, an effect reversed by the CRH antagonist, alpha-helical CRF.[447] Conversely, mice deficient in CRF-R_1 gene expression displayed increased exploratory behavior in both the elevated plus maze and black/white box, indicating that depletion of this receptor has an anxiolytic effect.[82] Genetic links to feeding have classically been studied utilizing animal models such as the Zucker obese rats, an animal model of genetic obesity.[406] In Chapter VI, two selectively bred rat lines, the Fast and Slow rats, known to differ in their response to stressor exposure,[309] were used to explore the impact of genetic factors on food intake. It was shown that the Slow rats consumed more of the palatable snack compared to the Fast rats, were more sensitive to the orexigenic effects of diazepam, and appeared more anxious (in the novel cage paradigm). These results are consistent with the earlier findings indicating that the Slow kindling rats are more anxious and fearful than their Fast kindling counterparts,[298,328] although the behavioral and neurochemical manifestations of their anxiety appear to be situation-specific.[312] Post mortem tissue analysis revealed that feeding-related fluctuations in CRH and BN-LP (GRP) levels, were higher in the VMH of the Slow animals (compared to the Fast animals). Also, while basal levels of BN-LP (GRP) were similar in these two rat lines, basal CRH levels were elevated in the PVN of Slow animals, and feeding-induced levels of CRH were significantly higher in the medial amygdala. Further, basal levels of ACTH and corticosterone were similar, but the increase in plasma ACTH levels following feeding

was significantly greater in the Slow rats compared to their Fast counterparts, although this did not translate into greater (than Fast animals) levels of corticosterone.

Implications and future directions

The evidence presented in this dissertation indicates that a) CRH and BN-LPs are released in response to both aversive (restraint) and appetitive (anticipation and ingestion) events, b) this occurs in three interconnected nuclei (the CeA, mPFC and anterior pituitary) and c) factors that influence an organism's response to stressor exposure also impact feeding. While CRH has long been recognized (and studied) for its role as a key mediator of an organism's characteristic physiological response to stressors,[114,308,374,437] BN-LPs have classically been regarded as satiety peptides, capable of potently reducing food intake.[310] Interestingly, CRH has also been implicated in the control of food intake,[88,267] and recent work in our laboratory has revealed that BN-LPs are capable of stimulating the HPA axis by acting through CRH and/or AVP.[308] These observations are of particular interest in light of the fact that the HPA axis is not only a major regulator of the stress response but also a key regulator of energy flow.[5,97] As such, dysregulation of the HPA axis and hyperactivity of CRH may be involved in a number of feeding disorders such as anorexia nervosa and obesity[49,364] as well as neuropsychiatric disorders such as depression and anxiety.[175,318] Moreover, it has been suggested that certain anxiety disorders may represent antecedent risk factors for anorexia nervosa and bulimia nervosa.[70]

The first three chapters of this thesis used *in vivo* microdialysis and push-pull perfusion to assess dynamic changes in CRH and BN-LP release. It is important to note that, due to technical requirements (the permeability of the microdialysis probe and the quantity of sample required for RIA peptide analysis) we were restricted to 20-min sample pools. For certain events, such as response to stressors and cues for meal anticipation, initiation and termination, neurochemical fluctuations would be expected to occur very rapidly (in seconds or minutes). In future, in order to unmask such potentially rapid fluctuations, it would be of value to develop the technology that required smaller sample size (more sensitive detection methods) to gain a better time resolution. In this respect, Cook[84] has recently developed a new immunosensor probe (containing an internal antibody-coated platinum electrode), which substantially improves the time resolution of CRH and/or cortisol detection (2-min sampling periods) measurement in brain (sheep).[84] Further, given the interactive processes governing feeding and the stress response, it would be of interest to concurrently sample from multiple brain sites. This would not only facilitate a better understanding of the temporal dynamics within the circuits of interest, but would also help unravel potential interactions between distinct peptidergic cascades involved in the regulation of food ingestion and responses to stressor exposure.

Clinical Implications

It has been our contention that BN-LPs are released from the gastrointestinal tract in response to food ingestion to bridge the gut and brain through neurocrine means, to inhibit further food intake.[310] Given the role of CRH (and thus the HPA axis) in

anxiety,[175] it is interesting that BN-LPs appear to work in concert with CRH (and possibly AVP) to stimulate the HPA axis.[308] It is noteworthy that the father of William James, an early American psychologist, suffered his first (of many) panic attacks whilst lounging idly, "thinking of nothing", following a "comfortable" meal.[125] Any conclusions that one might draw from such a serendipitous observation remain highly speculative. However, it is tempting to consider the possibility that eating could be involved in the precipitation of a panic attack in individuals prone to such attacks. This notion also lends itself to the suggestion that, as well as there being a need for new antidepressant and anxiolytic drugs,[227] there may be a place for diet therapy as a possible adjunct to drug therapy in anxiety-related disorders.

Our findings may have other therapeutic implications as well. For instance, if the response to stressors and feeding comprise common neurochemical substrates, then one could envision commonalties in therapeutic targets for stress-and eating-related disorders. In keeping with this idea, selective serotonin reuptake inhibitors (which are known to effectively treat depression and some anxiety disorders) are effective in treating bulimic and anorexic patients (once normal weight has been restored).[49,124] Conversely, such drugs used in the treatment of depression, often affect appetite and weight gain. Similarly, CRH and/or BN receptor antagonists might also be effective in treating not only stress-related disorders but also eating disorders. In fact, phase 1 clinical trials currently in progress suggest a high efficacy and low side-effect profile for CRH-R_1 antagonists against mental and physical disorders that are exacerbated or precipitated by stressors.[175] As mentioned earlier, anxiety appears to be antecedent to the development

of eating disorders.[70] By way of illustration, when animals are exposed to a new environment (presumed to be anxiogenic) and given a familiar palatable snack, they will generally consume less than they would in their home cage. A study in our laboratory revealed that, when treated with a NMB antagonist (PD 176252), the feeding suppressant effects of the novel environment were attenuated.[308] Since eating disorders are so closely related to anxiety, these novel drugs might provide a new and effective strategy for targeting such disorders.

The research contained in this thesis challenges the traditionally held views regarding the physiological roles of CRH and BN-LPs. It adds to the growing body of evidence suggesting that both these peptide families are involved in stress/anxiety and feeding behaviors. These experiments extend our current knowledge of the mechanism(s) underlying the control of food intake and support our contention that, rather than simply evoking feelings of fear/anxiety or satiety, these systems may serve to draw an organism's attention to events or cues that are biologically relevant. In this regard, the present studies should provide new insights into the mechanism(s) underlying various stress and eating dysfunctions, including anorexia nervosa, anorexia associated with AIDS, cancer, sickness or stress, bulimia nervosa, obesity and depression.

Bibliography

1. Advokat, C. and Kutlesic, V., (1995). Pharmacotherapy of the Eating Disorders: A Commentary, *Neuroscience and Biobehavioral Reviews*, **19**[1], 59-65.

2. Aguilera, G., Rabadan-Diehl, C., and Nikodemova, M., (2001). Regulation of pituitary corticotropin releasing hormone receptors, *Peptides*, **22**, 769-774.

3. Ahn, S. and Phillips, A.G., (1999). Dopaminergic correlates of sensory-specific satiety in the medial prefrontal cortex and nucleus accumbens of the rat, *Journal of Neuroscience*, **19**, RC29.

4. Al-Naser, H.A.H.C., (1994). A-68930, a novel, potent dopamine D1 receptor agonist: a microstructural analysis of its effects on feeding and other behaviour in the rat, *Behavioural Pharmacology*, **5**, 210-218.

5. Akana, S.F., Strack, A.M., Hanson,E.S., and Dallman,M.F., (1994). Regulation of activity in the hypothalamo-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow, *Endocrinology*, **135**, 1125-1134.

6. Akeson, M., Sainz, E., Mantey,S.A., Jensen,R.T., and Battey,J.F., (1997). Identification of Four Amino Acids in the Gastrin-releasing Peptide Receptor That are Required for High Affinity Agonist Binding, *The Journal of Biological Chemistry*, **272**, 17405-17409.

7. Al-Damluji, S., Iveson, T., Thomas, J.M., Pendlebury, D.J., Rees, L.H., and Besser, G.M., (1987). Food-induced cortisol secretion is mediated by central alpha-1 adrenoreceptor modulation of pituitary ACTH secretion, *Clinical Endocrinology*, **26**, 629-636.

8. Anastasi, A., Erspamer, V., and Bucci, M., (1971). Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes, *Experientia*, **27**, 166-167.

9. Anisman, H., Kelly, O., Hayley, S., Borowski, T., Merali, Z., and McIntyre, D.C., (2000). Acoustic startle and fear-potentiated startle in rats selectively bred for fast and slow kindling rates: relation to monoamine activity, *European Journal of Neuroscience*, **12**, 4405-4416.

10. Anisman, H. and Merali, Z., (1999). Understanding stress: characteristics and caveats, *Alcohol Research & Health: The Journal of the National Institute on Alcohol Abuse & Alcoholism*, **23**, 241-249.

11. Anisman, H., Zaharia, M.D., Meaney, M.J., and Merali, Z., (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors?, *International Journal of Developmental Neuroscience*, **16**, 149-164.

12. Atkinson, H.C. and Waddell, B.J., (1997). Circadian Variation in Basal Plasma Corticosterone and Adrenocorticotropin in the Rat: Sexual Dimorphism and Changes across the Estrous Cycle, *Endocrinology*, **138**, 3842-3848.

13. Attia, E., Haiman, C., Walsh, B., and Flater, S.R., (1998). Does Fluoxetine Augment the Inpatient Treatment of Anorexia Nervosa, *American Journal of Psychiatry*, **155**, 548-551.

14. Au, C.L., Canny, B.J., Farnworth, P.G., and Giraud, A.S., (1997). Bombesin regulation of adrenocorticotropin release from ovine anterior pituitary cells, *Peptides*, **18**, 995-1000.

15. Avishai-Eliner, S., Yi, S. -J., Newth, C.J.L., and Baram, T.Z., (1995). Effects of maternal and sibling deprivation on basal and stress induced hypothalamic-pituitary-adrenal components in the infant rat, *Neuroscience Letters*, **192**, 49-52.

16. Babcock, A.M., Avery, D.D., and Maitland, K.L., (1985). Suppression of operant responding for food and water reinforcement following intraventricular injections of Bombesin in the rat, *Life Sciences*, **36**, 981-985.

17. Badiani, A., Jakob, A., Rodaros, D., and Stewart, J., (1996). Sensitization of stress-induced feeding in rats repeatedly exposed to brief restraint: the role of corticosterone, *Brain Research*, **710**, 35-44.

18. Bakshi, V.P. and Kalin, N.H., (2000). Corticotropin-releasing hormone and animal models of anxiety: gene-environmental interactions, *Biological Psychiatry*, **48**, 1175-1198.

19. Balagura, S., (1972). Neurophysiologic Aspects: Hypothalamic Factors in the Control of Eating Behavior, *Advances in Psychosomatic Medicine*, **7**, 25-48.

20. Balasubramaniam, A., (2002). Clinical potentials of neuropeptide Y family of hormones, *The American Journal of Surgery*, **183**, 430-434.

21. Baldwin, B.A., Ebenezer, I.S., and De La Riva C., (1990). Effects of intracerebroventricular injection of muscimol or GABA on operant feeding in pigs, *Physiology & Behavior*, **48**, 417-421.

22. Bale, T.L., Contarino, A., Smith, G.W., Chan, R., Gold, L.H., Sawchenko, P.E., Koob, G.F., Vale, W.W., and Lee, K.F., (2000). Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress, *Nature Genetics*, **24**, 410-414.

23. Banks, W.A. and Kastin, A.J., (1990). Exchange of peptides between the circulation and the nervous system: role of the blood-brain barrier, *Advances in Experimental Medicine & Biology*, **274**, 59-70.

24. Bannon, A.W., Seda, J., Carmouche, M., Francis, J.M., Norman, M.H., Karbon, B., and McCaleb, M.L., (2000). Behavioral characterization of neuropeptide Y knockout mice, *Brain Research*, **868**, 79-87.

25. Barden, N., Reul, J.M.H.M., and Holsboer, F., (1995). Do antidepressants stabilize mood through actions on the hypothalamic-pituitary-adrenocortical system? *Trends in Neurosciences*, **18**, 6-11.

26. Bartanusz, V., Jezova, D., Bertini, L.T., Tilders, F.J., Aubry, J.M., and Kiss, J.Z., (1993). Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons, *Endocrinology*, **132**, 895-902.

27. Bassareo, V. and Di Chiara, G., (1997). Differential Influence of Associative and Nonassociative Learning Mechanisms on the Responsiveness of Prefrontal and Accumbal Dopamine Transmission to Food Stimuli in Rats Fed *Ad Libitum*, *The Journal of Neuroscience*, **17**, 851-861.

28. Basso, A.M. and Kelley, A.E., (1999). Feeding induced by GABA(A) receptor stimulation within the nucleus accumbens shell: regional mapping and characterization of macronutrient and taste preference, *Behavioral Neuroscience*, **113**, 324-336.

29. Baumann, M.H., Gendron, T.M., Becketts, K.M., Henningfield, J.E., Gorelick, D.A., and Rothman, R.B., (1995). Effects of intravenous cocaine on plasma cortisol and prolactin in human cocaine abusers, *Biological Psychiatry*, **38**, 751-755.

30. Beck, B., (2000). Neuropeptides and obesity, *Nutrition*, **16**, 916-923.

31. Beck, B., Burlet, A., Bazin, R., Nicolas, J.P., and Burlet, C., (1993). Elevated Neuropeptide Y in the Arcuate Nucleus of Young Obese Zucker Rats May Contribute to the Development of Their Overeating, *Journal of Nutrition*, **123**, 1172.

32. Behan, D.P., Grigoriadis, D.E., Lovenberg, T., Chalmers, D., Heinrichs, S., Liaw, C., and De Souza, E.B., (1996). Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders, *Molecular Psychiatry*, **1**, 255-277.

33. Beinfeld, M.C., (2001). An introduction to neuronal cholecystokinin, *Peptides*, **22**, 1197-1200.

34. Bell, M.E., Bhatnagar, S., Akana, S.F., Choi, S., and Dallman, M.F., (2000). Disruption of arcuate/paraventricular nucleus connections changes body energy balance and response to acute stress, *Journal of Neuroscience*, **20**, 6707-6713.

35. Berridge, K.C., (1996). Food reward: brain substrates of wanting and liking, *Neuroscience & Biobehavioral Reviews*, **20**, 1-25.

36. Berridge, K.C. and Pecina, S., (1995). Benzodiazepines, appetite, and taste palatability, *Neuroscience & Biobehavioral Reviews*, **19**, 121-131.

37. Beverly, J.L. and Martin, R.J., (1989). Increased GABA shunt activity in VMN of three hyperphagic rat models, *American Journal of Physiology*, **256**, R1225-R1231.

38. Bielajew, C. and Trzcinska, M., (1998). Activation of reward-relevant neurons in the caudate-putamen influences the development of medial prefrontal cortex self-stimulation: a moveable electrode mapping study, *Acta Neurobiologiae Experimentalis*, **58**, 189-198.

39. Billington, C.J., Levine, A.S., and Morley, J.E., (1983). Are peptides truly satiety agents? A method of testing for neurohumoral satiety effects, *American Journal of Physiology*, **245**, R920-R926.

40. Birk, J. and Noble, R.G., (1982). Bicuculline blocks diazepam-induced feeding in Syrian hamsters, *Life Sciences*, **30**, 321-325.

41. Bittencourt, J.C. and Sawchenko, P.E., (2000). Do Centrally Administered Neuropeptides Access Cognate Receptors?: An Analysis in the Central Corticotropin-Releasing Factor System, *The Journal of Neuroscience*, **20**, 1142-1156.

42. Blackburn, J.R., Phillips, A.G., Jakubovic, A., and Fibiger, H.C., (1989). Dopamine and preparatory behavior: II. A neurochemical analysis, *Behavioral Neuroscience*, **103**, 15-23.

43. Blood, A.J.Z.R.J., (2001). Intensely pleasurable responses to music correlate with activity in brain regions implicated in reward and emotion, *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 11818-11823.

44. Blum, K., Braverman, E.R., Holder, J.M., Lubar, J.F., Monastra, V.J., Miller, D., Lubar, J.O., Chen, T.J., and Comings, D.E., (2001). Reward deficiency syndrome: a biogenetic model for the diagnosis and treatment of impulsive, addictive, and compulsive behaviors, *Journal of Psychoactive Drugs*, **32**, Suppl i-iv.

45. Blundell, J., (1991). Pharmacological approaches to appetite suppression, *Trends in Pharmacological Science*, **12**, 147-157.

46. Blundell, J.E. and Halford, J.C.G., (1998). Serotonin and Appetite Regulation, *Pharmacology and Pathophysiology*, **9**, 473-495.

47. Bodnoff, S.R., Suranyi-Cadotte, B., Quirion, R., and Meaney, M.J., (1987). Postnatal handling reduces novelty-induced fear and increases [3H]flunitrazepam binding in rat brain, *European Journal of Pharmacology*, **144**, 105-107.

48. Bodnoff, S.R., Suranyi-Cadotte, B.E., Quirion, R., and Meaney, M.J., (1989). Role of the central benzodiazepine receptor system in behavioral habituation to novelty, *Behavioral Neuroscience*, **103**, 209-212.

49. Bolis, C.L., Abraham, G., Araki, S., Brambilla, F., Brewerton, T., Casper, R., Halmi, K., Herholz, K., Kaye, W., Kuboki, T., Licinio, J., Magistretti, P., Müller, E., Negrao, A., N'Diaye, P., Pirke, K., Swematsu, H., Thomas, P.K., Wakeling, T., and Zhou, D., (1995). Anorexia Nervosa: Directions for Future Research, *International Journal of Eating Disorders*, **17**, 235-241.

50. Bonavera, J.J., Dube, M.G., Kalra, P.S., and Kalra, S.P., (1994). Anorectic effects of estrogen may be mediated by decreased neuropeptide-Y release in the hypothalamic paraventricular nucleus, *Endocrinology*, **134**, 2367-2370.

51. Bonsall, R.W., Emery, M.S., and Weiss, J.M., (1996). Corticosterone levels in rat brain microdialysates following exposure to noise stress or lipopolysaccharide, *Society for Neuroscience Abstracts.*

52. Bonson, K.R., Grant, S.J., Contoreggi, C.S., Links, J.M., Metcalfe, J., Weyl, H.L., Kurian, V., Ernst, M., and London, E.D., (2002). Neural systems and cue-induced cocaine craving, *Neuropsychopharmacology*, **26**, 376-386.

53. Boushaki, F.Z., Rasio, E., and Serri, O., (1997). Hypothalamic-pituitary-adrenal axis in abdominal obesity: effects of dexfenfluramine, *Clinical Endocrinology*, **46**, 461-466.

54. Bradbury, M.J., McBurnie, M.I., Denton, D.A., Lee, K.F., and Vale, W.W., (2000). Modulation of Urocortin-Induced Hypophagia and Weight Loss by Corticotropin-Releasing Factor Receptor 1 Deficiency in Mice, *Endocrinology*, **141**, 2715-2724.

55. Bradwejn, J. and Koszycki, D., (1994). The cholecystokinin hypothesis of anxiety and panic disorder, *Annals of the New York Academy of Sciences*, **713**, 273-282.

56. Bradwejn, J., Koszycki, D., and Payeur, R., (1991). Cholecystokinin Tetrapeptide: a Provocation Agent for Research in Panic Disorder? In: Briley, M., File, S.E. (Eds.), *New Concepts in Anxiety*, The Macmillan Press Ltd, Boca Raton, FL, 313-319.

57. Bradwejn, J. and de Montigny, C., (1984). Benzodiazepines antagonize cholecystokinin-induced activation of rat hippocampal neurones, *Nature*, **312**, 363-364.

58. Brake, W.G., Sullivan, R.M., and Gratton, A., (2000). Perinatal distress leads to lateralized medial prefrontal cortical dopamine hypofunction in adult rats, *Journal of Neuroscience*, **20**, 5538-5543.

59. Bray, G.A., (1995). Obesity, Fat Intake, and Chronic Disease. In: Bloom, F.E., Kapfer, D.J. (Eds.), *Psychopharmacology: The Fourth Generation of Progress*, Raven Press, New York, 1591-1608.

60. Bray, G.A., (2000). Afferent signals regulating food intake, *Proceedings of the Nutrition Society*, **59**, 373-384.

61. Bray, G.A., (2000). Reciprocal relation of food intake and sympathetic activity: experimental observations and clinical implications, *International Journal of Obesity & Related Metabolic Disorders*, **24**, Suppl 2, S8-17.

62. Britton, D.R., Koob, G.F., Rivier, J., and Vale, W., (1982). Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty, *Life Sciences*, **31**, 363-367.

63. Britton, K.T., Akwa, Y., Spina, M.G., and Koob, G.F., (2000). Neuropeptide Y blocks anxiogenic-like behavioral action of corticotropin-releasing factor in an operant conflict test and elevated plus maze, *Peptides*, **21**, 37-44.

64. Britton, K.T. and Southerland, S., (2001). Naloxone blocks 'anxiolytic' effects of neuropeptide Y, *Peptides*, **22**, 607-612.

65. Broqua, P.P.W., (1995). Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures, *Behavioural Pharmacology*, **6**, 215-222.

66. Brown, C.M., Coscina, D.V., and Fletcher, P.J., (2000). The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens, *Peptides*, **21**, 1279-1287.

67. Brown, M.R., Marki, W., and Rivier, J., (1980). Is Gastrin Releasing Peptide Mammalian Bombesin? *Life Sciences*, **27**, 125-128.

68. Brown, P. and Molliver, M.E., (2000). Dual Serotonin (5-HT) Projections to the Nucleus Accumbens Core and Shell: Relation of the 5-HT Transporter to

Amphetamine-Induced Neurotoxicity, *The Journal of Neuroscience*, **20**, 1952-1963.

69. Buijs, R.M. and Van Eden, C.G., (2000). The integration of stress by the hypothalamus, amygdala and prefrontal cortex: balance between the autonomic nervous system and the neuroendocrine system, *Progress in Brain Research*, **126**, 118-132.

70. Bulik, C.M., Sullivan, P.F., Fear, J.L., and Joyce, P.R., (1997). Eating disorders and antecedent anxiety disorders: a controlled study, *Acta Psychiatrica Scandinavica*, **96**, 101-107.

71. Burlet, A.J., Jhanwar-Uniyal, M., Chapleur-Chateau, M., Burlet, C.R., and Leibowitz, S.F., (1992). Effect of food deprivation and refeeding on the concentration of vasopressin and oxytocin in discrete hypothalamic sites, *Pharmacology, Biochemistry & Behavior*, **43**, 897-905.

72. Bushnik, T., Bielajew, C., Konkle, A.T., and Merali, Z., (1999). Influence of bombesin on threshold for feeding and reward in the rat, *Acta Neurobiologiae Experimentalis*, **59**, 295-302.

73. Campfield, L.A., Smith, F.J., and Burn, P., (1998). Strategies and Potential Molecular Targets for Obesity Treatment, *Science*, **280**, 1383-1387.

74. Carlson, J.N., Fitzgerald, L.W., Keller, R.W., Jr., and Glick, S.D., (1993). Lateralized changes in prefrontal cortical dopamine activity induced by controllable and uncontrollable stress in the rat, *Brain Research*, **630**, 178-187.

75. Carlson, N.R., (1999). Psychopharmacology. In: Merrill, C. (Ed.), *Foundations of Physiological Psychology*, Allyn and Bacon, Boston, 86-112.

76. Carr, D.B. and Sesack, S.R., (2000). GABA-Containing Neurons in the Rat Ventral Tegmental Area Project to the Prefrontal Cortex, *Synapse*, **38**, 114-123.

77. Carr, D.B. and Sesack, S.R., (2000). Projections from the Rat Prefrontal Cortex to the Ventral Tegmental Area: Target Specificity in the Synaptic Associations with Mesoaccumbens and Mesocortical Neurons, *The Journal of Neuroscience*, **20**, 3864-3873.

78. Chronwall, B.M., Pisano, J.J., Bishop, J.F., Moody, T.W., and O'Donohue, T.L., (1985). Biochemical and Histochemical Characterization of Ranatensin Immunoreactive Peptides in Rat Brain: Lack of Coexistence with Bombesin/GRP, *Brain Research*, **338**, 97-113.

79. Clarke, S.N. and Ossenkopp, K.P., (1998). Taste reactivity responses in rats: influence of sex and the estrous cycle, *American Journal of Physiology*, **274**, R718-R724.

80. Commons, K.G., Kow, L.M., Milner, T.A., and Pfaff, D.W., (1999). In the ventromedial nucleus of the rat hypothalamus, GABA-immunolabeled neurons are abundant and are innervated by both enkephalin- and GABA-immunolabeled axon terminals, *Brain Research*, **816**, 58-67.

81. Cone, R.D., (2000). The corticotropin-releasing hormone system and feeding behavior--a complex web begins to unravel. *Endocrinology*, **141**, 2713-2714.

82. Contarino, A., Delprete, E., Koob, G.F., Smith, G.W., Lee, K.F., Vale, W., and Gold, L.H., (1999). Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1, *Brain Research*, **835**, 1-9.

83. Cook, C., Gatchair-Rose, A., Herminghuysen, D., Nair, R., Prasad, A., Mizuma, H., and Prasad, C., (1994). Individual differences in the macronutrient preference profile of outbred rats: implications for nutritional, metabolic, and pharmacologic studies, *Life Sciences*, **55**, 1463-1470.

84. Cook, C.J., (2001). Measuring of extracellular cortisol and corticotropin-releasing hormone in the amygdala using immunosensor coupled microdialysis, *Journal of Neuroscience Methods*, **110**, 95-101.

85. Cooper, S.J., (1989). Benzodiazepine receptor-mediated enhancement and inhibition of taste reactivity, food choice, and intake, *Annals of the New York Academy of Sciences*, **575**, 321-336.

86. Cooper, S.J. and Moores, W.R., (1985). Benzodiazepine-induced hyperphagia in the nondeprived rat: comparisons with CL 218,872, zopiclone, tracazolate and phenobarbital, *Pharmacology, Biochemistry & Behavior*, **23**, 169-172.

87. Cooper, S.J., (1996). Cholecystokinin Modulation of Serotonergic Control of Feeding Behavior, *Annals of the New York Academy of Sciences*, **780**, 213-222.

88. Coste, S.C., Murray, S.E., and Stenzel-Poore, M.P., (2001). Animal models of CRH excess and CRH receptor deficiency display altered adaptations to stress, *Peptides*, **22**, 733-741.

89. Costela, C., Tejedor-Real, P., Mico, J.A., and Gibert-Rahola, J., (1995). Effect of Neonatal Handling on Learned Helplessness Model of Depression. *Physiology and Behavior* **57**, 407-410.

90. Costello, J.F., Brown, M.R., and Gray, R.S., (1991). Bombesin immunoreactive neurons in the hypothalamic paraventricular nucleus innervate the dorsal vagal complex in the rat, *Brain Research*, **542**, 77-82.

91. Craig, A.D., (1992). Spinal and trigeminal lamina I input to the locus coeruleus anterogradely labeled with phaseolus vulgaris leucoagglutinin (PHA-L) in the cat and the monkey, *Brain Research*, **584**, 325-328.

92. Crawley, J.N., (1985). Clarification of the Behavioral Functions of Peripheral and Central Cholecystokinin: Two Separate Peptide Pools, *Peptides*, **6**, 129-136.

93. Critchley, H.D., Mathias, C.J., and Dolan, R.J., (2001). Neural activity in the human brain relating to uncertainty and arousal during anticipation, *Neuron*, **29**, 537-545.

94. Cullinan, W.E., (2000). GABA(A) receptor subunit expression within hypophysiotropic CRH neurons: a dual hybridization histochemical study, *Journal of Comparative Neurology*, **419**, 344-351.

95. Cummings, S., Elde, R., Ells, J., and Lindall, A., (1983). Corticotropin-Releasing Factor Immunoreactivity is Widely Distributed within the Central Nervous System of the Rat: An Immunohistochemical Study, *The Journal of Neuroscience*, **3**, 1355-1368.

96. D'Amore, A., Mazzucchelli, A., and Loizzo, A., (1995). Long-Term Changes Induced by Neonatal Handling in the Nociceptive Threshold and Body Weight in Mice, *Physiology and Behavior*, **57**, 1195-1197.

97. Dallman, M.F., Akana, S.F., Strack, A.M., Hanson, E.S., and Sebastian, R.J., (1995). The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site

proximal to CRF neurons, *Annals of the New York Academy of Sciences*, **771**, 730-742.

98. Dallman, M.F., Darlington, D.N., Suemaru, S., Cascio, C.S., and Levin, N., (1989). Cortosteriods in homeostasis, *Acta Physiologica Scandinavia*, **136**, 27-34.

99. Dautzenberg, F.M. and Hauger, R.L., (2002). The CRF peptide family and their receptors: yet more partners discovered, *Trends in Pharmacological Science*, **23**, 71-77.

100. Dautzenberg, F.M., Kilpatrick, G.J., Hauger, R.L., and Moreau, J., (2001). Molecular biology of the CRH receptors - in the mood. *Peptides*, **22**, 753-760.

101. Davis, M., Rainnie, D., and Cassell, M., (1994). Neurotransmission in the rat amygdala related to fear and anxiety, *Trends in Neurosciences*, **17**, 208-214.

102. De Goeij, D.C., Binnekade, R., and Tilders, F.J., (1992). Chronic stress enhances vasopressin but not corticotropin-releasing factor secretion during hypoglycemia, *American Journal of Physiology*, **263**, E394-E399.

103. De Goeij, D.C., Dijkstra, H., and Tilders, F.J., (1992). Chronic psychosocial stress enhances vasopressin, but not corticotropin-releasing factor, in the external zone of the median eminence of male rats: relationship to subordinate status, *Endocrinology*, **131**, 847-853.

104. De Goeij, D.C., Jezova, D., and Tilders, F.J., (1992). Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus, *Brain Research*, **577**, 165-168.

105. De Goeij, D.C., Kvetnansky, R., Whitnall, M.H., Jezova, D., Berkenbosch, F., and Tilders, F.J., (1991). Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats, *Neuroendocrinology*, **53**, 150-159.

106. De Lecea, L. and Sutcliffe, J.G., (1999). The hypocretins/orexins: novel hypothalamic neuropeptides involved in different physiological systems, *Cellular & Molecular Life Sciences*, **56**, 473-480.

107. De Souza, E.B., Insel, T.R., Perrin, M.H., Rivier, J., Vale, W., and Kuhar, M.J., (1985). Corticotropin-releasing Factor Receptors Are Widely Distributed within the Rat Central Nervous System: An Autoradiographic Study, *Journal of Neuroscience*, **5**, 3189-3203.

108. Degen, L., Matzinger, D., Drewe, J., and Beglinger, C., (2001). The effect of cholecystokinin in controlling appetite and food intake in humans, *Peptides*, **22**, 1265-1269.

109. Demitrack, M.A., Lesem, M.D., Brandt, H.A., Pigott, T.A., Jimerson, D.C., Altemus, M., and Gold, P.W., (1989). Neurohypophyseal dysfunction: implications for the pathophysiology of eating disorders, *Psychopharmacology Bulletin*, **25**, 439-443.

110. Denbow, D.M., (1991). Induction of food intake by a GABAergic mechanism in the turkey, *Physiology & Behavior*, **49**, 485-488.

111. Desousa, N.J., Wunderlich, G.R., De Cabo, C., and Vaccarino, F.J., (1998). Individual differences in sucrose intake predict behavioral reactivity in rodent models of anxiety, *Pharmacology, Biochemistry & Behavior*, **60**, 841-846.

112. Diorio, D., Viau, V., and Meaney, M.J., (1993). The Role of The Medial Prefrontal Cortex (Cingulate Gyrus) in the Regulation of Hypothalamic-Pituitary-Adrenal Responses to Stress, *The Journal of Neuroscience*, **13**, 3839-3847.

113. Domin, J., Polak, J.M., and Bloom, S.R., (1988). The distribution and biological effects of neuromedins B and U, *Annals of the New York Academy of Sciences*, **547**, 391-403.

114. Drolet, G. and Rivest, S., (2001). Corticotropin-releasing hormone and its receptors; an evaluation at the transcription level in vivo, *Peptides*, **22**, 761-767.

115. Dube, M.G., Horvath, T.L., Kalra, P.S., and Kalra, S.P., (2000). Evidence of NPY Y_5 receptor involvement in food intake elicited by orexin A in sated rats, *Peptides*, **21**, 1557-1560.

116. Dube, M.G., Kalra, S.P., and Kalra, P.S., (1999). Food intake elicited by central administration of orexins/hypocretins: identification of hypothalamic sites of action, *Brain Research*, **842**, 473-477.

117. Dunn, A.J., (2000). Footshock-induced changes in brain catecholamines and indoleamines are not mediated by CRF or ACTH, *Neurochemistry International*, **37**, 61-69.

118. Dunn, A.J. and Berridge, C.W., (1990). Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Research - Brain Research Reviews*, **15**, 71-100.

119. Dunn, A.J., Berridge, C.W., Lai, Y.I., and Yachabach, T.L., (1987). CRF-Induced Excessive Grooming Behavior in Rats and Mice, *Peptides*, **8**, 841-844.

120. Eckel, L.A., Langhans, W., Kahler, A., Campfield, L.A., Smith, F.J., and Geary, N., (1998). Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats, *American Journal of Physiology*, **275**, R186-R193.

121. Eckel, L.A. and Geary, N., (2001). Estradiol treatment increases feeding-induced c-Fos expression in the brains of ovariectomized rats, *American Journal of Physiology*, **281**, R746.

122. Elmquist, J.K., (2001). Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin, *International Journal of Obesity & Related Metabolic Disorders*, **25**, S78-S82.

123. Ely, D., Dapper, V., Marasca, J., Correa, J., Gamaro, G., and Xavier, M., (1997). Effect of restraint stress on feeding behavior of rats, *Physiology & Behavior*, **61**, 395-398.

124. Ericsson, M., (1996). Common Biological Pathways in Eating Disorders and Obesity, *Addictive Behaviors*, **21**, 733-743.

125. Fancher, R.E., (1990). William James and Psychology in America. In: *Pioneers of Psychology*, W.W. Norton & Company, New York, 239-274.

126. Feenstra, M.G., (2000). Dopamine and noradrenaline release in the prefrontal cortex in relation to unconditioned and conditioned stress and reward, *Progress in Brain Research*, **126**, 133-163.

127. Feenstra, M. and Botterbom, M., (1996). Rapid sampling of extracellular dopamine in the rat prefrontal cortex during food consumption, handling and exposure to novelty, *Brain Research*, **742**, 17-24.

128. Fernandez-Teruel, A., Escorihuela, R.M., Nunez, J.F., Goma, M., Driscoll, P., and Tobena, A., (1992). Early stimulation effects on novelty-induced behavior in two psychogenetically-selected rat lines with divergent emotionality profiles, *Neuroscience Letters*, **137**, 185-188.

129. Ferre, P., Nunez, J.F., Garcia, E., Tobena, A., Escorihuela, R.M., and Fernandez-Teruel. (1995). Postnatal Handling Reduces Anxiety as Measured by Emotionality Rating and Hyponeophagia Tests in Female Rats, *Pharmacology Biochemistry and Behavior*, **51**, 199-203.

130. Fletcher, P.J. and Davies, M., (1990). Effects of 8-OH-DPAT, buspirone and ICS 205-930 on feeding in a novel environment: comparisons with chlordiazepoxide and FG 7142, *Psychopharmacology*, **102**, 301-308.

131. Flood J.F. and Morley, J.E., (1991). Increased Food Intake by Neuropeptide Y is Due to an Increased Motivation to Eat, *Peptides*, **12**, 1329-1332.

132. Flood J.F., Silver, A.J., and Morley, J.E., (2001). Do peptide-induced changes in feeding occur because of changes in motivation to eat? *Peptides*, **11**, 265-270.

133. Flynn, F.W., (1989). Fourth ventricle bombesin injection suppresses ingestive behaviors in rats, *American Journal of Physiology*, **256**, R590-R596.

134. Flynn, F.W. and Robillard, L., (1992). Inhibition of ingestive behavior following fourth ventricle bombesin injection in chronic decerebrate rats, *Behavioral Neuroscience*, **106**, 1011-1014.

135. Flynn, F.W., Schulkin, J., and Havens, M., (1993). Sex differences in salt preference and taste reactivity in rats, *Brain Research Bulletin*, **32**, 91-95.

136. Follenius, M., Brandenberger, G., and Hietter, B., (1982). Diurnal cortisol peaks and their relationships to meals, *Journal of Clinical Endocrinology & Metabolism*, **55**, 757-761.

137. Foote, S.L., Bloom, F.E., and Aston-Jones, G., (1983). Nucleus locus coeruleus: new evidence of anatomical and physiological specificity, *Physiological Reviews*, **63**, 844-914.

138. Francis, D., Diorio, J., LaPlante, P., Weaver, S., Seckl, J.R., and Meaney, M.J. (1996). The Role of Early Environmental Events in Regulating Neuroendocrine Development: Moms, Pups, Stress, and Glucocorticoid Receptors, *Annals of the New York Academy of Sciences*, **794**, 136-152.

139. Fukuda, M. and Ono, T., (1993). Amygdala-hypothalamic control of feeding behavior in monkey: single cell responses before and after reversible blockade of temporal cortex or amygdala projections, *Behavioral Brain Research*, **55**, 233-241.

140. Gaffan, E.A., Gaffan, D., and Harrison, S., (1988). Disconnection of the amygdala from visual association cortex impairs visual reward association in monkeys, *The Journal of Neuroscience*, **8**, 3144-3150.

141. Ganesan, R., (1994). The aversive and hypophagic effects of estradiol, *Physiology & Behavior*, **55**, 279-285.

142. Garrido, M.M., Martin, S., Ambrosio, E., Fuentes, J.A., and Manzanares, J., (1998). Role of corticotropin-releasing hormone in gastrin-releasing peptide-mediated regulation of corticotropin and corticosterone secretion in male rats, *Neuroendocrinology*, **68**, 116-122.

143. Geary, N., (1997). Estradiol and the control of eating, *Appetite*, **29**, 386.

144. Geary, N., (2001). Estradiol, CCK and satiation, *Peptides*, **22**, 1251-1263.

145. Geary, N. and Asarian, L., (1999). Cyclic estradiol treatment normalizes body weight and test meal size in ovariectomized rats, *Physiology & Behavior*, **67**, 141-147.

146. Geary, N., Asarian, L., Korach, K.S., Pfaff, D.W., and Ogawa, S., (2001). Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice, *Endocrinology* , **142**, 4751-4757.

147. Geary, N., Smith, G.P., and Corp, E.S., (1996). The increased satiating potency of CCK-8 by estradiol is not mediated by upregulation of NTS CCK receptors, *Brain Research*, **719**, 179-186.

148. Geary, N., Trace, D., McEwen, B., and Smith, G.P., (1994). Cyclic estradiol replacement increases the satiety effect of CCK-8 in ovariectomized rats, *Physiology & Behavior*, **56**, 281-289.

149. Gehlert, D.R., (1999). Role of hypothalamic neuropeptide Y in feeding and obesity, *Neuropeptides*, **33**, 329-338.

150. Getz, R., Merchant, C., Rosenstein, J., Merali, Z., and Moody, T.W., (1992). Ontogeny of Bombesin/Gastrin-Releasing Peptide Binding Sites in Rat Brain, *Molecular & Cellular Neurosciences*, **3**, 162-170.

151. Gibbs, J., (1985). Effect of bombesin on feeding behavior, *Life Sciences*, **37**, 147-153.

152. Gibbs, J., Fauser, D.J., Rowe, E.A., Rolls, B.J., Rolls, E.T., and Maddison, S.P., (1979). Bombesin suppresses feeding in rats, *Nature*, **282**, 208-210.

153. Gibbs, J., Kulkosky, P.J., and Smith, G.P., (1981). Effects of peripheral and central bombesin on feeding behavior of rats, *Peptides*, **2**, 179-183.

154. Gibbs, J. and Smith, G.P., (1988). The Actions of Bombesin-Like Peptides on Food Intake, *Annals of the New York Academy of Sciences*, **547**, 210-216.

155. Gibbs, J. and Smith, G.P., (2001). Satiety: the roles of peptides from the stomach and the intestine, *Federation Proceedings*, **45**, 1391-1395.

156. Gibbs, J., Young, R.C., and Smith, G.P., (1973). Cholecystokinin decreases food intake in rats, *Journal of Comparative & Physiological Psychology*, **84**, 488-495.

157. Gillati, M.T. and Moody, T.W., (1984). The development of rat brain bombesin-like peptides and their receptors, *Developmental Brain Research*, **15**, 286-289.

158. Girard, C.L., Seoane, J.R., and Matte, J.J., (1985). Studies of the role of gamma-aminobutyric acid in the hypothalamic control of feed intake in sheep, *Canadian Journal of Physiology & Pharmacology*, **63**, 1297-1301.

159. Giraud, A.S., Smith, A.I., and Rundle, S.E., (1987). Distribution and molecular forms of immunoreactive bombesin in the ovine median eminence, *Molecular & Cellular Endocrinology*, **53**, 245-250.

160. Giraudo, S.Q., Billington, C.J., and Levine, A.S., (1998). Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the central nucleus of the amygdala and in the paraventricular nucleus in the rat, *Brain Research*, **782**, 18-23.

161. Gold, P.W., Gwirtsman, H., Avgerinos, P.C., Nieman, L.K., Gallucck, W.T., Kaye, W., Jimerson, D., Ebert, M., Rittmaster, R., Loriaux, L., and Chrousos, G.P., (1986). Abnormal Hypothalamic-Pituitary-Adrenal Function in Anorexia Nervosa, *The New England Journal of Medicine*, **314**, 1335-1342.

162. Gold, R.M., (1973). Hypothalamic Obesity: The Myth of the Ventromedial Nucleus, *Science*, **182**, 488-490.

163. Goldstein, L.E., Rasmusson, A.M., Bunney B.Steve, and Roth, R.H., (1996). Role of the Amygdala in the Coordination of Behavioral, Neuroendocrine, and Prefrontal Cortical Monoamine Responses to Psychological Stress in the Rat, *The Journal of Neuroscience*, **16**, 4787-4798.

164. Gosnell, B.A., Krahn, D.D., and Majchrzak, M.J., (1990). The effects of morphine on diet selection are dependent upon baseline diet preferences, *Pharmacology, Biochemistry & Behavior*, **37**, 207-212.

165. Gray T.S., (1991). Amygdala: role in autonomic and neuroendocrine responses to stress. In: McCubbin, J.A., Kauffman, G., Nemeroff, C.B. (Eds.), *Stress, Neuropeptides and Systemic Disease*, Academic, New York, 37-53.

166. Gray T.S., Carney, M.E., and Magnuson, D.J., (1989). Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release, *Neuroendocrinology*, **50**, 433-446.

167. Gray, R.W. and Cooper, S.J., (1995). Benzodiazepines and palatability: taste reactivity in normal ingestion, *Physiology & Behavior*, **58**, 853-859.

168. Grigoriadis, D.E., Lovenberg, T.W., Chalmers, D.T., Liaw, C., and De Souze, E.B., (1996). Characterization of corticotropin-releasing factor receptor subtypes, *Annals of the New York Academy of Sciences*, **780**, 60-80.

169. Grino, M., Paulmyer-Lacroix, O., Anglade, G., and Oliver, C., (1995). Molecular Aspects of the Regulation of the Hypothalamo-pituitary-adrenal Axis during Development in the Rat, *Annals of the New York Academy of Sciences* **771**, 339-351.

170. Grogan, S.C., Bell, R., and Conner, M., (1997). Eating sweet snacks: gender differences in attitudes and behaviour, *Appetite*, **28**, 19-31.

171. Guillemin, R. and Rosenberg, B., (1955). Humoral hypothalamic control of anterior pituitary: a study with combined tissue cultures, *Endocrinology*, **57**, 599-607.

172. Gunion, M.W., Tache, Y., Rosenthal, M.J., Miller, S., Butler, B., and Zib, B., (1989). Bombesin microinfusion into the rat hypothalamic paraventricular nucleus increases blood glucose, free fatty acids and corticosterone, *Brain Research*, **478**, 47-58.

173. Gutzwiller, J.P., Drewe, J., Hildebrand, P., Rossi, L., Lauper, J.Z., and Beglinger, C., (1994). Effect of intravenous human gastrin-releasing peptide on food intake in humans, *Gastroenterology*, **106**, 1168-1173.

174. Guyard, B., Fricker, J., Brigant, L., Betoulle, D., and Apfelbaum, M., (1991). Effects of ovarian steroids on energy balance in rats fed a highly palatable diet, *Metabolism: Clinical & Experimental*, **40**, 529-533.

175. Habib, K.E., Gold, P.W., and Chrousos, G.P., (2001). Neuroendocrinology of stress, *Endocrinology & Metabolism Clinics of North America*, **30**, 3-695.

176. Hajnal, A. and Lénárd, L., (1997). Feeding-related dopamine in the amygdala of freely moving rats, *Neuroreport*, **8**, 2817-2820.

177. Halford, J.C. and Blundell, J.E., (2000). Pharmacology of appetite suppression, *Progress in Drug Research*, **54**, 25-58.

178. Hampton, L.L., Ladenheim, E.E., Akeson, M., Way, J.M., Weber, H.C., Sutliff, V.E., Jensen, R.T., Wine, L.J., Arnheiter, H., and Battey, J.F., (1998). Loss of bombesin-induced feeding suppression in gastrin-releasing peptide receptor-deficient mice, *Proceedings National Academy Science USA*, **95**, 3188-3192.

179. Handa, R.J., Burgess, L.H., Kerr, J.E., and O'Keefe, J.A., (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis, *Hormones & Behavior*, **28**, 464-476.

180. Hansen, T.V.O., (2001). Cholecystokinin gene transcription: promoter elements, transcription factors and signaling pathways, *Peptides*, **22**, 1201-1211.

181. Harro, J., Kiivet, R.-A., Lang, A., and Vasar, E., (1990). Rats with anxious or non-anxious type of exploratory behaviour differ in their brain CCK-8 and benzodiazepine receptor characteristics, *Behavioural Brain Research*, **39**, 63-71.

182. Hashimoto, K., Murakami, K., Takao, T., Makino, S., Sugawara, M., and Ota, Z., (1989). Effect of acute ether or restraint stress on plasma corticotropin-releasing hormone, vasopressin and oxytocin levels in the rat, *Acta Medica Okayama*, **43**, 161-167.

183. Haynes, A.C., Chapman, H., Taylor, C., Moore, G.B., Cawthorne, M.A., Tadayyon, M., Clapham, R.C., and Arch, J.R., (2002). Anorectic, thermogenic and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice, *Regulatory Peptides*, **104**, 153-159.

184. Haynes, A.C., Jackson, B., Overend, P., Buckingham, R.E., Wilson, S., Tadayyon, M., and Arch, J.R., (1999). Effects of single and chronic intracerebroventricular administration of the orexins on feeding in the rat, *Peptides*, **20**, 1099-1105.

185. Heilig, M., Koob, G.F., Ekman, R., and Britton, K.T., (1994). Corticotropin-releasing factor and neuropeptide Y: role in emotional integration, *Trends in Neurosciences*, **17**, 80-85.

186. Heilig, M., McLeod, S., Koob, G.F., and Britton, K.T., (1992). Anxiolytic-like effect of neuropeptide Y (NPY), but not other peptides in an operant conflict test, *Regulatory Peptides*, **41**, 61-69.

187. Heinrichs, S.C., Cole, B.J., Pich, E.M., Menzaghi, F., Koob, G.F., and Hauger, R.L., (1992). Endogenous corticotropin-releasing factor modulates feeding induced by neuropeptide Y or a tail-pinch stressor, *Peptides*, **13**, 879-884.

188. Heinrichs, S.C., Menzaghi, F., Pich, E.M., Britton, K.T., and Koob, G.F., (1995). The Role of CRF in Behavioral Aspects of Stress, *Annals of the New York Academy of Sciences*, **771**, 92-104.

189. Herman, J.P. and Cullinan, W.E., (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis, *Trends in Neurosciences*, **20**, 78-84.

190. Hernandez, L. and Hoebel, B.G., (1988). Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens, *Physiology & Behavior*, **44**, 599-606.

191. Higgs, S. and Cooper, S.J., (1996). Effects of the benzodiazepine receptor inverse agonist Ro 15-4513 on the ingestion of sucrose and sodium saccharin solutions: a microstructural analysis of licking behavior, *Behavioral Neuroscience*, **110**, 559-566.

192. Higgs, S. and Cooper, S.J., (1996). Hyperphagia induced by direct administration of midazolam into the parabrachial nucleus of the rat, *European Journal of Pharmacology*, **313**, 1-9.

193. Hilakivi-Clarke, L.A., Turkka, J., Lister, R.G., and Linnoila, M., (1991). Effects of early postnatal handling on brain B-adrenoreceptors and behavior in tests related to stress, *Brain Research*, **542**, 286-292.

194. Hill, J.O. and Peters, J.C., (1998). Environmental Contributions to the Obesity Epidemic, *Science*, **280**, 1371-1374.

195. Hoebel, B.G., Hernandez, L., Schwartz, D.H., Mark, G.P., and Hunter, G.A., (1989). Microdialysis studies of brain norepinephrine, serotonin, and dopamine release during ingestive behavior. Theoretical and clinical implications, *Annals of the New York Academy of Sciences*, **575**, 171-191.

196. Hoebel, B.G., Leibowitz, S.F., and Hernandez, L., (1992). Neurochemistry of Anorexia and Bulimia. In: Anderson, G.H., Kennedy, S.H. (Eds.), *The Biology of Feast and Famine: Relevance to Eating Disorders*, Academic Press, Inc., San Diego, 21-45.

197. Hoffman, B.J., Hansson, S.R., Mezey, E., and Palkovits, M., (1998). Localization and dynamic regulation of biogenic amine transporters in the mammalian central nervous system, *Frontiers in Neuroendocrinology*, **19**, 187-231.

198. Hotta, M., Shibasaki, T., Arai, K., and Demura, H., (1999). Corticotropin-releasing factor receptor type 1 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats, *Brain Research*, **823**, 221-225.

199. Hotta, M., Shibasaki, T., Masuda, A., Imaki, T., Demura, H., Ling, N., and Shizume, K., (1986). The responses of plasma adrenocorticotropin and cortisol to corticotropin-releasing hormone (CRH) and cerebrospinal fluid immunoreactive CRH in anorexia nervosa patients, *Journal of Clinical Endocrinology & Metabolism*, **62**, 319-324.

200. Houben, H., Vandenbroucke, A.T., Verheyden, A.M., and Denef, C., (1993). Expression of the genes encoding bombesin-related peptides and their receptors in anterior pituitary tissue, *Molecular & Cellular Endocrinology*, **97**, 159-164.

201. Hrupka, B.J., Smith, G.P., and Geary, N., (1997). Ovariectomy and estradiol affect postingestive controls of sucrose licking, *Physiology & Behavior*, **61**, 243-247.

202. Hulsey, M.G., Pless, C.M., and Martin, R.J., (1995). ICV administration of anti-corticotropin-releasing factor antisense oligonucleotide: effects on feeding behavior and body weight, *Regulatory Peptides*, **59**, 241-246.

203. Ida, T., Nakahara, K., Kuroiwa, T., Fukui, K., Nakazato, M., Murakami, T., and Murakami, N., (2000). Both corticotropin releasing factor and neuropeptide Y are involved in the effect of orexin (hypocretin) on the food intake in rats, *Neuroscience Letters*, **293**, 119-122.

204. Imaki, T., Katsumata, H., Miyata, M., Naruse, M., Imaki, J., and Minami, S., (2001). Expression of corticotropin-releasing hormone type 1 receptor in paraventricular nucleus after acute stress, *Neuroendocrinology*, **73**, 293-301.

205. Jarry, J.L. and Vaccarino, F.J., (1996). Eating Disorder and Obsessive-Compulsive Disorder: Neurochemical and Phenomenological Commonalties, *Journal of Psychiatry Neuroscience*, **21**, 36-48.

206. Jodo, E., Suzuki, Y., and Kayama, Y., (2000). Selective responsiveness of medial prefrontal cortex neurons to the meaningful stimulus with a low probability of occurrence in rats, *Brain Research*, **856**, 68-74.

207. Johnson, E.O., Kamilaris, T.C., Chrousos, G.P., and Gold, P.W., (1992). Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis, *Neuroscience & Biobehavioral Reviews*, **16**, 115-130.

208. Johnston, S.A. and Merali, Z., (1988). Specific neuroanatomical and neurochemical correlates of grooming and satiety effects of bombesin, *Peptides*, **9**, Suppl 1, 233-244.

209. Johnston, S.A. and Merali, Z., (1988). Specific neuroanatomical and neurochemical correlates of locomotor and grooming effects of bombesin, *Peptides*, **9**, Suppl 1, 245-256.

210. Jones, B. and Mishkin, M., (1972). Limbic lesions and the problem of stimulus-reinforcement associations, *Experimental Neurology*, **36**, 362-377.

211. Jones, P.M., Withers, D.J., Ghatei, M.A., and Bloom, S.R., (1992). Evidence for neuromedin-B synthesis in the rat anterior pituitary gland, *Endocrinology*, **130**, 1829-1836.

212. Jones, S. and Kauer, J.A., (1999). Amphetamine depresses excitatory synaptic transmission via serotonin receptors in the ventral tegmental area, *Journal of Neuroscience*, **19**, 9780-9787.

213. Joyner, K., Smith, G.P., and Gibbs, J., (1993). Abdominal vagotomy decreases the satiating potency of CCK-8 in sham and real feeding, *American Journal of Physiology*, **264**, R912-R916.

214. Kaga, T., Fujimiya, M., and Inui, A., (2001). Emerging functions of neruopeptide Y Y_2 receptors in the brain, *Peptides*, **22**, 501-506.

215. Kalin, N.H., Takahashi, L.K., and Chen, F.L., (1994). Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus, *Brain Research*, **656**, 182-186.

216. Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., and Kalra, P.S., (1999). Interacting appetite-regulating pathways in the hypothalamic regulation of body weight, *Endocrine Reviews*, **20**, 68-100.

217. Kamatchi, G.L., Bhakthavatsalam, P., Chandra, D., and Bapna, J.S., (1984). Inhibition of insulin hyperphagia by gamma aminobutyric acid antagonists in rats, *Life Sciences*, **34**, 2297-2301.

218. Kandel, E.R. and Schwartz, J.H., (1991). Directly Gated Transmission at Central Synapses. In: Kandel, E.R., Schwartz, J.H., Jessell, T.M. (Eds.), *Principles of Neural Science*, Appleton & Lange, Norwalk, Connecticut, 153-172.

219. Karbonitis, M., Trainer, P.J., Nelson, M.L., Howse, I., Kopelman, P.G., Besser, G.M., Grossman, A.B., and Svec, F., (1996). Differential stimulation of cortisol and dehydroenpiandrosterone levels by food in obese and normal subjects: relation to body fat distribution, *Clinical Endocrinology*, **45**, 699-706.

220. Kasckow, J.W., Hagan, M., Mulchahey, J.J., Baker, D.G., Ekhator, N.N., Strawn, J.R., Nicholson, W., Orth, D.N., Loosen, P.T., and Geracioti, T.D., (2001). The effect of feeding on cerebrospinal fluid corticotropin-releasing hormone levels in humans, *Brain Research*, **904**, 218-224.

221. Kask, A., Rago, L., and Harro, J., (1998). Evidence for involvement of neuropeptide Y receptors in the regulation of food intake: studies with Y_1-selective antagonist BIBP3226, *British Journal of Pharmacology*, **124**, 1507-1515.

222. Kask, A., Rago, L., and Harro, J., (1998). Anxiogenic-like effect of the NPY Y1 receptor antagonist BIBP3226 administered into the dorsal periaqueductal gray matter in rats, *Regulatory Peptides*, **75-76**, 255-262.

223. Kask, A., Rago, L., and Harro, J., (1998). Anxiolytic-like effect of neuropeptide Y (NPY) and NPY13-36 microinjected into vicinity of locus coeruleus in rats, *Brain Research*, **788**, 345-348.

224. Kateb, C.C. and Merali, Z., (1992). A single meal elicits regional changes in bombesin-like peptide levels in the gut and brain, *Brain Research*, **596**, 10-16.

225. Kaur, G.K.S.K., (2002). Studies on modulation of feeding behavior by atypical antipsychotics in female mice, *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, **26**, 277-285.

226. Kaye, W.H., Rubinow, D., Gwirtsman, H.E., George, D.T., Jimerson, D.C., and Gold, P.W., (1988). CSF Somatostatin in Anorexia Nervosa and Bulimia: Relationship to the Hypothalamic-Pituitary-Adrenal Cortical Axis, *Psychoneuroendocrinology*, **13**, 265-272.

227. Keck, M.E. and Holsboer, F., (2001). Hyperactivity of CRH neuronal circuits as a target for therapeutic interventions in affective disorders, *Peptides*, **22**, 835-844.

228. Kelly, J. and Grossman, S.P., (1979). GABA and hypothalamic feeding systems. II. A comparison of GABA, glycine and actylcholine agonists and their antagonists, *Pharmacology, Biochemistry & Behavior*, **11**, 647-652.

229. Kent, P., (2001). Peptidergic Mediation of Response to Stress: Focus on Bombesin-like Peptides. Thesis Dissertation, 118-144.

230. Kent, P., Anisman, H., and Merali, Z., (1998). Are bombesin-like peptides involved in the mediation of stress response? *Life Sciences*, **62**, 103-114.

231. Kent, P., Anisman, H., and Merali, Z., (2001). Central bombesin activates the hypothalamic-pituitary-adrenal axis. Effects on regional levels and release of corticotropin-releasing hormone and arginine-vasopressin, *Neuroendocrinology*, **73**, 203-214.

232. Kent, P., Bedard, T., Khan, S., Anisman, H., and Merali, Z., (2001). Bombesin-induced HPA and sympathetic activation requires CRH receptors, *Peptides*, **22**, 57-65.

233. Khan, S., Michaud, D.S., Anisman, H., and Merali, Z., (1999). Diazepam Reverses Novel Environment-Induced Feeding Suppression Without Blocking Amygdaloid CRH Release, *Society for Neuroscience Abstracts*, **25**, 1126.

234. Kim, E., Welch, C.C., Grace, M.K., Billington, C.J., and Levine, A.S., (1998). Effects of palatability-induced hyperphagia and food restriction on mRNA levels of neuropeptide-Y in the arcuate nucleus, *Brain Research*, **806**, 117-121.

235. King, J.A., (1996). Perinatal Stress and Impairment of the Stress Response; possible link to nonoptimal behavior, *Annals of the New York Academy of Sciences*, **794**, 104-111.

236. Kinzl, J.F., Traweger, C., Guenther, V., and Biebl, W., (1994). Family Background and Sexual Abuse Associated with Eating Disorders, *American Journal of Psychiatry* **151**, 1127-1131.

237. Kirkham, T.C., Gibbs, J., and Smith, G.P., (1991). Satiating effect of bombesin is mediated by receptors perfused by celiac artery, *American Journal of Physiology*, **261**, R614-R618.

238. Kiss, A., Palkovits, M., and Aguilera, G., (1996). Neural regulation of corticotropin releasing hormone (CRH) and CRH receptor mRNA in the hypothalamic paraventricular nucleus in the rat, *Journal of Neuroendocrinology*, **8**, 103-112.

239. Klitenick, M.A. and Wirtshafter, D., (1988). Comparative studies of the ingestive behaviors produced by microinjections of muscimol into the midbrain raphe nuclei of the ventral tegmental area of the rat, *Life Sciences*, **42**, 775-782.

240. Klitenick, M.A. and Wirtshafter, D., (1989). Elicitation of feeding, drinking, and gnawing following microinjections of muscimol into the median raphe nucleus of rats, *Behavioral & Neural Biology*, **51**, 436-441.

241. Knoll, E., Muller, F.W., Ratge, D., Bauersfeld, W., and Wisser, H., (1984). Influence of food intake on concentrations of plasma catecholamines and cortisol, *Journal of Clinical Chemistry & Clinical Biochemistry*, **22**, 597-602.

242. Knutson, B., Fong, G.W., Adams, C.M., Varner Jerald L., and Hommer, D., (2001). Dissociation of reward anticipation and outcome with event-related fMRI, *Neuroreport*, **12**, 3683-3687.

243. Koob, G.F., Heinrichs, S.C., Menzaghi, F., Pich, E.M., and Britton, K.T., (1994). Corticotropin releasing factor, stress and behavior, *Seminars in The Neurosciences*, **6**, 221-229.

244. Kotz, C.M., Teske, J.A., Levine, J.A., and Wang, C., (2002). Feeding and activity induced by orexin A in the lateral hypothalamus in rats, *Regulatory Peptides*, **104**, 27-32.

245. Kovács, K.J. and Sawchenko, P.E., (1996). Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons, *Journal of Neuroscience*, **16**, 262-273.

246. Krahn, D.D., Gosnell, B.A., Grace, M., and Levine, A.S., (1986). CRF antagonist partially reverses CRF- and stress-induced effects on feeding, *Brain Research Bulletin*, **17**, 285-289.

247. Krahn, D.D., Gosnell, B.A., Levine, A.S., and Morley, J.E., (1988). Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects, *Brain Research*, **443**, 63-69.

248. Krysiak, R., Obuchowicz, E., and Herman, Z.S., (2000). Conditioned fear-induced changes in neuropeptide Y-like immunoreactivity in rats: the effect of diazepam and buspirone, *Neuropeptides*, **34**, 148-157.

249. Kulkosky, P.J., Gibbs, J., and Smith, G.P., (1982). Behavioral Effects of Bombesin Administration in Rats, *Physiology & Behavior*, **28**, 505-512.

250. Kuo, D.Y., (2002). Co-administration of dopamine d(1) and d(2) agonists additively decreases daily food intake, body weight and hypothalamic neuropeptide y level in rats, *Journal of Biomedical Science*, **9**, 126-132.

251. Kurose, T., Ueta, Y., Yamamoto, Y., Serino, R., Ozaki, Y., Saito, J., Nagata, S., and Yamachshita, H., (2002). Effects of restricted feeding on the activity of hypothalamic Orexin (OX)-A containing neurons and OX2 receptor mRNA level in the paraventricular nucleus of rats, *Regulatory Peptides*, **104**, 145-151.

252. Kyrkouli, S.E., Stanley, B.G., and Leibowitz, S.F., (1987). Bombesin-Induced Anorexia: Sites of Action in the Rat Brain, *Peptides*, **8**, 237-241.

253. Laban, O., Dimitrijevic, M., von Hoersten, S., Markovic, B.M., and Jankovic, B.D., (1995). Experimental allergic encephalomyelitis in adult DA rats subjected to neonatal handling or gentling, *Brain Research*, **676**, 133-140.

254. Ladd, C.O., Owens, M.J., and Nemeroff, C.B., (1996). Persistent Changes in Corticotropin-Releasing Factor Neuronal Systems Induced by Maternal Deprivation, *Endocrinology*, **137**, 1212-1218.

255. Ladenheim, E. and Ritter, R.C., (1993). Caudal hindbrain participation in the suppression of feeding by central and peripheral bombesin, *American Journal of Physiology*, **264**, R1229-R1234.

256. Ladenheim, E.E., Taylor, J.E., Coy, D.H., Moore, K.A., and Moran, T.H., (1996). Hindbrain GRP receptor blockade antagonizes feeding suppression by peripherally administered GRP, *American Journal of Physiology*, **271**, R180-R184.

257. Ladenheim, E.E., Taylor, J.E., Coy, D.H., and Moran, T.H., (1994). Blockade of feeding inhibition by neuromedin B using a selective receptor antagonist, *European Journal of Pharmacology*, **271**, R7-R9.

258. Laeng, B., Berridge, K.C., and Butter, C.M., (1993). Pleasantness of a sweet taste during hunger and satiety: effects of gender and "sweet tooth", *Appetite*, **21**, 247-254.

259. Langhans, W., Delprete, E., and Scharrer, E., (1991). Mechanisms of vasopressin's anorectic effect, *Physiology & Behavior*, **49**, 169-176.

260. Laviano, A., Meguid, M.M., Gleason, J.R., Yang, Z.J., and Renvyle, T., (1996). Comparison of long-term feeding pattern between male and female Fischer 344 rats: influence of estrous cycle, *American Journal of Physiology*, **270**, R413-R419.

261. Lawrence, C.B., Turnbull, A.V., and Rothwell, N.J., (1999). Hypothalamic control of feeding, *Current Opinion in Neurobiology*, **9**, 778-783.

262. Lee, C.C. and Miller R.J., (1998). Is there really an NPY Y3 receptor? *Regulatory Peptides*, **75-76**, 71-78.

263. Lee, Y. and Davis, M., (1997). Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex, *Journal of Neuroscience*, **17**, 6434-6446.

264. Leibowitz, S.F., (1986). Brain monoamines and peptides: role in the control of eating behavior, *Federation Proceedings*, **45**, 1396-1403.

265. Leibowitz, S.F., (1991). Brain Neurochemical Systems Controlling Appetite and Body Weight Gain. In: Rothwell, N.J., Stock, M.J. (Eds.), *Obesity and Cachexia: Physiological Mechanisms and New Approaches to Pharmacological Control*, John Wiley & Sons, Chichester, 33-48.

266. Levine, A.S. and Billington, C.J., (1997). Why do we eat? A neural systems approach, *Annual Review of Nutrition*, **17**, 597-619.

267. Levine, A.S., Rogers, B., Kneip, J., Grace, M., and Morley, J.E., (1983). Effect of centrally administered corticotropin releasing factor (CRF) on multiple feeding paradigms, *Neuropharmacology*, **22**, 337-339.

268. Levine, A.S. and Billington, C.J., (1989). Opioids: Are They Regulators of Feeding?, *Annals of the New York Academy of Sciences*, **575**, 209-219.

269. Levine, S., (1957). Infantile Experience and Resistance to Physiological Stress, *Science*, **126**, 405.

270. Levine, S., (1994). The Ontogeny of the Hypothalamic-Pituitary-Adrenal Axis: The Influence of Maternal Factors, *Annals of the New York Academy of Sciences*, **746**, 275-288.

271. Levine, S., Haltmeyer, G.C., Karas, G.G., and Denenberg, V.H., (1967). Physiological and Behavioral Effects of Infantile Stimulation, *Physiology and Behavior*, **2**, 55-59.

272. Levine, S., Huchton, D.M., Wiener, S.G., and Rosenfeld, P., (1992). Time Course of the Effect of Maternal Deprivation on the Hypothalamic-Pituitary-Adrenal Axis in the Infant Rat, *Developmental Psychobiology*, **24**[8], 547-558.

273. Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P.E., and Vale, W.W., (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor, *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 7570-7575.

274. Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilenzikjian, L., Rivier, J., Sawchenko, P.E., and Vale, W.W., (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor, *Proceedings of the National Academy of Sciences*, **98**, 7570-7575.

275. Lin, J. and Pan, J., (1994). Stimulatory Effects of Bombesin-like Peptides on Hypothalamic Arcuate Neurons in Rat Brain Slices, *Brain Research Bulletin*, **35**, 241-246.

276. Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., and Meaney, M.J., (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress, *Science*, **277**, 1659-1662.

277. Lopez-Garcia, J.C., Bermudez-Rattoni, F., and Tapia, R., (1990). Release of acetylcholine, gamma-aminobutyrate, dopamine and glutamate, and activity of some related enzymes, in rat gustatory neocortex, *Brain Research*, **523**, 100-104.

278. Lopez, J.F., Akil, H., and Watson, S.J., (1999). Role of Biological and Psychological Factors in Early Development and Their Impact on Adult Life: Neural Circuits Mediating Stress, *Biological Psychiatry*, **46**, 1461-1471.

279. Lutz, T.A., Del Prete, E., Szabady, M.M., and Scharrer, E., (1996). Attenuation of the Anorectic Effects of Glucagon, Cholecystokinin, and Bombesin by the Amylin Receptor Antagonist CGRP(8-37), *Peptides*, **17**, 119-123.

280. Lydiard, R.B., (1994). Neuropeptides and Anxiety: Focus on Cholecystokinin, *Clinical Chemistry*, **40**, 315-318.

281. Lydiard, R.B., Ballenger, J.C., Laraia, M.T., Fossey, M.D., and Beinfeld, M.C., (1992). CSF Cholecystokinin Concentrations in Patients with Panic Disorder and in Normal Comparison Subjects, *American Journal of Psychiatry*, **149**[5], 691-693.

282. Ma, X.M., Lightman, S.L., and Aguilera, G., (1999). Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint, *Endocrinology*, **140**, 3623-3632.

283. Macey, D.J., Koob, G.F., and Markou, A., (2000). CRF and urocortin decreased brain stimulation reward in the rat: reversal by a CRF receptor antagonist, *Brain Research*, **866**, 82-91.

284. Magiakou, M.A., Mastorakos, G., Webster, E., and Chrousos, G.P., (1997). The hypothalamic-pituitary-adrenal axis and the female reproductive system, *Annals of the New York Academy of Sciences*, **816**, 42-56.

285. Maidment, N.T. and Evans, C.J., (1991). Measurement of extracellular neuropeptides in the brain: microdialysis linked to solid-phase radioimmunoassays with sub-femtomole limits of detection. In: Robinson, T.E., Justice, J.B. (Eds.), *Microdialysis in the Neurosciences*, Elsevier, Amsterdam, 275-303.

286. Malendowicz, L.K., Nussdorfer, G.G., Miskowiak, B., and Majchrzak, M., (1995). Effects of bombesin on the morphology and function of the rat adrenal cortex: comparison of the acute and chronic responses, *Histology & Histopathology*, **10**, 11-15.

287. Mansbach, R.S., Stanley, J.A., and Barrett, J.E., (1984). Ro 15-1788 and beta-CCE selectively eliminate diazepam-induced feeding in the rabbit, *Pharmacology, Biochemistry & Behavior*, **20**, 763-766.

288. Matos, F.F., Guss, V., and Korpinen, C., (1996). Effects of neuropeptide Y (NPY) and [D-Trp^{32}]NPY on monoamine and metabolite levels in dialysates from rat hypothalamus during feeding behavior, *Neuropeptides*, **30**, 391-394.

289. Matsumoto, R.R., (1989). GABA receptors: are cellular differences reflected in function? *Brain Research - Brain Research Reviews*, **14**, 203-225.

290. McBride, W.J., Murphy, J.M., Gatto, G.J., Levy, A.D., Yoshimoto, K., Lumeng, L., and Li, T.K., (1993). CNS mechanisms of alcohol self-administration, *Alcohol & Alcoholism*, **Suppl 2**, 463-467.

291. McCormick, C.M., Smythe, J.W., Sharma, S., and Meaney, M.J., (1995). Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats, *Developmental Brain Research*, **84**, 55-61.

292. McCoy, J.G. and Avery, D.D., (1990). Bombesin: Potential Integrative Peptide for Feeding and Satiety, *Peptides*, **11**, 595-607.

293. McDonald, T.J., Jornvall, H., Nilsson, G., Vagne, M., Ghatei, M.A., Bloom, S.R., and Mutt, V., (1979). Characterization of a gastrin releasing peptide from porcine non-antral gastic tissue, *Biochemical and Biophys. Research Communication*, **90**, 227-237.

294. McElroy, S.L. and Keck, P.E., (1995). Misattribution of Eating and Obsessive-Compulsive Disorder Symptoms to Repressed Memories of Childhood Sexual or Physical Abuse, *Biological Psychiatry*, **37**, 48-51.

295. McEwen, B., (2000). The neurobiology of stress: from serendipity to clinical relevance, *Brain Research*, **886**, 172-189.

296. McGregor, G.P., Woodhams, P.L., O'Shaughnessy, D.J., Ghatel, M.A., Polak, J.M., and Bloom, S.R., (1982). Developmental Changes in Bombesin, Substance P, Somatostatin and Vasoactive Intestinal Polypeptide in the Rat Brain, *Neuroscience Letters*, **28**, 27.

297. McIntyre, D.C., Kelly, M.E., and Dufresne, C., (1999). FAST and SLOW amygdala kindling rat strains: comparison of amygdala, hippocampal, piriform and perirhinal cortex kindling, *Epilepsy Research*, **35**, 197-209.

298. McIntyre, D.C., Kent, P., Hayley, S., Merali, Z., and Anisman, H., (1999). Influence of psychogenic and neurogenic stressors on neuroendocrine and central monoamine activity in fast and slow kindling rats, *Brain Research*, **840**, 65-74.

299. Meaney, M.J., (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations, *Annual Review of Neuroscience*, **24**, 1161-1192.

300. Meaney, M.J., Bhatnagar, S., Larocque, S., McCormick, C., Shanks, N., Sharma, S., Smythe, J., Viau, V., and Plotsky, P.M., (1993). Environmental regulation of the development of glucocorticoid receptor systems in the rat forebrain, *Annals of the New York Academy of Sciences*, **697**, 70-85.

301. Meaney, M.J., Diorio, J., Francis, D., Widdowson, J., LaPlante, P., Caldji, C., Sharma, S., Seckl, J.R., and Plotsky, P.M., (1996). Early environmental

regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress, *Developmental Neuroscience*, **18**, 49-72.

302. Meaney, M.J., Aitken, D.H., van Berkel, C., Bhatnagar, S., and Sapolsky, R.M., (1988). Effect of Neonatal Handling on Age-Related Impairments Associated with the Hippocampus, *Science*, **239**, 766-768.

303. Meaney, M.J., Aitken, D.H., Viau, V., Sharma, S., and Sarrieau, A., (1989). Neonatal Handling Alters Adrenocortical Negative Feedback Sensitivity and Hippocampal Type II Glucocorticoid Receptor Binding in the Rat, *Neuroendocrinology*, **50**, 597-604.

304. Meaney, M.J., Bhatnagar, S., Larocque, S., McCormick, C., Shanks, N., Sharma, S., Smythe, J., Viau, V., and Plotsky, P.M., (1993). Individual Differences in the Hypothalamic-Pituitary-Adrenal Stress Response and the Hypothalamic CRF System, *Annals of the New York Academy of Sciences*, **697**, 70-85.

305. Menzaghi, F., Heinrichs, S., Pich, E.M., Weiss, F., and Koob, G.F., (1993). The role of the limbic and hypothalamic corticotropin-releasing factor in behavioral responses to stress,. *Annals of the New York Academy of Sciences*, **697**, 142-154.

306. Merali, Z., Johnston, S., and Zalcman, S., (1983). Bombesin-induced behavioural changes: antagonism by neuroleptics, *Peptides*, **4**, 693-697.

307. Merali, Z. and Kateb, C.C., (1993). Rapid alterations of hypothalamic and hippocampal bombesin-like peptide levels with feeding status, *American Journal of Physiology*, **265**, R420-R425.

308. Merali, Z., Kent, P., and Anisman, H., (2002). Role of bombesin-related peptides in the mediation or integration of the stress response, *Cellular & Molecular Life Sciences*, **59**, 272-287.

309. Merali, Z., Kent, P., Michaud, D., McIntyre, D., and Anisman, H., (2001). Differential impact of predator or immobilization stressors on central corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rat, *Brain Research*, **906**, 60-73.

310. Merali, Z., McIntosh, J., and Anisman, H., (1999). Role of bombesin-related peptides in the control of food intake, *Neuropeptides*, **33**, 376-386.

311. Merali, Z., McIntosh, J., Kent, P., Michaud, D., and Anisman, H., (1998). Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala, *Journal of Neuroscience*, **18**, 4758-4766.

312. Merali, Z., Michaud, D., Kent, P., McIntyre, D.C., McIntosh, J., and Anisman, H., (1997). Effects of Psychological (Ferret Exposure) & Restraint Stressors on Amygdalar CRF Release & Endocrine Responses in Two Differentially stress-Sensitive Rat Strains, *Society for Neuroscience Abstracts*, **23**[2], 1077.

313. Merali, Z., Moody, T., Kateb, P., and Piggins, H., (1988). Antagonism of Satiety and Grooming Effects of Bombesin by Antiserum to Bombesin and by [Try^{4}, D-Phe^{12}}Bombesin: Central versus Peripheral Effects, *Annals of the New York Academy of Sciences*, **547**, 489-492.

314. Merali, Z., Moody, T.W., and Coy, D., (1993). Blockade of brain bombesin/GRP receptors increases food intake in satiated rats, *American Journal of Physiology*, **264**, R1031-R1034.

315. Merali, Z., Moody, T.W., and Coy, D., (1994). Corticotropin-releasing factor (CRF) antagonist blocks the autonomic, endocrine, ingestive and behavioral effects of central bombesin (BN), *Society for Neuroscience Abstracts*, **1**, 528.

316. Mercer, J.G. and Speakman, J.R., (2001). Hypothalamic neuropeptide mechanism for regulating energy balance: from rodent models to human obesity, *Neuroscience & Biobehavioral Reviews*, **25**, 101-116.

317. Merchenthaler, I., Vigh, S., Schally, A.V., Stumpf, W.E., and Arimura, A., (1984). Immunocytochemical localization of corticotropin releasing factor (CRF)-like immunoreactivity in the thalamus of the rat, *Brain Research*, **323**, 119-122.

318. Merola, B., Longobardi, S., Colao, A., Di Soma, C., Ferone, D., Rossi, E., Covelli, V., and Lombardi, G., (1994). Hypothalamic-Pituitary-Adrenal Axis in Neuropsychiatric Disorders, *Annals of the New York Academy of Sciences*, **741**, 263-270.

319. Michaud, D., Anisman, H., and Merali, Z., (1999). Capsaicin-sensitive fibers are required for the anorexic action of systemic but not central bombesin, *American Journal of Physiology*, **276**, R1617-R1622.

320. Minamino, N., Kangawa, K., and Matsuo, H., (1983). Neuromedin B: A Novel Bombesin-like Peptide Identified in Porcine Spinal Cord, *Biochemical and Biophysical Research Communications*, **114**, 541-548.

321. Minamino, N., Kangawa, K., and Matsuo, H., (1984). Neuromedin B is a major bombesin-like peptide in rat brain: regional distribution of neuromedin B and neuromedin C in rat brain, pituitary and spinal cord, *Biochemical & Biophysical Research Communications*, **124**, 925-932.

322. Minamino, N., Kangawa, K., and Matsuo, H., (1984). Neuromedin C: a bombesin-like peptide identified in porcine spinal cord, *Biochemical & Biophysical Research Communications*, **119**, 14-20.

323. Minamino, N., Kangawa, K., and Matsuo, H., (1988). Neuromedin B and Neuromedin C: Two Mammalian Bombesin-Like Peptides Identified in Porcine Spinal Cord and Brain, *Annals of the New York Academy of Sciences*, **547**, 373-390.

324. Minano, F.J., Meneres Sancho, M.S., Sancibrian, M., Salinas, P., and Myers, R.D., (1992). GABAA receptors in the amygdala: role in feeding in fasted and satiated rats, *Brain Research*, **586**, 104-110.

325. Mirenowicz, J. and Schultz, W., (1996). Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli, *Nature*, **379**, 449-451.

326. Mistlberger, R.E. and Marchant, E.G., (1999). Enhanced food-anticipatory circadian rhythms in the genetically obese Zucker rat, *Physiology & Behavior*, **66**, 329-335.

327. Moghaddam, B. and Bunney, B., (1989). Ionic composition of microdialysis perfusing solution alters the pharmacological responsiveness and basal outflow of striatal dopamine, *Journal of Neurochemistry*, **53**, 652-654.

328. Mohapel, P. and McIntyre, D.C., (1998). Amygdala kindling-resistant (SLOW) or -prone (FAST) rat strains show differential fear responses, *Behavioral Neuroscience*, **112**, 1402-1413.

329. Moody, T.W., Getz, R., O'Donohue, T.L., and Rosenstein, J.M., (1988). Localization of Receptors for Bombesin-like Peptides in the Rat Brain, *Annals of the New York Academy of Sciences*, **547**, 114-130.

330. Moody, T.W., Korman, L.Y., and O'Donohue, T.L., (1986). Neuromedin B-like peptides in rat brain: biochemical characterization, mechanism of release and localization in synaptosomes, *Peptides*, **7**, 815-820.

331. Moody, T.W., Merali, Z., and Crawley, J.N., (1988). The effects of anxiolytics and other agents on rat grooming behavior, *Annals of the New York Academy of Sciences*, **525**, 281-290.

332. Moody, T.W., O'Donohue, T.L., and Jacobowitz, D.M., (1981). Biochemical Localization and Characterization of Bombesin-like Peptides in Discrete Regions of Rat Brain, *Peptides*, **2**, 75-79.

333. Moran, T.H. and Ladenheim, E., (1998). Identification of Receptor Populations Mediating the Satiating Actions of Brain and Gut Peptides. In: Smith, G.P. (Ed.), *Satiation: From Gut to Brain*, Oxford University Press, New York, 126-163.

334. Morley, J.E. and Levine, A., (1980). Bombesin Inhibits Stress-Induced Eating, *Pharmacology, Biochemistry & Behavior*, **14**, 149-151.

335. Morley, J.E. and Levine, A.S., (1982). Corticotrophin Releasing Factor, Grooming and Ingestive Behavior, *Life Sciences*, **31**, 1459-1464.

336. Morley, J.E., Levine, A.S., Gosnell, B.A., and Billington, C.J., (1984). Neuropeptides and appetite: contribution of neuropharmacological modeling, *Federation Proceedings*, **43**, 2903-2907.

337. Muglia, L.J., Jacobson, L., Weninger, S.C., Karalis, K.P., Jeong, K., and Majzoub, J.A., (2001). The physiology of corticotropin-releasing hormone deficiency in mice, *Peptides*, **22**, 725-731.

338. Mullett, M.A., Billington, C.J., Levine, A.S., and Kotz, C.M., (2000). Hypocretin I in the lateral hypothalamus activates key feeding-regulatory brain sites, *Neuroreport*, **11**, 103-108.

339. Mullett, M.A., Billington, C.J., Levine, A.S., and Kotz, C.M., (2000). Hypocretin I in the lateral hypothalamus activates key feeding-regulatory brain sites, *Neuroreport*, **11**, 103-108.

340. Muurahainen, N.E., Kissileff, H.R., and Pi-Sunyer, F.X., (1993). Intravenous infusion of bombesin reduces food intake in humans, *American Journal of Physiology*, **264**, R350-R354.

341. Myers, R.D., Lankford, M.F., and Paez, X., (1992). Norepinephrine, Dopamine, and 5-HT Release from Perfused Hypothalamus of the Rat During Feeding Induced by Neuropeptide Y, *Neurochemical Research*, **17**, 1123-1132.

342. Naimi, N., Rivest, S., Racotta, I., and Richard, D., (1997). Neuronal activation of the hypothalamic magnocellular system in response to oropharyngeal stimuli in the rat, *Journal of Neuroendocrinology*, **9**, 329-340.

343. Nakahara, D., Nakamura, M., Furukawa, H., and Furuno, N., (2000). Intracranial self-stimulation increases differentially in vivo hydroxylation of tyrosine but similarly in vivo hydroxylation of tryptophan in rat medial prefrontal cortex, nucleus accumbens and striatum, *Brain Research*, **864**, 124-129.

344. Nielsen, D.M., Crosley, K.J., Keller Jr.Richard, W., Glick, S.D., and Carlson, J.N., (1999). Left and right 6-hydroxydopamine lesions of the medial prefrontal cortex differentially affect voluntary ethanol consumption, *Brain Research*, **823**, 59-66.

345. Noble, F. and Roques, B.P., (1999). CCK-B receptor: chemistry, molecular biology, biochemistry and pharmacology, *Progress in Neurobiology*, **58**, 349-379.

346. Nunez, J.F., Ferre, P., Garcia, E., Escorihuela, R.M., Fernandez-Teruel, A., and Tobena, A., (1995). Postnatal Handling Reduces Emotionality Ratings and Accelerates Two-Way Active Avoidance in Female Rats, *Physiology and Behavior*, **57**, 831-835.

347. Ogawa, T., Mikuni, M., Kuroda, Y., Muneoka, K., Mori, K.J., and Takahashi, K. (1994). Periodic Maternal Deprivation Alters Stress Response in Adult Offspring: Potentiates the Negative Feedback Regulation of Restraint Stress-Induced Adrenocortical Response and Reduces the Frequencies of Open Field-Induced Behaviors, *Pharmacology Biochemistry and Behavior*, **49**, 961-967.

348. Ohki-Hamazaki, H., (2000). Neuromedin B., *Progress in Neurobiology*, **62**, 297-312.

349. Ohki-Hamazaki, H., Sakai, Y., Kamata, K., Ogura, H., Okuyama, S., Watase, K., Yamada, K., and Wada, K., (1999). Functional properties of two bombesin-like peptide receptors revealed by the analysis of mice lacking neuromedin B receptor, *Journal of Neuroscience*, **19**, 948-954.

350. Oiry, C., Pannequin, J., Bernad, N., Artis, A., Galleyrand, J., Devin, C., Cristau, M., Fehrentz, J., and Martinez, J., (2000). A synthetic glycine-extended bombesin analogue interacts with the GRP/bombesin receptor, *European Journal of Pharmacology*, **403**, 17-25.

351. Olsen, L., Knigge, U., and Warberg, J., (1992). Gastrin-releasing peptide stimulation of corticotropin secretion in male rats, *Endocrinology*, **130**, 2710-2716.

352. Olson, G.A., Olson, R.D., Vaccarino, A.L., and Kastin, A.J., (1998). Endogenous Opiates: 1997, *Peptides*, **19**, 1791-1843.

353. Ono, M., Shibata, S., Minamoto, Y., and Watanabe, S., (1996). Effect of the noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 on food-anticipatory activity rhythm in the rat, *Physiology & Behavior*, **59**, 585-589.

354. Paez, X. and Leibowitz, S.F., (1993). Changes in Extracellular PVN Monoamines and Macronutrient Intake After Idazoxan or Fluoxetine Injection, *Pharmacology Biochemistry and Behavior*, **46**, 933-941.

355. Paez, X., Stanley, B.G., and Leibowitz, S.F., (1993). Microdialysis analysis of norepinephrine levels in the paraventricular nucleus in association with food intake at dark onset, *Brain Research*, **606**, 167-170.

356. Palanza, P., (2001). Animal models of anxiety and depression: how are females different? *Neuroscience & Biobehavioral Reviews*, **25**, 219-233.

357. Palkovits, M. and Brownstein, M.J., (1988). Maps and Guide to Microdissection of the Rat Brain, Elsevier, New York, **223**, 1-23.

358. Panula, P., Neiminen, O., Falkenberg, M., and Auvinen, S., (1988). Localization and Development of Bombesin/GRP-like Immunoreactivity in the Rat Central Nervous System, *Annals of the New York Academy of Sciences*, **547**, 54-69.

359. Pare, W.P., (1994). Hyponeophagia in Wistar Kyoto (WKY) rats, *Physiology & Behavior*, **55**, 975-978.

360. Pare, W.P., Blair, GR., Kluczynski, J., and Tejani-Butt, S., (1999). Gender differences in acute and chronic stress in Wistar Kyoto (WKY) rats, *Integrative Physiological & Behavioral Science*, **34**, 227-241.

361. Patchev, V.K., Hayashi, S., Orikasa, C., and Almeida, O.F.X., (1995). Implications of estrogen-dependent brain organization for gender differences in hypothalamo-pituitary-adrenal regulation, *The FASEB Journal*, **9**, 419-423.

362. Paxinos, G. and Watson C., (1986). The brain in stereotaxic coordinates, Academic, New York.

363. Pecina, S. and Berridge, K.C., (1996). Brainstem mediates diazepam enhancement of palatability and feeding: microinjections into fourth ventricle versus lateral ventricle, *Brain Research*, **727**, 22-30.

364. Peeke, P.M. and Chrousos, G.P., (1995). Hypercortisolism and Obesity, *Annals of the New York Academy of Sciences*, **771**, 665-676.

365. Pehek, E.A., McFarlane, H.G., Maguschak, K., Price, B., and Pluto, C.P., (2001). M100,907, a selective 5-HT_{2A} antagonist, attenuates dopamine release in the rat medial prefrontal cortex, *Brain Research*, **888**, 51-59.

366. Persons, J.E., Stephan, F.K., and Bays, M.E., (1993). Diet-induced obesity attenuates anticipation of food access in rats, *Physiology & Behavior*, **54**, 55-64.

367. Pert, A., Moody, T.W., Pert, C.B., Dewald, L.A., and Rivier, J., (1980). Bombesin: Receptor Distribution in Brain and Effects on Nociception and Locomotor Activity, *Brain Research*, **193**, 209-220.

368. Peyron, C., Tighe, D.K., van den Pol, A.N., de Lecea, L., Heller, H.C., Sutcliffe, J.G., and Kilduff, T.S., (1998). Neurons Containing Hypocretin (Orexin) Project to Multiple Neuronal Systems, *The Journal of Neuroscience*, **18**, 9996-10015.

369. Piazza, P.V. and Le Moal, M., (1997). Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications, *Brain Research - Brain Research Reviews*, **25**, 359-372.

370. Pich, E.M., Lorang, M., Yeganeh, M., Rodriguez, D.F., Raber, J., Koob, G.F., and Weiss, F., (1995). Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis, *Journal of Neuroscience*, **15**, 5439-5447.

371. Pinel, J.P., (1997). Digestion and Energy Flow. In: *Biopsychology*, Allyn and Bacon, Boston, 234-254.

372. Pinnock, R.D., Reynolds, T., and Woodruff, G.N., (1994). Different types of bombesin receptors on neurons in the dorsal raphe nucleus and the rostral hypothalamus in rat brain slices in vitro, *Brain Research*, **653**, 119-124.

373. Pinnock, S.B. and Herbert, J., (2001). Corticosterone differentially modulates expression of corticotropin releasing factor and arginine vasopressin mRNA in the hypothalamic paraventricular nucleus following either acute or repeated restraint stress, *European Journal of Neuroscience*, **13**, 576-584.

374. Pisarska, M., Mulchahey, J.J., Sheriff, S., Geracioti, T.D., Jr., and Kasckow, J.W., (2001). Regulation of corticotropin-releasing hormone in vitro, *Peptides*, **22**, 705-712.

375. Pivac, N. and Pericic, D., (1993). Inhibitory effect of diazepam on the activity of the hypothalamic-pituitary-adrenal axis in female rats, *Journal of Neural Transmission - General Section*, **92**, 173-186.

376. Plamondon, H. and Merali, Z., (1993). Effects of central neuromedin B and related peptides on blood glucose, *Regulatory Peptides*, **47**, 133-140.

377. Plamondon, H. and Merali, Z., (1994). Push-pull perfusion reveals meal-dependent changes in the release of bombesin-like peptides in the rat paraventricular nucleus, *Brain Research*, **668**, 54-61.

378. Plamondon, H. and Merali, Z., (1997). Regulation of ingestion by CRF and bombesin-like peptides: distinct meal-related peptide level changes, *American Journal of Physiology*, **272**, R268-R274.

379. Plamondon, H., Moody, T.W., and Merali, Z., (1996). Central infusion of neuromedin B and gastrin-releasing peptide receptor antisense oligonucleotides reduce anxiety, bombesin (BN) binding, BN-elicited behaviors, *Society for Neuroscience Abstracts*, **22**, 456.

380. Plotsky, P.M. and Meaney, M.J., (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats, *Molecular Brain Research*, **18**, 195-200.

381. Pomonis, J.D., Jewett, D.C., Kotz, C.M., Briggs, J.E., Billington, C.J., and Levine, A.S., (2000). Sucrose consumption increases naloxone-induced c-Fos immunoreactivity in limbic forebrain, *American Journal of Physiology*, **278**, R719.

382. Potter, E., Behan, D.P., Linton, E.A., Lowry, P.J., Sawchenko, P.E., and Vale, W.W., (1992). The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF, *Proceedings National Academy Science USA*, **89**, 4192-4196.

383. Poulter, M.O., Brown, L.A., Tynan, S., Willick, G., William, R., and McIntyre, D.C., (1999). Differential expression of alpha1, alpha2, alpha3, and alpha5 GABAA receptor subunits in seizure-prone and seizure-resistant rat models of temporal lobe epilepsy, *Journal of Neuroscience* , **19**, 4654-4661.

384. Poyner, D., Cox, H., Bushfield, M., Treherne, J.M., and Demetrikopoulos, M.K., (2000). Neuropeptides in drug research, *Progress in Drug Research*, **54**, 121-149.

385. Pratt, W.E. and Mizumori, S.J., (2001). Neurons in rat medial prefrontal cortex show anticipatory rate changes to predictable differential rewards in a spatial memory task, *Behavioural Brain Research*, **123**, 165-183.

386. Pu, S., Jain, M.R., Horvath, T.L., Diano, S., Kalra, P.S., and Kalra, S.P., (1999). Interactions between neuropeptide Y and gamma-aminobutyric acid in stimulation of feeding: a morphological and pharmacological analysis, *Endocrinology*, **140**, 933-940.

387. Rabin, B.M., (1972). Ventromedial Hypothalamic Control of Food Intake and Satiety: A Reappraisal, *Brain Research*, **43**, 317-342.

388. Racine, R.J., Steingart, M., and McIntyre, D.C., (1999). Development of kindling-prone and kindling-resistant rats: selective breeding and electrophysiological studies, *Epilepsy Research*, **35**, 183-195.

389. Ravard, S. and Dourish, C.T., (1990). Cholecystokinin and anxiety, *Trends in Pharmacological Science*, **11**, 271-273.

390. Redrobe, J.P., Dumont, Y., St Pierre, J.A., and Quirion, R., (1999). Multiple receptors for neuropeptide Y in the hippocampus: putative roles in seizures and cognition., *Brain Research*, **848**, 153-166.

391. Rex, A., Stephens, D.N., and Fink, H., (1996). "Anxiolytic" action of diazepam and abecarnil in a modified open field test, *Pharmacology, Biochemistry & Behavior*, **53**, 1005-1011.

392. Richard, D., (1993). Involvement of Corticotropin-Releasing Factor in the Control of Food Intake and Energy Expenditure, *Annals of the New York Academy of Sciences*, **697**, 155-172.

393. Richardson, N.R. and Gratton, A., (1996). Behavior-relevant changes in nucleus accumbens dopamine transmission elicited by food reinforcement: an electrochemical study in rat, *Journal of Neuroscience*, **16**, 8160-8169.

394. Richardson, N.R. and Gratton, A., (1998). Changes in medial prefrontal cortical dopamine levels associated with response-contingent food reward: an electrochemical study in rat, *Journal of Neuroscience*, **18**, 9130-9138.

395. Rivier, C., (1999). Gender, Sex steroids, corticotropin-releasing factor, nitric oxide, and the HPA response to stress, *Pharmacology Biochemistry and Behavior*, **64**, 739-751.

396. Rivier, C., Brownstein, M.J., Spiess, J., Rivier, J., and Vale, W., (1982). *In Vivo* Corticotropin-Releasing Factor-Induced Secretion of Adrenocorticotropin, B-Endorphin, and Corticosterone, *Endocrinology*, **110**, 272-278.

397. Rodgers, R.J., Halford, J.C., Nunes de Souza, R.L., Canto de Souza, A.L., Piper, D.C., Arch, J.R., and Blundell, J.E., (2000). Dose-response effects of orexin-A on food intake and the behavioural satiety sequence in rats, *Regulatory Peptides*, **96**, 71-84.

398. Rodríguez de Fonseca, F., Carrera, M.R.A., Navarro, M., Koob, G.F., and Weiss, F., (1997). Activation of corticotropin-releasing factor in the limbic system during cannabinoid withdrawal, *Science*, **276**, 2050-2054.

399. Romero, L.M., Levine, S., and Sapolsky, R.M., (1995). Adrenocorticotropin secretagog release: stimulation by frustration and paradoxically by reward presentation, *Brain Research*, **676**, 151-156.

400. Rosmond, R., Holm, G., and Bjorntorp, P., (2000). Food-induced cortisol secretion in relation to anthropometric, metabolic and haemodynamic variables in men, *International Journal of Obesity & Related Metabolic Disorders*, **24**, 416-422.

401. Rothenberg, M.A. and Chapman, C.F., (1994). Dictionary of Medical Terms for the Nonmedical Person, Barron's Educational Series, Inc., Hauppauge. New York.

402. Rowland, N., (1991). Biological factors in eating and its disorders, *Bulletin of the Psychonomic Society*, **29**, 244-249.

403. Ryan, R.R., Weber, H.C., Hou, W., Sainz, E., Mantey, S.A., Battey, J., Coy, D.H., and Jensen, R.T., (1997). Ability of Various Bombesin Receptor Agonists and Antagonist to Alter Intracellular Signaling of the Human Orphan Receptor BRS-3, *The Journal of Biological Chemistry*, **273**, 13613-13624.

404. Rybkin, I.I., Zhou, Y., Volaufova, J., Smagin, G.N., Ryan, D.H., and Harris, R.B., (1997). Effect of restraint stress on food intake and body weight is determined by time of day, *American Journal of Physiology*, **273**, R1612-R1622.

405. Saffran, M. and Schally, A.V., (1955). The release of corticotropin by anterior pituitary tissue in vitro, *Canadian Journal of Biochemical Physiology*, **33**, 408-415.

406. Sakata, T., Yoshimatsu, H., and Kurokawa, M., (1997). Hypothalmic neuronal histamine: implications of its homeostatic control of energy metabolism, *Nutrition*, **13**, 403-411.

407. Sakurai, T., (1999). Orexins and orexin receptors: implication in feeding behavior, *Regulatory Peptides*, **85**, 25-30.

408. Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., and Yanagisawa, M., (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior, *Cell*, **92**, 573-585.

409. Salacinski, P.R., McLean, C., Sykes, J.E.C., Clement-Jones, V.V., and Lowry, P.J., (1981). Iodination of proteins, and peptides using solid-phase oxidizing agent, 1,3,4.6-tetrachloro-3,6-diphenyl glucoluril (iodogen) radioiodine into proteins and peptides without damage, *Anal Biochemistry*, **117**, 136-146.

410. Sand, P., Kavvadias, D., Feineis, D., Riederer, P., Schreier, P., Kleinschnitz, M., Czygan, F.C., Abou-Mandour, A., Bringmann, G., and Beckmann, H., (2000). Naturally occurring benzodiazepines: current status of research and clinical implications, *European Archives of Psychiatry & Clinical Neuroscience*, **250**, 194-202.

411. Sapolsky, R.M., (1997). The importance of a well-groomed child, *Science*, **277**, 1620-1621.

412. Sapolsky, R.M., Romero, L.M., and Munck, A.U., (2000). How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions, *Endocrine Reviews*, **21**, 55-89.

413. Sawchenko, P.E., Brown, E.R., Chan, R.K., Ericsson, A., Li, H.Y., Roland, B.L., and Kovacs, K.J., (1996). The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress, *Progress in Brain Research*, **107**, 201-222.

414. Schmidt, E.D., Binnekade, R., Janszen, A.W., and Tilders, F.J., (1996). Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats, *Journal of Neuroendocrinology*, **8**, 703-712.

415. Schulkin, J., McEwen, B.S., and Gold, P.W., (1994). Allostasis, amygdala, and anticipatory angst, *Neuroscience & Biobehavioral Reviews*, **18**, 385-396.

416. Schultz, W., (2001). Reward signaling by dopamine neurons, *Neuroscientist*, **7**, 293-302.

417. Schwartz, D.H., Hernandez, L., and Hoebel, B.G., (1990). Serotonin release in lateral and medial hypothalamus during feeding and its anticipation, *Brain Research Bulletin*, **25**, 797-802.

418. Schwartz, G.J., (2000). The role of gastrointestinal vagal afferents in the control of food intake: current prospects, *Nutrition*, **16**, 866-873.

419. Schwartz, M.W., Dallman, M.F., and Woods, S.C., (1995). Hypothalamic response to starvation: implications for the study of wasting disorders, *American Journal of Physiology*, **269**, R949-R957.

420. Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., and Baskin, D.G., (2000). Central nervous system control of food intake, *Nature*, **404**, 661-671.

421. Sclafani, A., Hertwig, H., Vigorito, M., and Feigin, M.B., (1987). Sex differences in polysaccharide and sugar preferences in rats, *Neuroscience & Biobehavioral Reviews*, **11**, 241-251.

422. Sclafani, A., Lucas, F., and Ackroff, K., (1996). The importance of taste and palatability in carbohydrate-induced overeating in rats, *American Journal of Physiology*, **270**, R1197-R1202.

423. Seasholtz, A.F., Burrows, H.L., Karolyi, I.J., and Camper, S.A., (2001). Mouse models of altered CRH-binding protein expression, *Peptides*, **22**, 743-751.

424. Shanks, N., Larocque, S., and Meaney, M.J., (1995). Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: Early illness and later responsivity to stress, *Journal of Neuroscience*, **15**, 376-384.

425. Shephard, R.A. and Broadhurst, P.L., (1982). Effects of diazepam and picrotoxin on hyponeophagia in rats, *Neuropharmacology*, **21**, 771-773.

426. Shi, B., Heavner, J.E., Liu, J., Wang, M.J., Lutherer, L.O., McIntyre, D.C., and Reigel, C.E., (1999). Two genetically selected strains of rats exhibit hypersensitivity or resistance to cocaine-induced fatal arrhythmias, *Journal of Pharmacology & Experimental Therapeutics*, **288**, 685-692.

427. Shibasaki, T., Yamauchi, N., Yumiko, K., Masuda, A., Imaki, T., Hotta, M., Demura, H., Ono, H., Ling, N., and Shizume, K., (1988). Involvement of

Corticotropin-Releasing Factor in Restraint Stress-Induced Anorexia and Reversion of the Anorexia by Somatostatin in the Rat, *Life Sciences*, **43**, 1103-1110.

428. Shibata, S., Ono, M., Fukuhara, N., and Watanabe, S., (1995). Involvement of dopamine, N-methyl-D-aspartate and sigma receptor mechanisms in methamphetamine-induced anticipatory activity rhythm in rats, *Journal of Pharmacology & Experimental Therapeutics*, **274**, 688-694.

429. Shimizu, H., Ohtani, K., Kato, Y., Tanaka, Y., and Mori, Masatomo, (1996). Estrogen increases hypothalamic neuropeptide Y (NPY) mRNA expression in ovariectomized obese rat, *Neuroscience Letters*, **204**, 81-84.

430. Shiraishi, I., Honma, K., Honma, S., and Hiroshige, T., (1984). Ethosecretogram: relation of behavior to plasma corticosterone in freely moving rats, *American Journal of Physiology*, **247**, R40-R45.

431. Sills, T.L., Onalaja, A.O., and Crawley, J.N., (1998). Mesolimbic dopaminergic mechanisms underlying individual differences in sugar consumption and amphetamine hyperlocomotion in Wistar rats, *European Journal of Neuroscience*, **10**, 1895-1902.

432. Silveira, P.P., Xavier, M.H., Souza, F.H., Manoli, L.P., Rosat, R.M., Ferreira, M.B., and Dalmaz, C., (2000). Interaction between repeated restraint stress and concomitant midazolam administration on sweet food ingestion in rats, *Brazilian Journal of Medical & Biological Research*, **33**, 1343-1350.

433. Simansky, K.J., (1998). Serotonin and the Structure of Satiation. In: Smith,G.P. (Ed.), *Satiation: From Gut to Brain*, Oxford University Press, New York, 217-262.

434. Simpson, J.R., Jr., Snyder, A.Z., Gusnard, D.A., and Raichle, M.E., (2001). Emotion-induced changes in human medial prefrontal cortex: I. During cognitive task performance, *Proceedings of the National Academy of Science USA*, **98**, 683-687.

435. Singh, L., Lewis, A.S., Field, M.J., Hughes, J., and Woodruff, G.N., (1991). Evidence for an involvement of the brain cholecystokinin B receptor in anxiety, *Proceedings National Academy Science USA*, **88**, 1130-1133.

436. Skelton, K.H., Nemeroff, C.B., Knight, D.L., and Owens, M.J., (2000). Chronic Administration of the Triazolobenzodiazepine Alprazolam Produces Opposite Effects on Corticotropin-Releasing Factor and Urocortin Neuronal Systems, *The Journal of Neuroscience*, **20**, 1240-1248.

437. Smagin, G.N., Heinrichs, S.C., and Dunn, A.J., (2001). The role of CRH in behavioral responses to stress, *Peptides*, **22**, 713-724.

438. Smith, G.P. and Gibbs, J., (1998). The Satiating Effects of Cholecystokinin and Bombesin-Like Peptides. In: Smith,G.P. (Ed.), *Satiation: From Gut to Brain*, Oxford University Press, New York, 97-125.

439. Smith, G.P., (1998). Introduction. In: Smith,G.P. (Ed.), *Satiation: From Gut to Brain*, Oxford University Press, New York, 3-9.

440. Smith, T.A., (2001). Type A gamma-aminobutyric acid (GABAA) receptor subunits and benzodiazepine binding: significance to clinical syndromes and their treatment, *British Journal of Biomedical Science*, **58**, 111-121.

441. Smythe, J.W., Rowe, W.B., and Meaney, M.J., (1994). Neonatal handling alters serotonin (5-HT) turnover and 5-HT2 receptor binding in selected brain regions: relationship to the handling effect on glucocorticoid receptor expression. *Developmental Brain Research*, **80**, 183-189.

442. Soderpalm, A.H. and Berridge, K.C., (2000). Food intake after diazepam, morphine or muscimol: microinjections in the nucleus accumbens shell, *Pharmacology, Biochemistry & Behavior*, **66**, 429-434.

443. Spanagel, R. and Weiss, F., (1999). The dopamine hypothesis of reward: past and current status, *Trends in Neurosciences*, **22**, 521-527.

444. Spiegler, G.J. and Mishkin, M., (1981). Evidence for the sequential participation of inferior temporal cortex and amygdala in the acquisition of stimulus-reward associations, *Behavioral Brain Research*, **3**, 303-317.

445. Spina, M., Merlo-Pich, E., Chan, R.K., Basso, A.M., Rivier, J., Vale, W., and Koob, G.F., (1996). Appetite-suppressing effects of urocortin, a CRF-related neuropeptide, *Science*, **273**, 1561-1564.

446. Steckler, T. and Holsboer, F., (1999). Corticotropin-releasing hormone receptor subtypes and emotion, *Biological Psychiatry*, **46**, 1480-1508.

447. Stenzel-Poore, M.P., Heinrichs, S.C., Rivest, S ., Koob, G.F., and Vale, W.W., (1994). Overproduction of Corticotropin-releasing Factor in Transgenic Mice: A Genetic Model of Anxiogenic Behavior, *The Journal of Neuroscience*, **14**, 2579-2584.

448. Strand, F.L., (1999). Neuropeptides: Regulators of Physiological Processes, MIT Press, Cambridge, Massachusetts.

449. Stratakis, C.A. and Chrousos, G.P., (1995). Neuroendocrinology and Pathophysiology of the Stress System, *Annals of the New York Academy of Sciences*, **771**, 1-54.

450. Stratford, T.R., Gibbs, J., and Smith, G.P., (1996). Simultaneous Administration of Neuromedin B-10 and Gastrin-Releasing Peptide(1-27) Reproduces the Satiating and Microstructural Effects of Bombesin, *Peptides*, **17**, 107-110.

451. Stratford, T.R. and Kelley, A.E., (1997). GABA in the nucleus accumbens shell participates in the central regulation of feeding behavior, *Journal of Neuroscience*, **17**, 4434-4440.

452. Stratford, T.R. and Kelley, A.E., (1999). Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior, *Journal of Neuroscience*, **19**, 11040-11048.

453. Stratford, T.R., Kelley, A.E., and Simansky, K.J., (1999). Blockade of GABAA receptors in the medial ventral pallidum elicits feeding in satiated rats, *Brain Research*, **825**, 199-203.

454. Strohmayer, A.J. and Greenberg, D., (1996). Devazepide increases food intake in male but not female Zucker rats, *Physiology & Behavior*, **60**, 273-275.

455. Stuckey, J.A. and Gibbs, J., (1982). Lateral hypothalamic injection of bombesin decreases food intake in rats, *Brain Research*, **8**, 617-621.

456. Stuckey, J.A., Gibbs, J., and Smith, G.P., (1985). Neural disconnection of gut from brain blocks bombesin-induced satiety, *Peptides*, **6**, 1249-1252.

457. Suchecki, D., Nelson, D.Y., Van Oers, H., and Levine, S., (1995). Activation and Inhibition of the Hypothalamic-Pituitary-Adrenal Axis of the Neonatal Rat: Effects of Maternal Deprivation, *Psychoneuroendocrinology*, **20**, 169-182.

458. Sullivan, R.M. and Gratton, A., (1998). Relationships between stress-induced increases in medial prefrontal cortical dopamine and plasma corticosterone levels in rats: role of cerebral laterality, *Neuroscience*, **83**, 81-91.

459. Sullivan, R.M. and Gratton, A., (1999). Lateralized effects of medial prefrontal cortex lesions on neuroendocrine and autonomic stress responses in rats, *Journal of Neuroscience*, **19**, 2834-2840.

460. Sullivan, R.M. and Gratton, A., (2002). Behavioral effects of excitotoxic lesions of ventral medial prefrontal cortex in the rat are hemisphere-dependent, *Brain Research*, **927**, 69-79.

461. Sutanto, W., Rosenfeld, P., de Kloet, E.R., and Levine, S., (1996). Long-term effects of neonatal maternal deprivation and ACTH on hippocampal mineralocorticoid and glucocorticoid receptors, *Developmental Brain Research*, **92**, 156-163.

462. Sutcliffe, J.G. and de Lecea, L., (2000). The hypocretins: excitatory neuromodulatory peptides for multiple homeostatic systems, including sleep and feeding, *Journal of Neuroscience Research*, **62**, 161-168.

463. Sutton, R.E., Koob, G.F., Le Moal, M., Rivier, J., and Vale, W., (1982). Corticotropin releasing factor produces behavioural activation in rats, *Nature*, **297**, 331-333.

464. Swanson, L.W., Sawchenko, P.E., Rivier, J., and Vale, W.W., (1983). Organization of Ovine Corticotropin-Releasing Factor Immunoreactive Cells and Fibers in the Rat Brain: An Immunohistochemical Study, *Neuroendocrinology*, **36**, 165-186.

465. Swiergiel, A.H. and Dunn, A.J., (1999). CRF-deficient mice respond like wild-type mice to hypophagic stimuli, *Pharmacology, Biochemistry & Behavior*, **64**, 59-64.

466. Swiergiel, A.H., Takahashi, L.K., and Kalin, N.H., (1993). Attenuation of stress-induced behavior by antagonism of corticotropin-releasing factor receptors in the central amygdala in the rat, *Brain Research*, **623**, 229-234.

467. Szelényi, Z., (2001). Cholecystokinin and thermoregulation - a minireview, *Peptides*, **22**, 1245-1250.

468. Taber, M. and Fibiger, H., (1997). Activation of the mesocortical dopamine system by feeding: lack of a selective response to stress, *Neurocscience* **77**, 295-298.

469. Tanganelli, S., Fuxe, K., Antonelli, T., O'Connor, W.T., and Ferraro, L., (2001). Cholecystokinin/dopamine/GABA interactions in the nucleus accumbens: biochemical and functional correlates, *Peptides*, **22**, 1229-1234.

470. Tempel, D.L., McEwen, B., and Leibowitz, S.F., (1992). Effects of Adrenal Steroid Agonists on Food Intake and Macronutrient Selection, *Physiology & Behavior*, **52**, 1161-1166.

471. Thorsell, A., Michalkiewicz, M., Dumont, Y., Quirion, R., Caberlotto, L., Rimondini, R., Mathe, A.A., and Heilig, M., (2000). Behavioral insensitivity to restraint stress, absent fear suppression of behavior and impaired spatial learning in transgenic rats with hippocampal neuropeptide Y overexpression, *Proceedings of the National Academy of Science USA*, **97**, 12852-12857.

472. Tilders, F.J., Schmidt, E.D., and de Goeij, D.C., (1993). Phenotypic plasticity of CRF neurons during stress, *Annals of the New York Academy of Sciences*, **697**, 39-52.

473. Turnbull, A.V. and Rivier, C., (1997). Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides, *Experimental Biology & Medicine*, **215**, 1-10.

474. Vale, W., Spiess, J., Rivier, C., and Rivier, J., (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin, *Science*, **213**, 1394-1397.

475. Vale, W., Vaughan, J., Yamamoto, G., Bruhn, T., Douglas, C., Dalton, D., Rivier, C., and Rivier , J., (1983). Assay of corticotropin releasing factor, *Methods in Enzymology*, **103**, 565-577.

476. Valee, M., Mayo, W., Maccari, S., Le Moal, M., and Simon, H., (1996). Long-term effects of prenatal stress and handling on metabolic parameters: relationship to corticosterone secretion response, *Brain Research*, **712**, 287-292.

477. Valles, A., Marti, O., Garcia, A., and Armario, A., (2000). Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats, *American Journal of Physiology*, **279**, R1138-R1144.

478. Van der, K.D., Koda, L.Y., McGinty, J.F., Gerfen, C.R., and Bloom, F.E., (1984). The organization of projections from the cortex, amygdala, and hypothalamus to the nucleus of the solitary tract in rat, *Journal of Comparative Neurology*, **224**, 1-24.

479. Van Eden, C.G. and Buijs, R.M., (2000). Functional neuroanatomy of the prefrontal cortex: autonomic interactions, *Progress in Brain Research*, **126**, 49-62.

480. Varma, M., Laviano, A., Meguid, M.M., Gleason, J.R., Yang, Z.J., and Oler, A., (2000). Comparison of early feeding pattern dynamics in female and male rats after reversible ventromedial nucleus of hypothalamus block, *Journal of Investigative Medicine*, **48**, 417-426.

481. Vazquez, D.M., Van Oers, H., Levine, S., and Akil, H., (1996). Regulation of glucocorticoid and mineralocorticoid receptor mRNAs in the hippocampus of the maternally deprived infant rat, *Brain Research*, **731**, 79-90.

482. Vergoni, A.V. and Bertolini, A., (2000). Role of melanocortins in the central control of feeding, *European Journal of Pharmacology*, **405**, 25-32.

483. Vergoni, A.V., Poggioli, R., Marrama, D., and Bertolini, A., (1990). Inhibition of feeding by ACTH-(1-24): behavioral and pharmacological aspects, *European Journal of Pharmacology*, **179**, 347-355.

484. Wada, E., Watase, K., Yamada, K., Ogura, H., Yamano, M., Inomata, Y., Eguchi, J., Yamamoto, K., Sunday, M.E., Maeno, H., Mikoshiba, K., Ohki-Hamazaki, H., and Wada, K., (1997). Generation and Characterization of Mice Lacking Gastrin-Releasing Peptide Receptor, *Biochemical & Biophysical Research Communications*, **239**, 28-33.

485. Wada, E., Way, J., Lebacq-Verheyden, A.M., and Battey, J., (1990). Neuromedin B and Gastrin-Releasing Peptide mRNAs Are Differentially Distributed in the Rat Nervous System, *The Journal of Neuroscience*, **10**, 2917-2930.

486. Wada, E., Way, J., Lebacq-Verheyden, A.M., and Battey, J.F., (1990). Neuromedin B and gastrin-releasing peptide mRNAs are differentially distributed in the rat nervous system, *Journal of Neuroscience*, **10**, 2917-2930.

487. Walker, C.D., (1995). Chemical sympathectomy and maternal separation affect neonatal stress responses and adrenal sensitivity to ACTH, *American Journal of Physiology*, **268**, R1281-R1288.

488. Walsh, B.T. and Devlin, M.J., (1998). Eating Disorders: Progress and Problems, *Science*, **280**, 1387-1390.

489. Weinberg, D.H., Sirinathsinghji, D.J., Tan, C.P., Shiao, L.L., Morin, N., Rigby, M.R., Heavens, R.H., Bayne, M.L., Cascieri, M.A., Strader, C.D., Linemeyer, D.L., and MacNeil, D.J., (1996). Cloning and expression of a novel neuropeptide Y receptor, *Journal of Biomedical Chemistry*, **271**, 16435-16438.

490. Weinstock, M., (1997). Does Prenatal Stress Impair Coping and Regulation of Hypothalamic-Pituitary-Adrenal Axis? *Neuroscience and Biobehavioral Reviews* **21**[1], 1-10.

491. Wenkstern, D., Pfaus, J.G., and Fibiger, H.C., (1993). Dopamine transmission increases in the nucleus accumbens of male rats during their first exposure to sexually receptive female rats, *Brain Research*, **618**, 41-46.

492. Wickelgren, I., (1997). Getting the brain's attention, *Science*, **278**, 35-37.

493. Wickelgren, I., (1998). Obesity: How Big a Problem? *Science*, **280**, 1364-1367.

494. Wieland, H.A., Hamilton, B.S., Krist, B., and Doods, H.N., (2000). The role of NPY in metabolic homeostasis: implications for obesity therapy, *Expert Opinion on Investigational Drugs*, **9**, 1327-1346.

495. Wise, R.A., (1996). Neurobiology of addiction, *Current Opinion in Neurobiology*, **6**, 243-251.

496. Wise, R.A., (2000). Interactions between medial prefrontal cortex and mesolimbic components of brain reward circuitry, *Progress in Brain Research*, **126**, 255-262.

497. Wolf, S.S., Moody, T.W., O'Donohue, T.L., Zarbin, M.A., and Kuhar, M.J., (1983). Autoradiographic Visualization of Rat Brain Binding Sites for Bombesin-like Peptides, *European Journal of Pharmacology*, **87**, 163-164.

498. Woodruff, G.N., Hill, D.R., Boden, P., Pinnock, R., Singh, L., and Hughes, J., (1991). Functional Role of Brain CCK Receptors, *Neuropeptides*, **19**, 45-56.

499. Woods, S.C. and Seeley, R.J., (2000). Adiposity signals and the control of energy homeostasis, *Nutrition*, **16**, 894-902.

500. Woods, S.C., Seeley, R.J., Porte, D., Jr., and Schwartz, M.W., (1998). Signals that regulate food intake and energy homeostasis, *Science*, **280**, 1378-1383.

501. Wurtman, J., Wurtman, R., Berry, E., Gleason, R., Goldberg, H., McDermott, J., Kahne, M., and Tsay, R., (1993). Dexfenfluramine, fluoxetine, and weight loss among female carbohydrate cravers, *Neuropsychopharmacology*, **9**, 201-210.

502. Yan, X., Day, P., Hollis, T., Monzingo, A.F., Schelp, E., Robertus, J.D., Milne, G.W.A., and Wang, S., (1998). Recognition and Interaction of Small Rings With the Ricin A-Chain Binding Site, *Proteins: Structure, Function, and Genetics*, **31**, 33-41.

503. Zarbin, M.A., Kuhar, M.J., O'Donohue, T.L., Wolf, S.S., and Moody, T.W., (1985). Autoradiographic localization of (^{125}I-Tyr^{4})Bombesin-binding Sites in Rat Brain, *The Journal of Neuroscience*, **5**, 429-437.

504. Zoeller, R.T., Lebacq-Verheyden, A.M., and Battey, J., (1989). Distribution of Two Distinct Messenger Ribonucleic Acids Encoding Gastrin-Releasing Peptide in Rat Brain, *Peptides*, **10**, 415-422.

505. Zola-Morgan, S., Squire, L.R., Alvarez-Royo, P., and Clower, R.P., (1991). Independence of memory functions and emotional behavior: separate contributions of the hippocampal formation and the amygdala, *Hippocampus*, **1**, 207-220.

506. Zukowska-Grojec, Z., (1995). Neuropeptide Y: a Novel Sympathetic Stress Hormone and More, *Annals of the New York Academy of Sciences*, **771**, 219-233.

507. Zylan, K.D. and Brown, S.D., (1996). Effect of Stress and Food Variety on Food Intake in Male and Female Rats, *Physiology & Behavior*, **59**, 165-169.

508. Zylan, K.D., (1996). Gender differences in the reasons given for meal termination, *Appetite*, **26**, 37-44.