

**Physiological Role of Bombesin-like Peptides in the Regulation of Food Intake:
Ontogenic Profile and Mechanisms of Action.**

A Doctoral Dissertation

by

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for the degree of Doctor of Philosophy to the
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Abstract

This research aimed to characterize the physiological role of bombesin-like peptides (BN-LP) in the control of ingestion. The first experiment assessed the developmental profile of BN response in rats, and demonstrated that BN effectively suppressed feeding from postnatal day (PD) 1 through PD 15. Pretreatment with BN receptor antagonist blocked this suppression, suggesting that BN receptors are functional and may participate in feeding regulation from the first hours following birth. We then examined whether endogenous levels of BN-LP in the brain changed in a meal-dependent manner. Of the 15 distinct nuclei examined, meal-related alterations in BN-LP were observed at the hypothalamic paraventricular (PVN), arcuate and dorsomedial nuclei and at the nucleus accumbens. These alterations appeared site and peptide specific since changes in CRF levels were restricted to the hypothalamic lateral and ventromedial nuclei and the central nucleus of the amygdala. To determine what these changes meant in terms of peptide utilization, we then monitored the *in vivo* release of BN-LP at the PVN. Food ingestion was associated with marked suppression in the release of BN-LP as compared to the preprandial and/or postprandial conditions, where the interstitial levels of BN-LP were relatively high. The next study examined whether sustained central exposure to a BN agonist affected spontaneous feeding, ingestive response to acute BN, or the density of BN receptors within the CNS. Feeding was suppressed over the initial 48 h of BN infusion, however, tolerance to this effect was apparent by 72 h and was associated with receptor down-regulation at the PVN and dentate gyrus. Acute BN administration suppressed feeding in both the chronic BN exposed and control groups indicating lack of tolerance to the acute fluctuations of BN. These findings imply the existence of different neural mechanism(s) mediating the acute versus long-term effects of BN. Finally, the potential interactions of BN with other satiety peptides were investigated. These studies revealed that BN partly mediates its satiety effects through interactions with CRF. The specificity of this interaction was supported by the lack of interaction between BN and/or CRF with oxytocin. This series of experiments provide novel data supporting the view that BN-LP play an important role in the regulation of food intake, and provide some new insights into their possible mechanism(s) of action.

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Summary

Bombesin (BN), a tetradecapeptide of amphibian origin and its mammalian counterparts, gastrin-releasing peptides (GRP₁₋₂₇ and GRP₁₈₋₂₇) and neuromedin Bs (NMB₁₋₃₂ and NMB₂₃₋₃₂) and their receptors have been identified in numerous sites within the central and peripheral nervous system. Among various biological actions, central or systemic administration of BN has been shown to induce satiety in numerous animal species, including man. Studies demonstrating enhanced feeding following central blockade of BN receptors together with reported increases in BN-like peptide content in postmortem brain and gut tissue of food deprived rats following feeding suggest the physiological participation of these peptides in the regulation of food intake. Thus, the overall objective of the studies performed and presented in this thesis was to elucidate, using a multidisciplinary approach, some of the behavioral, pharmacological and physiological mechanisms underlying actions of BN-like peptides on ingestive behavior. Together, these studies have brought strong and novel evidence supporting the contention that BN-like peptides play a role in the endogenous control of ingestion. This summary highlights our main findings.

1. We first attempted to determine how soon after birth do rats become sensitive to the satiating effects of BN-like peptides. To attain this objective rat pups were treated with various doses of BN on postnatal day (PD) 1, 5, 10 and 15. Our findings indicated that subcutaneous administration of BN dose-dependently suppressed

feeding in neonatal rats from postnatal day 1 through 15. Pretreatment with BN antagonist, DesMet, reversed BN-induced satiety at all ages, indicating that this effect was mediated through BN-receptor interactions. In general, DesMet failed to alter milk consumption when administered alone. However, at PD 15, DesMet slightly enhanced feeding. These findings 1) suggest that some physiological mechanisms involving BN receptors are potentially functional in appetite control from the first hours following birth, a period when the expression of these peptides is rather limited, and 2) replicate in the neonates the well-documented food suppression elicited in adult rats by BN administration. Parallel studies using oxytocin (OX), revealed that like BN, this peptide also suppressed food intake. Similarly, the OX antagonist vasotocin failed to alter milk consumption except at PD 1 and 10 when vasotocin enhanced feeding.

2. In light of reported meal-related fluctuations of BN-like immunoreactivity (BLI) noted in response to refeeding following food deprivation, the aims of the present study were to determine whether 1) similar changes in BLI would also be observed following a spontaneous (non imposed) meal of *ad libitum* fed (non deprived) rats, and 2) changes in BLI immunoreactivity previously observed in entire brain regions (i.e. the hypothalamus and hippocampus) could be anatomically localized to precise brain nuclei? Endogenous levels of BN were measured in 15 hypothalamic and extrahypothalamic brain nuclei before, during and after the initial spontaneous meal of the dark phase, using brain micropunch and radioimmunoassay. Feeding-related

alterations in BN concentrations were observed at the hypothalamic PVN, arcuate and dorsomedial nuclei and at the nucleus accumbens. At all three hypothalamic nuclei, BN content was significantly elevated during ingestion (by about two fold) as compared to before or after the meal while at the nucleus accumbens, meal-related fluctuations in BLI were characterized by increased preprandial levels accompanied by reduced BN content both during and after meal intake. These endogenous alterations appeared site- and peptide-specific since alterations in CRF content (as measured in the same study) were distinct and observed at the hypothalamic lateral and ventromedial nuclei and at the central amygdaloid nucleus.

3. In order to determine what these changes in terms of peptide availability at the synapse meant, we performed a push-pull perfusion study at the PVN to monitor the *in vivo* release of BN-like peptides in relationship to spontaneous feeding. Our findings revealed that food intake was associated with a marked suppression of the extracellular levels of BN-like peptides at the PVN. Conversely, the peptide release was significantly elevated preceding and following food ingestion. These peptidergic fluctuations appeared specific as they were observed at the anterior parvocellular portion of the PVN but not at more posterior PVN implants nor at extrahypothalamic perfusion sites within the caudate putamen.
4. The above set of experiments supports the physiological involvement of BN-like peptides in appetite control. Thus, would chronic disturbance of this peptidergic

system through sustained central exposure to BN agonist have any influence on a) spontaneous feeding, b) behavioral profile, c) ingestive response to acute BN, and d) changes in receptor in BN receptor density within the CNS? Our findings revealed significant reduction of spontaneous food intake in BN infused rats over the initial 48 h of chronic infusion. However, tolerance to this effect was apparent by 72 h post-infusion. An acute challenge with a bolus injection of BN (0.25 μ g; i.c.v.) elicited grooming and suppressed food intake in both the chronic BN exposed and control groups, by similar magnitude, indicating lack of tolerance to the acute effects. Rats chronically infused with BN also appeared more anxious as they spent less time in the open zones of the elevated plus maze and through tunnel oval maze. Following this 7-day exposure to BN, significant down-regulation of BN receptors was observed at the PVN and dentate gyrus. These findings represent the first demonstration of changes in receptor based and behavioral changes consequent to sustained exposure to BN in relationship to feeding behavior and suggest that gradually developing tolerance to the feeding suppressant effects of BN are partly mediated by receptor down-regulation at specific brain loci. Furthermore, these findings imply the existence of different neural substrate(s) mediating the acute “emergency-like” versus the long-term effects of BN.

5. Finally, we investigated whether interactions with other satiety peptides may play a role in the mediation of BN effects. In light of similarities between the effects of corticotropin-releasing factor (CRF) and BN, we first attempted to elucidate the potential role of CRF in the mediation and/or modulation of BN's effects. We

characterized the specificity of these effects through determination of pharmacological interactions between BN and OX as well as CRF and OX. Central pretreatment with CRF antagonist significantly attenuated or blocked BN's behavioral and ingestive effects. However, the OX antagonist, vasotocin, failed to alter BN-induced behaviors suggesting the absence of pharmacological interaction between these two peptidergic systems. Finally, CRF antagonist failed to reverse OX-induced suppression of food intake. Thus, the present experiments support the contention that BN partly mediates its satiety effects through interactions with CRF. The specificity of this interaction is supported by the lack of interaction between BN and/or CRF with OX.

In conclusion, our findings from this multiple faceted study provide novel and strong support for the contention that BN-like peptides may act as physiological satiety signals, and shed some light on the potential mechanism(s) by which BN-like peptides elicit their effects.

Introduction

Over the last two decades, substantial progress has been made in identifying the different signals involved in the regulation of feeding. Traditional views based on hunger and satiety centers in the hypothalamus have gradually been replaced by more dynamic models based on interactions between physiological processes in the central and peripheral nervous systems and social and physical environments. Thus, biopsychological approaches that emphasize the synchronous participation of psychological events (hunger perception, cravings, hedonic sensations), behavioral operations (food intake *per se*), peripheral physiological and metabolic processes as well as central neurotransmitter and metabolic processes are now believed to provide an integrative representation of the complex matrix of factors involved in feeding regulation^{41,182}.

The discontinuity of the feeding process, characterized by periods of meal intake interspaced by periods of non feeding (i.e. inter-meal intervals), has also made useful the distinction between satiation and satiety in these models. Satiation refers to the process which brings a period of ingestion to an end, while satiety refers to the inhibition of hunger and eating that arises as a consequence of food consumption. Satiating efficiency is the term applied to the capacity of the ingested food to suppress hunger and to inhibit the onset of a further period of eating^{42,171}. This efficiency appears largely dependent on the composition and total energy of the food consumed. The satiating power of the ingested food is important as it directly influences the biological drive to eat through its impact on physiological and biochemical mechanisms mediating satiation and satiety. For example, high-carbohydrate meals or preloads have been shown to significantly reduce the subject's

hunger ratings and food intake in a subsequent test meal compared to low-carbohydrate meals or preloads ^{41,309}.

The control of meal size (satiety) and the length of intermeal interval (satiety) are thought to be separately influenced by the nature and timing of physiological processes related to sensory, cognitive, post-ingestive and post-absorptive stimuli which individually contribute to the time course of satiety (see Fig.1) ⁴⁰. Thus, even before any food is consumed, physiological signals are generated by hedonic factors such as the thought, sight and/or smell of food, habits, learned factors, the social situation as well as other environmental influences that reaches cortical and hypothalamic brain regions ³⁹². The concomitant alterations in neurotransmitter activity further affects hunger and induces peripheral responses such as salivation, gastric secretion and insulin release in the anticipation of food ingestion ²⁹⁹. Afferent information is thus crucial and provides the major control over appetite both during and immediately after feeding. Thus, upon ingestion, the brain is informed of the physico-chemical nature of the ingested food through gastrointestinal mechanisms which reach the central nervous system (CNS) principally (but not exclusively) via the vagus nerve. These circulating factors and gastrointestinal neural afferents are affected by the meal itself, and their activity is proportional to the meal's nutritional, biochemical, and physical properties independent of adipose tissue mass ³⁹². These gut peptides which directly or indirectly reach the CNS form part of the post-ingestive mechanisms that control appetite and represent one class of "satiety signals" influencing the size and duration of a meal. Finally, post-absorptive processes also generate metabolic "satiety signals" which come into play once the

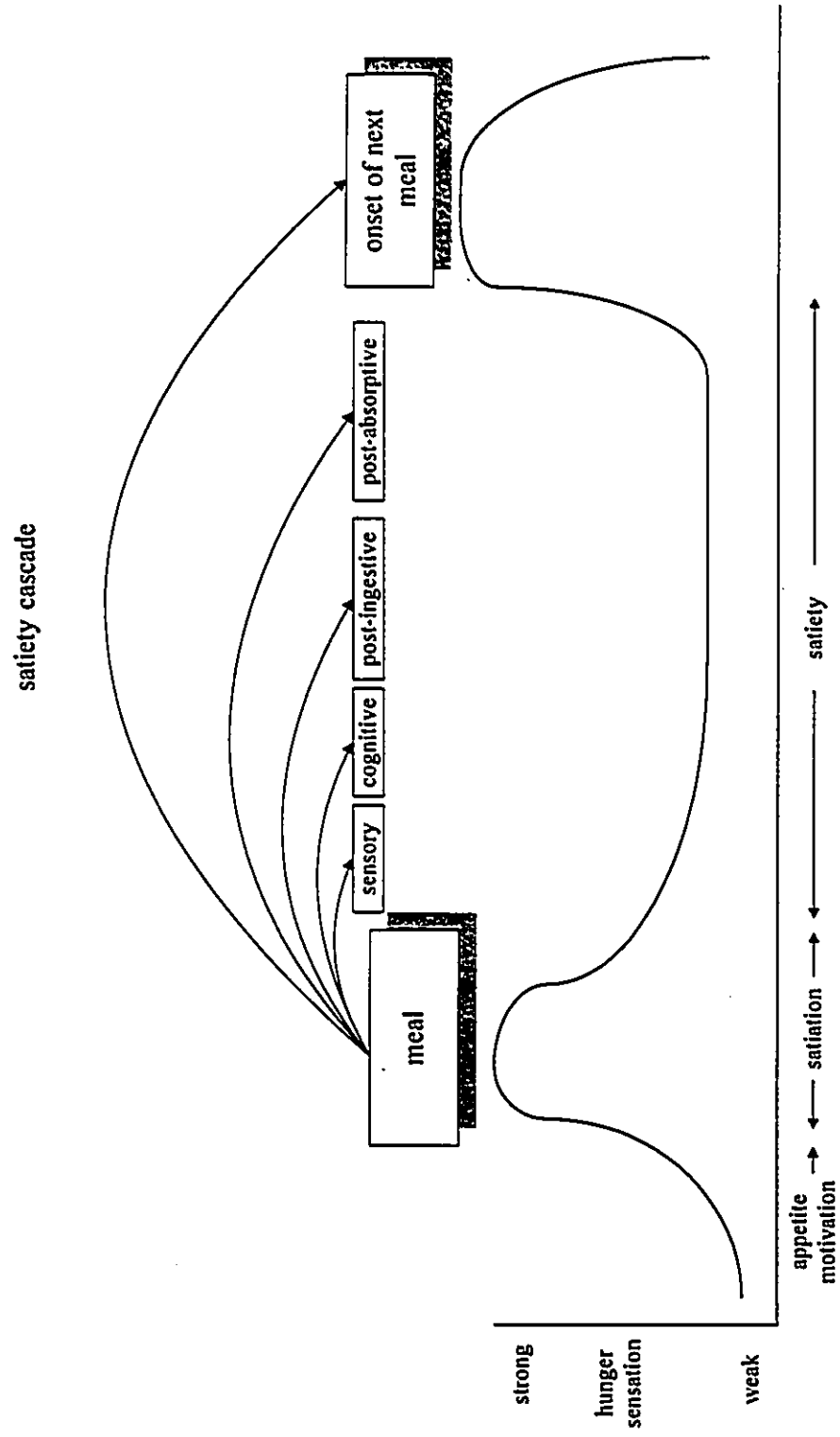


Fig.1 The Satiety Cascade: Mediating processes involved in the regulation of food intake ⁴⁰

ingested food has been digested and the nutrients have entered the blood circulation⁴⁰. These digested nutrients may enter the brain directly or may be further metabolized by peripheral tissues prior to influencing the CNS. The concentration of some of these circulating factors is influenced by the more slowly occurring changes in adiposity. Thus, these signals are generally thought as having a relatively long latency in influencing the initiation and termination of meals. Insulin is a good prototype candidate to serve as an adiposity signal. Thus, while post-ingestive secretion of insulin is influenced by levels of glucose and circulating hormones such as corticosterone (and considered among the primary satiety signals) its secretion is also dependent upon body adiposity. Supportive of this, are findings revealing that basal and total daily plasma insulin levels and secretion are directly proportional to the body adipose mass^{299,390}. Moreover, the recent demonstrations of *in vivo* fluctuations of hypothalamic insulin release in relation to food intake and obesity further support the participation of brain insulin in the short and long-term regulation of food intake and body weight²⁷⁶.

The physiological determinants of feeding are diverse and include simple nutrients in the blood (i.e. glucose, fatty acids and amino acids), classical neurotransmitters and numerous peptides and hormones. Close interactions between nutrients, endocrine and peptidergic signals from the periphery and neurochemical activity in the brain are believed to regulate the affective, physiological and metabolic properties of feeding. Due to its demonstrated importance as the principal fuel for the CNS, the participation of glucose in the control of feeding has been extensively studied. Evidence for the role of glucose in the initiation of eating has been established through the observation that transient decline in

blood glucose precedes meal intake⁶⁵. Similarly, injections of 2-deoxy-d-glucose have been associated with increased energy intake while glucose infusion in the hepatic portal system was shown to inhibit food ingestion²⁷⁰. In this context, glucose-detecting interoreceptors in the upper gastrointestinal tract linked to visceral afferent fibers in the vagus nerve, glucose-sensitive neurons and glucoreceptors in the brain are thought to provide an interface between the presence of available glucose and aminergic or peptidergic neural pathways^{40,224,275}. Classical neurotransmitters, namely the catecholamines and serotonin, have also been extensively studied as potential regulators of food ingestion^{10,117,147}. Furthermore, dopamine has been implicated in the mediation of reinforcing effects of food intake¹⁴⁸. Under some circumstances, acetylcholine²¹⁹ and γ -Aminobutyric acid⁹⁰ have also been shown to influence food intake. Moreover, subsequent to the demonstration by Gibbs and his colleagues of the ability of the peptide cholecystokinin (CCK) to reduce food intake, numerous brain and gut peptides were also shown to regulate food intake. Several studies have revealed extensive interplay of the various peptidergic signals in peripheral and CNS sites. The contribution of some selected anorectic and orexigenic peptides will be reviewed next.

Bombesin (BN)-like peptides constitute one family of peptides known to powerfully influence food ingestion. The principal objective of the research endeavor presented in this dissertation was to further elucidate the role and the mechanism(s) of action of these peptides in the control of food intake. Prior to a detailed presentation of this research, a brief review of the role of some putative neurochemical and peptidergic mediators of ingestion will be presented. Among the peptides reviewed, neuropeptide Y

and galanin are designated as “appetite peptides” for their stimulating properties on food ingestion while CCK and corticotropin-releasing factor (CRF) are among the more numerous peptides shown to inhibit food intake and referred to as “satiety peptides” (for a complete list of peptides involved in the control of ingestion see table 1). These peptides will be reviewed in terms of their distribution in the peripheral and central nervous systems, their documented role in the control of food ingestion, the proposed mechanisms of action as well as their interactions with classical neurotransmitters or other peptides. Following this preamble, an extensive review of the literature on BN-like peptides will be presented supporting a role for this family of peptides, and for BN in particular, in the control of ingestion. Finally, the overall objective of the present thesis will be outlined together with a set of five specific objectives highlighting the principal directions and avenues pursued by the present research. These objectives will be used to circumscribe the thesis research work into sections leading the reader through the different studies reported in this thesis.

Table 1. Peptides involved in the control of ingestion

<i>Peptides that inhibit feeding</i>	<i>Peptides that enhance feeding</i>
Bombesin Caerulein Calcitonin-Gen Related Peptide Cholecystokinin Cyclo-histidylproline-diketopiperazine Corticotropin Releasing Factor Glucagon Insulin Insulin Growth Factor Neurotensin Oxytocin Sauvagine Somatostatin Thyrotropin Releasing Hormone Vasoactive Intestinal Peptide	<i>Opioid Peptide Family</i> β -endorphin Dynorphin α -neo-endorphin <i>Pancreatic Polypeptide Family</i> Human pancreatic polypeptide Neuropeptide Y Peptide YY Galanin

The role of peptides in the modulation of food intake

1. Peptides as stimulators of appetite

Neuropeptide Y

Isolated from porcine brain in 1982, neuropeptide Y (NPY) is a 36-amino-acid peptide which belongs to the pancreatic polypeptide family³⁶⁰. Neuropeptide Y immunoreactivity, receptor sites and mRNA expression are distributed throughout the CNS^{97,104} with particularly high concentrations in hypothalamic nuclei, principally the paraventricular nucleus (PVN)^{97,216}. Administration of NPY into different brain areas has been shown to dose-dependently and specifically enhance food intake in satiated rats^{69,208}. Moreover, NPY is most potent when directly infused into the PVN³³⁹. When administered systemically, NPY failed to enhance food intake at doses shown effective when given centrally²⁰⁸. The orexigenic effects of NPY have been observed in various species including mouse²⁵³, sheep²⁴¹, squirrel²⁶⁷, pig²⁶⁷ and dog²⁸³ and this peptide is considered the most potent stimulator of feeding when injected into the brain of satiated animals^{69,208,341}.

Initial studies clearly demonstrated the strong pharmacological potency and efficacy of exogenous NPY to stimulate food intake (i.e. maximal feeding response induced by an optimal dose of NPY in satiated rats is 15 g in 1 h, which represents approximately 50% of the rat's daily food intake). However, more recent work further supports the view that NPY may play a physiological role in appetite control. In this context, initial studies using NPY receptor antagonists and antisera have shown that antagonism of endogenous NPY action is accompanied by a significant reduction of daily

food intake^{206,330,343}. Of particular interest is a recent study using infusion of oligonucleotide antisense aimed at diminishing endogenous NPY synthesis in the arcuate nucleus. In this study, NPY synthesis was diminished by about 30% at that brain site, and was accompanied by a significant reduction of spontaneous nutrient intake in the first 1.5 hr following dark onset⁶.

The levels of NPY immunoreactivity have also been shown to fluctuate with different feeding states: elevated NPY levels at the arcuate nucleus and PVN have been observed in relation to prolonged food deprivation (3–4 days) while a 24 h refeeding period has been associated with the return to normal of NPY immunoreactivity at the PVN but not the arcuate nucleus³¹⁴. In contrast, Beck et al's more recent findings, obtained subsequent to a shorter deprivation period (48 hr versus 3–4 days), revealed that while a 2–4 day deprivation induced similar increases in NPY concentrations at the arcuate nucleus and PVN, a 6 h refeeding (upon 48 hr deprivation) failed to alter NPY concentrations at the PVN but reversed the elevations noted at the arcuate nucleus²⁹. These two studies identified the PVN and arcuate nuclei as being specifically associated with food ingestion as compared to other sampled hypothalamic nuclei which failed to show feeding-related fluctuations. Authors of the former study concluded that NPY might be a physiological neurochemical signal in the PVN that normally evokes feeding in the rat. Alternatively, authors of the latter study proposed that the rapid fluctuations observed at the arcuate nucleus reflect a local cessation of NPY synthesis in that structure and/or increased axonal transport of NPY from the arcuate nucleus to the PVN. These authors also suggested that the elevated NPY concentration at the PVN after refeeding may be linked to NPY's

modulatory action on body weight (which is not back to baseline after 6 h refeeding) rather than food ingestion *per se*. The observed feeding-related NPY fluctuations at these two brain nuclei await further clarification.

Similar to what has been observed in food deprived animals, endogenous NPY concentrations have been shown to peak approximately 90 min preceding the initiation of the natural feeding cycle associated with dark onset¹⁶¹. The failure of adrenalectomized rats to demonstrate similar fluctuations in NPY levels argues for its dependence on circulating corticosterones²⁹⁶. Indeed, glucocorticoids have been shown to stimulate NPY mRNA synthesis at the ARC and food ingestion³⁴⁶. In an attempt to associate the feeding state related fluctuations in NPY tissue levels with the actual release of this peptide, Stricker-Krongrad et al.³⁴⁸ have used push-pull perfusion combined with time specific neuronal stimulation using high concentration of potassium (55 mM KCl) as a tool to assess the physiological role of NPY. Their results demonstrated, first, that NPY is indeed released from the PVN under basal conditions and, second, that increased K⁺-stimulated NPY release from this structure is associated with significant increase in food ingestion, suggesting the physiological participation of NPY at the PVN in feeding. However, in addition to NPY, KCl might have stimulated the release of various other compounds. In this context, the observation of dynamic changes in NPY release at the PVN in rats trained to eat only for 4 hr daily overrode this problem. In these rats, NPY release was significantly higher before food was available and during food presentation. The release of NPY at the PVN fell significantly 2 h after food consumption and stayed low for the remaining 2 h of the sampling period. These changes in NPY release at the

PVN seem attributable to food consumption as rats maintained on the same feeding regimen but not given food did not show fluctuations of NPY release which remained elevated for the entire observation period ¹⁶⁵. Also of interest in terms of potential physiological importance are findings revealing the existence of elevated hypothalamic NPY immunoreactivity and mRNA in genetically obese Zucker rats as compared to their lean littermates ^{318,369}. Finally, average *in vivo* NPY release from the PVN has also been found significantly higher in streptozotocin-induced diabetic rats as compared to controls, suggesting that PVN NPY secretion perhaps contributes to the hyperphagia observed in diabetic rats ³¹⁶.

The mechanisms underlying NPY action on food intake are not fully understood. Using receptor agonists, researchers have recently demonstrated NPY's orexigenic action on feeding to be primarily mediated by the Y1, rather than Y2, receptor subtype ^{166,201}. The strong stimulatory influence of NPY on the release of diverse hormones such as corticosterone, insulin, adrenocorticotropin hormone (ACTH) and vasopressin suggests that NPY might exert its action on food intake through alteration of some of these endocrine signals ^{5,6,204,381}. In turn, it has been suggested that insulin and corticosterone provide feedback actions and regulate NPY synthesis, secretion and gene expression ^{164,242,329,342,365}. Moreover, glucose injections have been shown to attenuate NPY stimulated feeding ³¹¹. Due to the coexistence of norepinephrine (NE) and NPY in locus ceruleus neurons innervating the hypothalamus and to similarities in their effects on feeding (both stimulate carbohydrate intake), numerous studies have also examined potential interactions between these two neurotransmitters. Despite the facts that

1) NPY's effects on feeding have been shown independent of its interaction with a NE receptor¹⁸⁵, 2) the response latency and duration of these two compounds differ³⁴⁰ and, 3) no synergistic or additive effects on feeding is observed upon simultaneous infusion of NPY and NE in the ventromedial hypothalamus (VMH)²⁵⁶, diverse findings nonetheless suggest indirect relationships between the two substances which elicit a similar maximal feeding response when infused at the PVN^{204,362}. However, the complex nature of such interactions between NPY and NE can be appreciated through studies showing its dependence on selected feeding conditions as well as brain sites. In this context, Kyrkouli et al. have demonstrated a different NE release profile upon intra PVN administration of NPY in the presence or absence of food during testing¹⁸⁸. While NPY administration enhanced NE release in the presence of food, it decreased extracellular NE concentrations in the absence of food. Furthermore, some studies have demonstrated site-dependent differential effects in NE turnover. Thus, while Vallejo et al.'s findings revealed a decreased NE turnover after low dose NPY infusion in the parvocellular portion of the PVN³⁷², Shimizu and Bray's findings demonstrated that NPY infusion in the ventromedial hypothalamus of *ad libitum* fed rats reduced NE turnover at that site, whereas NPY injection had the opposite effect at the lateral hypothalamus³³². Interestingly, infusion of NE into the lateral hypothalamus inhibited food ingestion while its administration into the PVN or VMH enhanced food intake^{199,200}. Thus, Shimizu and Bray suggest that the distinct NPY-elicited changes in NE release at the lateral and ventromedial hypothalamic sites might reveal counterregulatory mechanisms cycling through the hyperphagic action of NPY, suppression of food intake and restoration of homeostasis³³². Alternatively, other

researchers have proposed that the reduction of NE release at the PVN upon NPY administration may simply reflect the independence of these two neurotransmitter systems in eliciting their effects on ingestion. The former hypothesis suggesting some interactions between NE and NPY draws additional support from studies demonstrating that pharmacological inhibition of catecholamine synthesis at the PVN or bilateral brainstem nerve transection (both procedures resulting in a significant reduction of hypothalamic NE concentrations) enhanced NPY's stimulatory effect on food ingestion^{185,315}. More research is required to clarify the potential interactions between NPY and NE and the exact nature of these links.

Contrary to NPY and NE, the indolamine serotonin (5-HT) inhibits carbohydrate ingestion when injected into the PVN²⁰⁵ and, the 5-HT neuronal fibers have been shown to directly innervate NPY-immunoreactive hypothalamic neurons¹³⁷. Administration of the serotonergic agonist, fenfluramine, 1 h prior dark onset has been reported to reduce the NPY concentrations in different hypothalamic sites involved in feeding, including the PVN³⁰⁸. Additionally, NPY administration into the LH and VMH inhibit 5-HT release at both these brain sites³³². It is of interest to note that while Dube et al.⁹⁶ also found a rapid decrease in the levels of NPY at the PVN 2 h following fenfluramine administration in food deprived rats, NPY release remained unaffected in these rats over the same period. These results might suggest that the anorectic properties of fenfluramine are not mediated by changes in NPY release. Nonetheless, it is also possible that on a long-term basis, a deficiency in NPY stores could eventually attenuate NPY release and feeding in food deprived rats. At present, the exact nature of interactions between 5-HT and NPY is not

fully understood. It however remains possible that these neurotransmitters regulate feeding partly through mutual inhibition.

The majority of studies on the feeding effects of NPY have been conducted using satiated animals. In this context, one might question the possibility that NPY acts to attenuate the satiety signals rather than to stimulate food ingestion *per se*. Interestingly, recent studies have demonstrated that central NPY administration in fasted rats did not increase food intake initially but postponed the reduction of feeding that normally occur with satiation^{215,323}. While studies looking at potential interactions between NPY and the satiety agent CCK have generated conflicting results in terms of feeding behavior^{164,256,311}, Gourch et al.'s findings¹³² support a negative relationship between NPY and CCK. These authors measured NPY concentrations in the hypothalamus and plasma consequent to CCK agonists or antagonist pretreatment prior to CCK administration. Their results indicated that CCK agonist decreased NPY concentration in both the hypothalamus and plasma while CCK antagonist pretreatment blocked CCK-induced decrease of NPY concentrations. A study by Morley et al.²⁵⁶ also demonstrated the ability of peripherally administered BN, as well as central calcitonin and CRF administrations to attenuate NPY effects on ingestion. In this context, central NPY administration has also been shown to induce CRF release²⁵². It has been hypothesized that this phenomenon might take place as part of a counterregulatory response to NPY-induced hyperphagia. At present, the physiological significance and mechanisms underlying the counteractive effects of CCK and other peptides on NPY-induced feeding remain unknown. However, this variety of

indirect effects suggest that NPY may facilitate ingestion by blocking or attenuating normal satiety signals.

Galanin

Similar to NPY, the 29-amino acid neuropeptide galanin has also been isolated from porcine brain ³⁶¹. The peptide and its receptors are distributed throughout the gastrointestinal tract and brain of animals including humans ^{46,119,225}. Microinjection of galanin into the PVN or the amygdala stimulates consumption of high fat nutrients in a dose-dependent manner ^{80,184}. This response appears anatomically specific as infusion of GAL into the anterior, perifornical, ventromedial and posterior hypothalamic nuclei, the nucleus accumbens or the fourth ventricle failed to increase food intake in satiated rats ¹⁸⁴. Furthermore, galanin antagonists have been shown to block feeding induced by intra-PVN or intra-amygdala galanin injections in the rat suggesting that galanin's stimulatory effects on food intake is indeed mediated through specific activation of galanin receptors ⁷⁵. No changes in drinking, grooming, and resting behaviors are observed following central galanin infusion ^{75,183}.

Stimulatory effect of galanin on food ingestion appears to be primarily directed towards fat intake over other macronutrients. The magnitude of this effect has been reported to be similar to that of NPY's effect of carbohydrate intake ¹⁸³. In light of localization of both NPY and galanin in the PVN and of their stimulatory effects on food ingestion, a similar approach has been applied in the exploration of their mechanisms of action. In this context, study of the influence of galanin on endocrine release revealed

that, in contrast to NPY, galanin in general seems to have an inhibitory effect on endocrine release. Thus, galanin microinjection into the PVN is associated with a reduction of insulin and pituitary ACTH secretions as well as blood levels of corticosterone³⁶⁴. At present, the physiological relevance of such hormonal changes remains unclear but the observation of an intact ingestive response to intra-PVN administration of galanin in adrenalectomized rats suggest that galanin functions independently of corticosterone and ACTH to stimulate feeding³⁶⁵. This contention is further supported by findings that galanin gene expression or peptide levels are unaffected by adrenalectomy or corticosterone administration⁷. Additionally, the failure of both type I and type II steroid receptor antagonists to affect galanin-induced food intake suggest that galanin acts independently of the adrenal steroids and their receptors in the PVN³⁶⁵.

In terms of potential interactions of galanin with other neurotransmitters, research is still at an early stage. The coexistence of galanin and NE in brainstem noradrenergic neurons which project and innervate the PVN, combined to the potentiation of food intake obtained from administration of these substances at that site, led researchers to investigate this peptide-amine interaction. In addition to galanin's main ingestive effect on fat intake, both NE and galanin have been shown to participate in carbohydrate intake occurring at the onset of the active ingestive period³⁶³. Interestingly, galanin and NE-elicited feeding are both antagonized by selective blockade of α_2 -adrenergic receptors and galanin-induced hyperphagia is attenuated by prior PVN administration of NE synthesis inhibitors¹⁸⁵. These findings suggest that galanin may partially mediate its action on feeding through NE release at the PVN. This is supported by a microdialysis study demonstrating endogenous

NE release consequent to intra-PVN administration of galanin ¹⁸⁷. Interestingly, coexistence of dopamine (DA) and galanin has also been demonstrated in hypothalamic neurons and *in vivo* microdialysis revealed galanin's inhibitory influence on DA release from the median eminence ²⁶⁹. Moreover, amphetamine (whose action is thought to be mediated in part through its activation of the hypothalamic DA system ^{147,199}) had an inhibitory influence on galanin-induced fat ingestion ¹⁶⁷. Thus, it is proposed that galanin might stimulate fat intake partly through suppression of DA release. The participation of 5-HT in galanin-induced feeding is not clearly established. However, galanin is colocalized with 5-HT in dorsal raphe neurons projecting to hypothalamic areas ²²⁶, and galanin has been found to reduce 5-HT metabolism in the rat brain ³⁵². At present, more research is needed to elucidate whether galanin "disinhibits" feeding in part by controlling 5-HT release.

Potential interactions of galanin with other peptidergic systems have not been documented as yet. However, a recent study suggests that, contrary to what has been observed with NPY, galanin might not enhance feeding through suppression of satiety signals, peptidergic or not ³²⁴. Thus, while Schick et al. reported a diminution of the feeding rate 40 min after meal initiation in control animals, the rate of ingestion of galanin-treated rats appeared significantly suppressed after 20 min of ingestion ³²⁴. These results indicate an earlier activation of satiety signals in galanin-treated rats as compared to controls and perhaps suggest that, in contrast to NPY ³²³, galanin does not enhance feeding by postponing the onset of satiety. Interestingly, the enhanced cumulative food intake observed in galanin-treated rats over the 2 h post injection appeared mainly

attributable to the initial increase in food consumption occurring in the first 20 min of testing. Thus, it is also possible that the earlier onset of satiety observed in galanin-treated rats might principally be due to the very heavy gastric load during the first 20 min as compared to the slower but more steady eating pattern found in control animals. More studies are needed to ascertain these findings and clarify the exact physiological mechanisms underlying galanin stimulation of feeding.

The recent advances in the immunohistochemical techniques to quantify galanin expression will help to better understand the physiological role of galanin in the control of food intake. In this context, a recent micropunch study performed using *ad libitum* fed and food deprived lean and obese Zucker rats revealed a 100% increase in galanin-immunoreactivity in free feeding obese rats and a smaller increase in food deprived obese rats as compared to their respective control lean rats. This increase was highly specific and restricted to the parvocellular portion of the PVN and not observed in the magnocellular part of this nucleus as well as in the 5 other hypothalamic structures sampled. Thus, it is suggested that galanin might contribute to hyperphagia of the obese rat through its large increase in the parvocellular part of the PVN ²⁸. While this study is indicative of a role for galanin in the control of food intake, additional studies are required to determine the exact nature of its influence on feeding using more regular ingestive contexts and normal animal populations. In this context, the recent successful detection of galanin-immunoreactivity in *in vivo* microdialysis samples ⁷¹, might greatly assist in investigating the mechanisms of action and physiological relevance of endogenous galanin in the regulation of feeding.

2. Peptides as inhibitors of appetite

Corticotropin-releasing factor (CRF)

Corticotropin-releasing factor is a 41 amino-acid peptide that has been mainly studied for its role as the principal stimulator of ACTH secretion. However, the wide distribution pattern of this peptide suggests a physiological participation of CRF exceeding the unique regulation of pituitary secretions³⁵⁷. Interestingly, endogenous CRF-immunoreactivity has been detected in various hypothalamic structures, principally in the PVN, arcuate, dorsomedial and medial preoptic nuclei where the highest concentrations have been found. High CRF concentrations have also been observed in certain limbic areas, principally the bed nucleus of the stria terminalis and the central, cortical and medial amygdaloid nuclei, as well as in the raphe nucleus and the dorsal vagal complex of the brainstem^{334,357}. The areas of distribution of CRF-binding sites appear well correlated with the immunohistochemical distribution of endogenous CRF with the exception of a few mismatches between the peptide and its receptors⁸⁵.

In 1982, Britton et al.⁴⁸ and Morley and Levine²⁵⁵ simultaneously reported the potent anorectic properties of central CRF administration. Concurrently to its action on feeding, increased grooming was also observed following central CRF injection^{255,260,353}. These effects were first thought to be dependent on CRF-induced release of ACTH (which increases grooming) and beta-endorphin (which decreases feeding when administered peripherally) from the pituitary. However, since hypophysectomy failed to block the

effects of CRF on food ingestion and grooming, it would appear that these effects are independent of CRF's ability to release ACTH and/or β -endorphin³⁷⁰.

Intracerebroventricular (i.c.v.) administration of CRF has been shown to induce corticosterone release from the adrenal cortex secondary to release of pituitary ACTH¹³¹ as well as to stimulate NE and epinephrine release from the adrenal medulla⁵⁵. This CRF-stimulated catecholamine release has been shown to be independent of the presence of CRF in the hypophyseal portal system and may be the consequence of CRF action within the CNS to stimulate the target organs. This was convincingly demonstrated by Brown and Fisher through a series of experiments in which CRF maintained its ability to induce catecholamine release after intravenous infusion of CRF antiserum shown to neutralize blood borne CRF action and prevent elevations of ACTH plasma levels⁵⁴. In this context, Gosnell et al.'s findings revealed that adrenalectomy (contrary to hypophysectomy previously shown ineffective to alter CRF behavioral and ingestive effects) markedly attenuated CRF-induced suppression of food intake¹³¹. These results suggested that either some circulating corticosterone was necessary for CRF suppression of food intake, or, alternatively, that the adrenal medullary epinephrine secretion was required for the full expression of the CRF effects on feeding. Corticosterone replacement in adrenalectomized animals failed to restore the CRF effects on food intake, as could have been predicted from the inability of hypophysectomy to alter CRF-induced feeding. On the other hand, adrenal demedullation markedly attenuated the effect of centrally administered CRF on food intake suggesting that epinephrine or other adrenal medullary secretions play a role in the mediation of CRF effects on feeding¹³¹. This contention is

further supported by findings revealing inhibition of food intake consequent to peripheral epinephrine administration ¹⁴⁴. As for NE, Brown and Fisher's study demonstrated CRF-induced NE release to be mainly taking place in the kidney while CRF inhibition of NE release was observed in the brown fat and pancreas ⁵⁴. At the present time, the exact participation of peripheral NE release in central CRF suppression of food intake remains unknown.

Notwithstanding the fact that researchers have concentrated their investigative efforts on hypothalamic nuclei, largely neglecting the extrahypothalamic structures, in the search of potential effective CNS loci for CRF action on feeding, findings support CRF action on feeding to be anatomically very specific. In a study in which Krahn and his colleagues characterized the ingestive effects of CRF injections in 5 different brain structures, namely the PVN, the lateral and ventromedial hypothalamic nuclei, the globus pallidus and the striatum, the authors observed that CRF successfully inhibited food intake (by approximately 50% after 1 hr) only following injection into the PVN ¹⁷⁶. This peptide failed to alter feeding when injected in any of the other four structures tested. Similarly, a recent study has demonstrated grooming and locomotion to be specifically enhanced by CRF administration into the PVN but not into the lateral hypothalamus or into other medial hypothalamic areas ²⁶⁰. Of interest in terms of potential mechanisms for CRF-induced satiety are the important neuronal projections emanating from the PVN and projecting to other brain areas and connections to the autonomic nervous system ³⁵⁶. The involvement of these particular anatomical links in CRF-induced satiety need further investigation. However, due to the methodological difficulties involved in clearly

mapping physiological brain functioning, the approach to study CRF physiological participation in feeding has mainly been of pharmacological nature. Using the synthetic CRF antagonist, alpha-helical CRF₉₋₄₁ (α -CRF), Krahn et al. demonstrated the ability of this antagonist to partially reversed CRF-induced suppression of food intake in 24 hr deprived rats ¹⁷⁴. This effect appeared specific to blockade of CRF receptors as α -CRF failed to alter calcitonin-induced satiety. Interestingly, at the dose tested (50 μ g) in this study, α -CRF failed to significantly alter CRF-induced grooming. Further research is needed to test various dose combinations of CRF and α -CRF to determine whether feeding and grooming are mediated through different receptor subtypes or rather reflect the existence of different sensitivities of feeding and grooming to the effects of α -CRF. Moreover, it is possible that such a high dose of α -CRF exerts an agonistic effect reflected by increased grooming, a condition that has not been verified in Krahn et al's study ¹⁷⁴.

In terms of potential interaction of CRF with other neurotransmitters known to influence appetite, some studies have suggested that CRF may represent the feeding inhibitory substance within the PVN which is modulated by NE and 5-HT in order to produce their respective effects on feeding. The PVN has been attributed a role as a "satiety center" through studies demonstrating overeating and substantial body weight gain in rats consequent to lesioning of this brain structure ²⁰². Like CRF, NE is also quite potent when administered into the PVN ¹⁹⁸. Moreover, the stimulatory effects of NE on food intake have been shown significantly attenuated by PVN lesions ²⁰³. Interestingly, both NE and CRF induce their effects on feeding primarily by affecting carbohydrate intake ^{106,175}. In light of these findings, it was hypothesized that NE-induced hyperphagia

might in fact be due to the inhibition of CRF satiety signals by NE within the PVN. Supportive of that hypothesis are findings showing that adrenalectomy, a procedure shown to increase CRF-immunoreactivity in the PVN, markedly attenuates NE-induced feeding ²³. Additionally, NE has been shown to inhibit CRF release from the hypothalamus ⁶⁰. Reciprocally, CRF administration reduces NE turnover in the PVN ¹¹. Interestingly, 5-HT has been shown to enhance CRF release from the hypothalamus ⁶⁰ as well to inhibit NE-induced feeding when infused into the PVN ¹³⁰. These findings taken together support the contention that NE and 5-HT might interplay with CRF in the production of their effects on feeding.

As for the interaction of CRF with other peptides, while the potential candidates are numerous, studies of peptidergic interactions in the context of food ingestion are very few. Probably, one of the most documented of these peptidergic interactions involves NPY and CRF. Indeed, in a recent study, Heinrichs et al. have demonstrated the participation of CRF in NPY-induced increase in food intake ¹³⁸. Specifically, their findings revealed that central blockade of CRF receptors potentiated the hyperphagia induced by central NPY. In a second study by the same group, these effects were found to be specifically mediated by CRF neurons within the PVN ¹³⁹. This study also demonstrated that dexamethasone pretreatment, which increased glucocorticoid negative feedback on PVN CRF neurons and reduced CRF levels in this structure, significantly enhanced NPY-induced feeding. Finally, functional impairment of PVN CRF neurons using immunotargeting of toxins which led to 60% decrease in CRF content within the hypothalamus, markedly enhanced eating stimulated by intra-PVN NPY administration ²²⁷.

In a short paper, Levine et al. also reported that central CRF antagonized dynorphin- and insulin-induced feeding ²⁰⁹. Together, these results support the existence of a complex interplay between orexigenic and anorexigenic signals that control food intake. Potential interactions of BN and CRF have been assessed in the present thesis and will be presented later.

Cholecystokinin (CCK)

Cholecystokinin was the first peptide to be implicated in the modulation of food intake ¹²⁶. This peptide, in its 33 amino acid molecular form, was isolated from porcine small intestine in 1968 ²⁶². The carboxyl-terminal octapeptide (CCK-8), principally in its sulfated form, has been identified as the predominant biologically active moiety in the brain and periphery of several animal species, including man ^{63,91,154,261,347}. Nonetheless, a number of other circulating molecular forms of CCK also exist (CCK-58, CCK-39, CCK-25, CCK-22, CCK-18, CCK-7 and CCK-5) and CCK-58 has been identified as the major endocrine form of CCK in human postprandial plasma ¹⁰³.

High concentrations of CCK have been immunocytochemically localized in the gastrointestinal tract and in various central nuclei capable of affecting gastrointestinal functioning, including the vagus nerve, the nucleus tractus solitarius (NTS), PVN and dorsomedial hypothalamic nuclei ^{30,154,279}. Furthermore, CCK receptors have also been identified in the pancreas, pyloric sphincter and vagus nerve as well as in various brain structures including the olfactory bulb and tubercle, nucleus accumbens, amygdala, dentate gyrus, area postrema, NTS and hypothalamic nuclei including the ventromedial, PVN and

lateral nuclei³⁷³. The CCK receptors can be divided into two distinct subtypes namely the CCK-A (alimentary receptor) and CCK-B (brain receptor) receptors^{93,250}. The A-type receptors have been predominantly observed in the periphery, although their presence in specific brain structures such as the medial aspect of the NTS, the area postrema and the interpeduncular nucleus, have been reported^{143,250}. The precise function of these “peripheral-type” receptors in the brain is not fully understood but lesions of the NTS have been shown to prevent CCK-induced satiety⁷⁹. Furthermore, both the area postrema and NTS receive direct vagal inputs and are located in a brain region with a relatively permeable blood brain barrier. Thus, these areas might play a role in processing peripherally released CCK sensory afferent signals. As for CCK-B receptors, while principally located in brain areas, binding sites have also been observed in the vagus nerve^{73,250}.

Gibbs and his colleagues were the first to demonstrate that systemic administration of CCK suppressed food intake in rats¹²⁶. Interestingly, CCK ingestive effects were also found to be accompanied by a behavioral sequence normally observed following meal termination in spontaneously feeding animals, characterized by grooming, exploration, resting and sleeping^{13,125}. These findings have since been amply confirmed and extended. Thus, the satiety action of centrally or peripherally administered CCK in a variety of species is clearly established^{12,24,88,109,122,151}. In this context, it has been demonstrated that CCK, when slowly infused intravenously, decreased food ingestion in humans without producing overt malaise^{172,263,337}. While mechanisms of action have not been confirmed, peripheral CCK infusion is believed to suppress food intake principally through inhibition

of gastric emptying leading to gastric distention^{248,328}. Nonetheless, the potent suppression of sham feeding observed after peripheral CCK administration in rats with open gastric fistulas suggests that CCK effectively inhibits food intake even in situations where no food accumulates in the gut and when no gastric distention occurs¹²⁵. Interestingly however, Cox recently demonstrated the ability of duodenal sucrose infusion to potentiate suppression of sham feeding by otherwise subthreshold doses of CCK-8, indicating that peripheral CCK might induce satiety through synergistic interaction of the peptide with a signal generated by the ingested food, potentially within the small intestine⁷⁸. Findings from different studies also suggest a link between peripheral and central system mediating CCK satiety. This was initially demonstrated by failure of peripheral CCK to suppress feeding following selective lesions of the afferent, but not efferent, branches of the abdominal vagus nerve³³⁵. Furthermore, lesions of the vagal projections to the NTS or transection of the NTS projections to the PVN blocked CCK-induced satiety⁸¹.

A number of studies have attempted to clarify the physiological role of CCK in the control of ingestion through assessment of its release under various feeding states. In this context, Della-Fera et al.⁸⁹ demonstrated significant increases in CCK concentrations in the hindbrain dorsal motor nucleus of the vagus and the dorsal parabrachial nucleus following intra-duodenal infusion of a liquid diet. Moreover, using *in vivo* push-pull perfusion, elevations in endogenous CCK release from primate lateral hypothalamus was also observed in response to intragastric meal infusion³²². Interestingly, a subsequent article by the same authors demonstrated transient endogenous CCK release following

stomach distention with either saline or a carbohydrate-protein meal, suggesting that gastric distention might constitute an essential transduction mechanism responsible for CCK release in the lateral hypothalamus following feeding ³²⁶. This is consistent with Della-Fera's findings demonstrating that while central CCK administration decreased food intake in rats when the ingesta remained in the gastrointestinal tract, central CCK failed to inhibit sham feeding in rats with open gastric fistulas ⁸⁸. Finally, comparisons of meal-stimulated changes in CCK levels in lean and obese Zucker rats after a 6 h fast revealed equivalent postprandial increases in CCK levels within specific hypothalamic nuclei in both rat groups ²²². Thus, these results are concordant with the hypothesis that CCK in hypothalamic areas of the rat brain might be associated with satiety. On the other hand, increased food intake in obese Zucker rats appears unrelated to decreased postprandial hypothalamic CCK levels and might be related to a reduced affinity of CCK for its receptors.

Linden and Sodersten reported distinct time course of plasma CCK changes following exogenous CCK administration (i.p.) versus those noted during spontaneous food ingestion ²¹⁴. In contrast to the rapid and transient elevation of CCK plasma concentrations produced by exogenous CCK administration (CCK plasma levels peaked 5 min after CCK injection, drastically declined to reach baseline levels within 15 min), the endogenous CCK plasma levels of the free feeding animals increased gradually and only peaked 30 min after feeding onset. Thus, curiously, the hormonal CCK levels of the injected animals did not correlate well with their ingestive status and, while their CCK concentrations were rapidly back to baseline levels, feeding response of these animals

remained suppressed for an additional 45 min. Furthermore, these results suggest different mechanism(s) of action of endogenous versus exogenous CCK in producing satiety.

Evidence showing the enhancement of the feeding response following pharmacological blockade of endogenous CCK action, using CCK antibody or CCK receptor blockers, have also been crucial in supporting CCK's physiological participation in the control of food intake^{22,87,386}. In this context, the participation of the CCK-A versus -B receptor subtypes in the satiety response following CCK administration appears to be distinct. While selective blockade of CCK-A receptors prior to peripheral CCK administration dose-dependently reversed CCK-induced satiety in fasted animals, pretreatment with the CCK-B receptor antagonist, L365,260, failed to attenuate CCK's effect on food intake in neonatal and adult rats^{94,102,118,336}. These findings suggest that exogenous CCK exerts its action on feeding principally through activation of the CCK-A receptor subtype. However, when administered alone, these antagonists had no effect on food consumption in fasted rats, a finding perhaps attributable to low concentrations of endogenous circulating CCK present in that feeding state. Interestingly, studies using satiated animals to elucidate the physiological participation of the different CCK receptor subtypes suggest a more complex involvement of these receptors in response to endogenous CCK stimulation. In this context, Dourish et al.'s findings emphasized a more important physiological participation of CCK-B rather than -A receptor subtype in CCK-induced satiety. Thus, their study revealed that while blockade of either receptor subtypes increased food intake and postponed the onset of satiety in partially satiated rats, the CCK-B receptor antagonist L-365,260 was a 100 times more potent than CCK A receptor

antagonist devazepide (MK-329) ⁹⁵. Other studies have shown the ability of the CCK A antagonist L 364,718 to enhance feeding with similar or increased potency compared to MK-329 ^{142,303}. Alternatively, Weatherford et al. reported no enhancement in feeding upon L365,260 administration in mice while MK-329 significantly and dose-dependently increased feeding in these animals ³⁸². The recent development of a second generation of CCK-A and B receptor antagonists might shed more light on the differential participation of each subtype in CCK ingestive effects ^{43,134,394}.

Recent findings suggest that the satiety effects of exogenous CCK may depend on the activation of central monoaminergic systems. In this context, systemic CCK administration has been shown to restore DA levels in the CSF of mildly deprived animals and the DA antagonist cis-flupentixol, blocked CCK-induced satiety ²¹³. Interaction between CCK and 5-HT are more complex but Cooper and Dourish recently demonstrated that 5-HT-induced suppression of feeding could be antagonized by CCK-A receptors suggesting that enhanced 5-HT transmission may lead to an increase in endogenous CCK activity ⁷². Alternatively, serotonin receptor blockade attenuated CCK-induced anorexia suggesting the inhibitory action of CCK to depend on 5-HT release ³³⁸. Research on CCK interactions with other peptides has been rather limited. However, findings revealing the potentiation of satiety consequent to combined i.p. injections of glucagon, CCK and BN, at doses below the threshold for reliable food inhibition, suggest a synergistic collaboration of these peptides in postprandial satiety ¹⁴⁵. At present, these represent interesting research avenues that await further experimental investigations.

Bombesin (BN)

Immunoreactivity and receptor distribution in the brain and periphery.

Bombesin, a biologically active tetradecapeptide was initially isolated from the skin of the European frog *Bombina bombina*⁹. Subsequently, several structurally related peptides have been identified and are globally referred to as BN-like peptides (see Table 2). Three of these peptides are found in mammalian tissues: the 27-amino acid gastrin-releasing peptide (GRP) molecule (GRP₁₋₂₇ sometimes referred to as mammalian BN), a short form of the full GRP molecule, the decapeptide GRP₁₈₋₂₇ (also referred to as Neuromedin C) and Neuromedin B in both its molecular forms (NMB₁₋₃₂ and NMB₂₃₋₃₂). NMB belongs to the ranatensin subfamily²⁴⁰ while GRP is a member of the BN subfamily²²¹. The presence of endogenous BN-like peptides has been demonstrated in many different species ranging from mice to humans^{21,133,186,264,349,393}. Immunohistochemical studies have revealed a wide distribution pattern of BN-like immunoreactivity (BLI) in the peripheral and central nervous systems. Peripherally, highest levels of BN-like peptides have been detected in the gastrointestinal tract, particularly in the stomach, the jejunum-ileum and the duodenum of rats⁵⁰, guinea pig⁷⁶, dog³⁰¹ and man²⁹⁵. Additionally, BN-like peptides innervate fibers of the myenteric plexus (of both the stomach and intestine) as well as the submucosal plexus and mucosa of the stomach^{92,152}.

Table 2 Representative members of each BN-like peptide subfamily

<i>Bombesin subfamily</i>	
BN	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH ₂
GRP ₁₋₂₇	X-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
<i>Ranatensin subfamily</i>	
NMB ₁₋₃₂	X-Val-Hys-Pro-Arg-Gly-Asn-Leu-Trp-Ala-Trh-Gly-His-Phe-Met-NH ₂
NMB ₂₃₋₃₂	Gly-Asn-Leu-Trp-Ala-Trh-Gly-His-Phe-Met-NH ₂
Ranatensin	pGlu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH ₂
Ranatensin R	X-Ala-Leu-Arg-Arg-Tyr-Asn-Gln-Trp-Ala-Trh-Gly-His-Phe-Met-NH ₂
Ranatensin C	X-Glx-Thr-Pro-Gln-Trp-Ala-Trh-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
<i>Phyllolitorin subfamily</i>	
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-like peptides at the decapeptide C-terminal region (in bold). "X", represents the amino acid chain preceding the sequence indicated

In the CNS of the rat, the highest concentrations of BLI have been detected in certain hindbrain structures such as the NTS and the substantia gelatinosa, in hypothalamic nuclei (principally the arcuate, anterior, medial preoptic paraventricular and the ventromedial nuclei), in several midbrain structures including the interpeduncular nucleus and the central gray matter and in the amygdala at its medial and central nuclei. Moderate to low concentrations of endogenous BN-like peptides were also identified in the lateral preoptic, periventricular and dorsomedial hypothalamic nuclei and in the striatum, nucleus accumbens, olfactory tubercle, cingulate cortex, periventricular thalamus and hippocampus^{68,247,281}.

Interestingly, in situ hybridization studies revealed an heterogeneous distribution of NMB₁₋₃₂ and GRP₁₋₂₇ mRNAs in the rat nervous system³⁸⁰. Thus, in the forebrain, a strong hybridization signal for NMB mRNA was observed in the olfactory bulb and dentate gyrus while moderate signal expression was found in the pyramidal cell layer of Ammon's horn field and the central nucleus of the amygdala. Weaker signals were observed in the bed nucleus of the stria terminalis, shell part of the nucleus accumbens, lateral habenular nucleus, arcuate nucleus, medial preoptic nucleus and supramammillary nucleus. In contrast, GRP mRNA was much more widely distributed and generally more abundant in the rat forebrain. High mRNA densities were expressed in the subiculum, dentate gyrus, cortical amygdaloid nucleus and in the cingulate and perirhinal cortex. Moderate signals were obtained in the olfactory nucleus, presubiculum, pyramidal layer of Ammon's horn fields CA2 and CA3, medial and lateral nucleus of the amygdala, suprachiasmatic nucleus and the medial preoptic nucleus. Relatively weak signals were

finally expressed in the pyramidal layer of Ammon's horn fields CA1, anterior and basolateral amygdaloid nuclei, medial septal nucleus, median preoptic nucleus, anterior hypothalamus and in the parvocellular portion of the PVN. In the brainstem, moderate hybridization signals for NMB mRNA were observed in the dorsal motor nucleus of the vagus while moderate GRP mRNA expression was found in the inferior colliculus, NTS and parabrachial nucleus. Similar distribution patterns have been demonstrated for BN/GRP and NMB-like immunoreactivity using radioimmunoassays²⁴⁶. In light of the numerous bioactivities of BN-like peptides, the distinct distribution of GRP and NMB mRNAs and immunoreactivity potentially reflects the different physiological functions of these neuropeptides in the brain.

Similarly to its peptidergic distribution, BN receptors have been localized in numerous CNS sites^{245,286,389,396} and in the gastrointestinal tract^{249,378} of several mammalian species, including humans. Autoradiographic studies revealed high densities of ¹²⁵I-Tyr⁴ BN binding sites in forebrain areas such as the nucleus accumbens, the olfactory tubercle and the anterior olfactory nucleus, in the hippocampus and dentate gyrus, in the central and medial amygdaloid nuclei, in hypothalamic areas such as the suprachiasmatic, periventricular and paraventricular nuclei and in the thalamic medial and paraventricular nuclei. Moderate levels of BN binding were observed in the rhinal, cingulate and neo-cortices, caudate-putamen, septohippocampal nucleus, anterior hypothalamus, medial preoptic nucleus, locus coeruleus and in the NTS. Generally, the lowest density of BN receptors were observed in the globus pallidus and certain thalamic and brainstem areas. No BN receptors were found in the cerebellum^{245,396}. In the

periphery, BN binding sites are concentrated in the gastrointestinal tract and principally expressed in the circular muscle of the gastric fundus and antrum and in the submucosal layer of the small intestine and colon ^{249,265}. Autoradiographic studies also revealed the presence of BN receptors in the esophageal smooth muscle, carotid arteries and pancreatic acinar cells ^{160,302,378}.

Subsequent to the demonstration of an heterogeneous distribution of NMB and BN/GRP-like immunoreactivity and mRNA expression, binding studies were performed in the CNS and peripheral tissues to assess the possibility that these two BN-like peptides interact with distinct receptor populations. Indeed, detailed analyses of the relative affinity of rat brain receptors for NMB and BN/GRP revealed that the total population of BN receptors mapped by earlier studies using radiolabelled BN included both NMB- and BN/GRP-preferring binding sites. Moreover, concordant with the differential distribution of NMB and GRP in the rat CNS, the density of the different BN receptor subtypes throughout the brain was also distinct. While no important mismatches between the receptor subtype and the correspondent peptide were observed when the regional distribution of NMB- and BN/GRP-preferring binding sites with the presence of NMB- or BN/GRP-like immunoreactivity or mRNA expression were compared, on a few occasions, no anatomical correlation existed between the binding sites and peptidergic expression ^{26,189}. The most striking example is the nucleus accumbens which displayed a high density of BN/GRP preferring binding sites in the absence of BN/GRP-like immunoreactivity or mRNA expression in this structure ^{189,247,380}. At present, while mismatches have often

been observed ¹⁷⁸, the reason for such spatial disparity between the level of peptide and receptor is still not understood.

While studies on the central distribution of BN receptors have provided a detailed regional assessment of the BN/GRP- and NMB-preferring binding sites, some studies have also demonstrated the presence of different BN receptor subtypes in discrete peripheral loci. Thus, while the pancreatic tissue has high affinity for BN/GRP ³⁷⁹, it has no affinity for NMB. In contrast, receptors of the oesophageal tissue ³⁷⁹ and gastric fundus ²⁴⁹ displayed greater affinity for NMB than BN/GRP. The implication of these central and peripheral findings remains to be determined. The development of new antagonists with enhanced selectivity for the BN/GRP- or NMB-preferring binding sites as well as the use of oligonucleotide antisense aimed at these receptor subtypes should hopefully assist in the clarification of the particular physiological roles of these peptides in behavioral and endocrine regulation.

Bombesin: A peptide with multiple peripheral and central actions.

The widespread yet distinct distribution of BN-like peptides and its receptors within the brain and periphery together with the observed evolutionary conservation of the pharmacological action of BN across a range of animal species suggest important physiological role(s) for these peptides. In this context, central or systemic administration of BN potently affects a broad spectrum of activities and homeostatic processes. Thus, BN administration has been shown to induce various behavioral, metabolic and endocrine effects in experimental animals including hypothermia ^{15,16,19}, hyperglycemia ^{57,136},

grooming and locomotor activity ^{128,162,180}, adrenal medullary and pituitary hormone secretions ^{53,319,366}, reduced gastrointestinal transit and food intake ^{123,124,321}, enhanced memory retention ^{112,385}, decreased heart rate and cardiac output ¹¹⁰ and, cellular proliferation ^{66,312}. While the present thesis is principally concerned with the elucidation of the physiological role of BN-like peptides in the regulation of food intake and the underlying mechanism(s) of action, it is important to keep in mind that this family of peptides has diverse biological effects. Thus summarized below are the variety of effects of BN-like peptides.

Apart from its participation in the control of ingestion, other physiological and behavioral effects of BN suggest its role in controlling body homeostasis. Indeed, central administration of BN has been shown to induce hypothermia ^{4,16}. Bombesin poikilothermic effects were first thought to depend on the ambient temperature during testing and, while BN produces hypothermia in cold exposed rats, no changes in body temperature were observed in rats maintained in a thermoneutral environment and hyperthermia was observed in heat-stressed rats ³⁵⁸. However, recent studies suggest glucose availability as a permissive factor for BN thermoregulatory effects. This hypothesis is supported by evidence showing decrease in core body temperature following central infusions of BN in rats that are food deprived or made hypoglycemic with insulin or 2-deoxy-D-glucose but not in satiated rats ¹⁶⁻¹⁸.

Bombesin has also been proposed as a candidate in the central regulation of circulating metabolic fuels through studies demonstrating elevations of blood glucose, free fatty acids and corticosterone following BN microinfusion into the PVN ¹³⁶. The precise

mechanisms underlying such effects remains unknown but Gunion et al.¹³⁶ proposed that intra-PVN BN might affect the forebrain neural systems regulating the pituitary adrenal axis perhaps through BN activation of CRF neurons in the PVN and ultimately affecting ACTH release from the anterior pituitary and corticosterone release from the adrenal medulla. Interestingly, central administration of BN has been shown to stimulate pituitary hormone secretion^{319,366}. Peripheral administration of BN has also been shown to inhibit prolactin and thyroid-stimulating hormone while stimulating the release of luteinizing and follicle-stimulating hormones^{297,298}. Additional evidence supporting an integrative role for BN-like peptides, are findings revealing the ability of centrally administered BN to affect the autonomic nervous system functioning. In this context, BN-elicited hyperglycemia and hyperglucagonemia have been shown to mostly depend on BN stimulation of adrenomedullary epinephrine secretion^{52,53,57,153}. However, while the precise neuronal substrate(s) of BN-induced hyperglycemia remains unknown, Gunion et al.'s findings suggest that the NTS may be a potential activator of hypothalamic glucoregulatory systems¹³⁵.

Central administration of BN has also been shown to affect cardiovascular functions. These effects appear partially mediated through the adrenals and, adrenalectomy has been shown to prevent BN-induced increase in arterial pressure, while leaving BN-induced decrease in heart rate unaltered. Bombesin-induced decrease in heart rate is thought to depend on cardiac parasympathetic nervous activation¹¹¹.

The observation of higher levels of BN/GRP peptides in fetal and newborn lung compared to adult lungs suggests a trophic role for these peptides. Supportive of such

hypothesis are findings of reduced BN-like immunoreactivity in lungs of infants with respiratory distress and lung development problems ¹²⁰. Moreover, the demonstration that *in vivo* administration of BN will induce gastric hyperplasia in rats ¹⁹⁶ and pancreatic hyperplasia in humans ²⁵, while *in vitro*, BN increased the cell number of mouse 3T3 cells or human bronchial epithelial cell in cultures provides direct supportive evidence for a role of BN/GRP in cellular proliferation ^{223,313}.

Interestingly, Flood and Morley ¹¹² have demonstrated that BN and GRP enhanced retention test performance in weakly trained mice when administered immediately after training and one week before testing retention. This effect appeared to be mediated peripherally as higher doses of BN and GRP were required to enhance retention following i.c.v. administration. In this context, vagotomy blocked BN and GRP-induced enhanced retention indicating that vagal afferent stimulation might represent one mechanism by which these peptides improve memory ¹¹². Williams and McGaugh ³⁸⁵ have recently demonstrated improved performances in radial arm retention tests following microinjection of BN into the NTS, a brain nucleus which receives direct inputs from the periphery via afferent fibers of the vagus nerve ^{163,351}. In light of the documented improvements in retention following glucose administration, Flood and Morley also assessed whether BN-induced hyperglycemia played a role in BN's action on memory. At effective doses to enhance retention, however, BN and GRP failed to elevate circulating blood glucose suggesting that this factor does not play a critical role in BN's and GRP's mnemonic effects. Interestingly, feeding immediately following training also successfully enhanced retention in mice. This finding led the authors to propose that the passage of food through

the intestine might enhance memory retention via activation of a gastrointestinal peptidergic system¹¹². From an evolutionary perspective, such a system could play a role in the successful foraging for food.

Finally, BN administration has been associated with intense grooming and stimulation of locomotor activity. The ability of BN to elicit grooming by central but not peripheral administration suggests that this behavior is initiated through CNS sites^{128,162,180}. While the exact mechanisms underlying BN-induced behaviors are not fully understood, the selective ability of antagonists to dopamine D1 and D2 receptors subtypes to partially block BN-induced grooming and locomotion, respectively, suggest the involvement of the dopaminergic system in these behaviors but also support a dissociation of these behaviors^{288,327}. This is further supported by a study revealing attenuation of BN-induced grooming but not locomotion in rats pretreated with a muscarinic cholinergic receptor antagonist²³². These BN-induced behaviors also appear partially independent of other BN effects. Thus, as previously described, hypothermia is not observed following BN administration in rats maintained in a thermoneutral environment. Moreover, BN administration in the fourth ventricle, lateral hypothalamus or the NTS has been shown to inhibit food intake without increased grooming and locomotion^{162,191,349}. These results suggest the involvement of distinct neural substrates in the modulation of BN locomotor, ingestive and poikilothermic effects.

Bombesin: Satiety effects and suggested mechanisms of action

The mechanisms of action of BN-like peptides have not been clearly elucidated yet. Nonetheless, a growing body of evidence strongly supports the physiological participation of these peptides in the regulation of food intake. Numerous reports confirm the ability of central and peripheral administration of BN to dose-dependently induce satiety in animals^{113,123,124,180,186} and recently, inhibition of food intake has also been demonstrated following intravenous infusion of BN in humans with no reported side effects^{211,264}. The initial pharmacological demonstration of BN-induced satiety was obtained following intraperitoneal injection of the peptide in rats¹²³. In this study, BN selectively reduced intake of both solid and liquid food at doses that failed to affect water intake. The selective inhibition of food but not water intake suggests that BN is not acting through production of a general aversive state or through impairment of the animals motor abilities required for licking. Moreover, the observation that BN did not affect the initial rate of feeding but decreased feeding by shortening meal duration and prolonging intermeal interval is also supportive of a genuine satiety-like action of BN. Additionally, as in the case of another satiety peptide, CCK, BN-induced reduction in food intake was accompanied by the normal behavioral postprandial satiety sequence characterized by grooming, exploration followed by rest and sleep¹²⁴. This sequence contrast sharply with the behavioral repertoire of sick animals. Furthermore, Kulkosky et al. reported that the ID₅₀ of i.p. BN for the suppression of food intake failed to produce aversion for a novel taste upon its administration in a conditioned aversion test, while an equipotent dose of lithium chloride produced a clear aversion for the same novel flavor¹⁸¹. Furthermore, the

length of the deprivation period appears to significantly influence BN-induced suppression of food intake. Thus, at optimal doses, BN suppressive effects have been shown to be more pronounced after a short (12 h), than a prolonged (48 h), deprivation period. These results are supportive of a role for BN as a satiety factor, which should be less inhibiting of feeding in hungrier animals as opposed to aversive agents, such as lithium chloride, which retain their aversive properties independently of the magnitude of hunger³⁷. Together, these findings strongly suggest that BN may act as a putative satiety signal.

Numerous studies have followed Gibbs et al.'s initial report³⁹⁵ and further characterized, using different animal models, the suppressive effects of peripheral BN. Thus, BN was tested in a sham feeding paradigm, a preparation in which rats with open gastric fistulas eat almost continuously because of the absence of post-absorptive satiety signals. Findings revealed that BN significantly inhibited food intake of both sham-feeding and normal feeding in a dose-related manner. However, because the BN-induced suppression was of greater magnitude in the real feeding group compared to the sham feeding rats, other endogenous factors activated by accumulated food in the gut may interact with exogenous BN in producing satiety. Moreover, the enhanced ingestive effect observed after combined administration of BN and CCK support the existence of some synergism between the satiety signals¹²⁴. In light of the potential dependence of BN feeding effects on BN-induced glycemic effects, Gibbs et al. also investigated the ability of peripherally administered BN to produce satiety in adrenalectomized rats¹²⁴, as this procedure has been shown to completely abolish BN-induced hyperglycemia⁵⁷. Bilateral

adrenalectomy failed to alter BN-induced suppression of food intake across a wide range of BN doses, demonstrating the independence of BN glycemic from ingestive effects.

In the early 1980s, several studies focused on delineating the central versus peripheral effects of BN^{179,180,254}. These studies significantly enhanced our understanding of the potential mechanisms mediating BN-induced satiety. Studies also demonstrated that CCK-induced satiety was dependent on intact vagal connections. Furthermore, BN administration was found to increase CCK release in the dog^{108,159,173}. These observations prodded researchers to investigate the role and importance of vagal connections in BN-induced ingestive effects. Surprisingly, abdominal vagotomy failed to attenuate BN-induced satiety in rats suggesting that BN inhibition of feeding does not depend on the endogenous release of CCK and that vagal connections are not essential for BN effects on ingestion. In light of the attenuated response of other anorectic compounds such as glucagon and somatostatin following vagotomy, the physiological participation of BN in the control of food intake appears unique^{220,257}.

Importantly, Stuckey et al. have reported that a lesion consisting of subdiaphragmatic vagotomy combined with spinal cord transection and dorsal rhizotomy, a procedure which totally isolated the brain from all neural input from the gastrointestinal tract, completely abolished the satiety effects of systemic BN³⁵⁰. It is proposed that the critical damage of such lesions result from the interruption of sensory signals traveling from the gastrointestinal tract to the brain by both vagal and spinal routes. In an effort to further determine the participation of afferent fibers, Ladenheim and Ritter¹⁹² have used i.p. injection of capsaicin, a neurotoxin shown to selectively destroy small, unmyelinated

fibers in the vagus and spinal nerves³⁰⁵. Their findings revealed that capsaicin pretreatment significantly attenuated food intake suppression following peripheral BN administration, suggesting the participation of afferent fibers in BN ingestive effects. However, the absence of histochemical data in that study do not allow for definitive conclusions about the extent and/or nature of the capsaicin-induced damage to afferent fibers. These findings not only argue for BN-induced satiety to partially depend on central mechanisms for its complete expression but they also clearly suggest that systemically administered BN initially acts locally at peripheral sites. In this context, Kirkham et al.¹⁷⁰ have recently demonstrated the satiating potency of peripherally administered BN to be considerably larger when the peptide was directly infused into the celiac artery than intraperitoneally or into the superior mesenteric artery, suggesting the participation of the peripheral organs directly perfused by the celiac artery such stomach, pancreas, liver, spleen, and proximal duodenum and their receptors in the acute anorectic actions of BN. In light of high BN-like immunoreactivity and receptors within gastric tissue^{92,249}, and of BN-induced inhibition of gastric emptying^{107,320}, Hostetler et al. investigated the possibility that peripheral BN acts principally through a gastric site to inhibit feeding¹⁵⁰. Surprisingly, peripheral BN failed to inhibit gastric emptying of ingested glucose in their study. Moreover, while BN was more potent at inhibiting feeding than any other BN-like peptides tested, its affinity for gastric BN receptors was lower than the one of any of the other compounds tested. Thus, these results do not support a gastric mechanism or gastric site of action for systemically-induced BN satiety. Alternatively, significant increases in the concentration of endogenous BN-like peptides have been observed in the

antrum of the stomach in response to meal ingestion ¹⁶⁸. This response was very specific as none of the other gastrointestinal tract segments including the oesophagus, fundus, duodenum, jejunum, ileum and colon demonstrated similar fluctuations. Thus, while a particular site or receptor population for the peripheral action of BN on feeding remains to be clearly identified, a role for gastric receptors cannot yet be discounted.

Although Stuckey et al. and Kirkham et al.'s findings strongly support a peripheral initial site of action for systemically administered BN, these studies do not preclude the participation of central BN in a chain of events leading to satiety after peripheral administration of BN. Thus, numerous studies demonstrated food intake suppression after microinjections of BN into specific brain nuclei, including hypothalamic and extrahypothalamic structures ^{113,162,179,186,191}. In this context, caudal brainstem structures, the NTS in particular, which receives visceral vagal and spinal afferent inputs, have demonstrated great sensitivity to the suppressive effects of BN ^{86,162,193}. Moreover, Flynn's findings demonstrating the ability of peripheral BN administration to suppress sucrose intake in decerebrated rats, with transections extending ventrally from the anterior portion of the superior colliculus to the posterior mammillary nuclei, suggest that caudal brainstem sites, in isolation of the forebrain, are sufficient to mediate feeding suppression induced by peripheral BN administration ¹¹⁴. Nonetheless, recent observations clearly demonstrated that, in addition to neuronal activation in the area postrema and NTS, C-fos-like immunoreactivity was concurrently enhanced in the parvocellular portion of PVN following peripheral BN administration ^{45,190}. Moreover, postmortem studies have revealed alterations in hypothalamic and hippocampal levels of BN-like peptides in feeding

dependent manner²³⁴. Thus, while forebrain structures might not be necessary for exogenous BN to induce satiety, these studies provide evidence of a physiological participation of some forebrain areas in the regulation of food intake. This is consistent with the documented eating dysfunctions (hypo- and hyperphagia) observed in rats after lesions of the hypothalamic lateral and ventromedial nuclei, respectively²⁵¹. Interestingly, lesioning studies suggest that the ingestive effects of these hypothalamic structures may be mediated through axonal fibers originating from the PVN¹⁹⁹.

Recent studies using antagonists further support biological actions of endogenous BN-like peptides, particularly in the mediation of satiation and/or satiety. Thus, central blockade of BN/GRP receptors has been shown to enhance feeding in sated rats^{115,235} as well as to attenuate the effects of central or systemic administration of BN^{236,259}. Similarly, central pre-treatment with BN antiserum significantly attenuated the satiety effects of systemically injected BN²³⁵ suggesting the required participation of central mechanisms for the complete expression of BN satiety.

In search of a mechanism of action for BN-like peptides, some researchers have also investigated the potential functional interactions between these peptides and certain classical neurotransmitters and/or other peptides. Cholecystokinin was first thought as a likely candidate for peptide-peptide collaboration, as peripheral administration of BN is a potent secretagog of CCK in the human plasma¹⁵⁸. However, accumulating evidence strongly argue for a dissociation of the actions of these peptides on feeding. As mentioned earlier, while subdiaphragmatic vagotomy effectively blocked CCK-induced satiety, this procedure failed to attenuate satiety induced by systemically administered BN.

Furthermore, CCK antagonists, BIM18216 and L364 718, failed to reverse the effects of BN on food intake ¹⁹⁴. More recently, Lieve et al. also demonstrated the ability of intravenous BN to inhibit meal intake in man by a CCK-independent mechanism ^{210,211}. Finally, while lesion of the hypothalamic dorsomedial nucleus significantly reduced the effects of CCK on feeding suppression, such a lesion was ineffective in blocking BN-induced satiety ³¹. These findings combined with the reported synergistic actions of CCK and BN on satiety ¹²⁴ support independent mechanisms of action for these two peptides and suggest that BN does not inhibit food intake solely through CCK release. On the other hand, BN has been shown to stimulate gastric somatostatin secretion ¹⁰¹ and, Bado et al.'s recent findings suggest potential interactions between these two peptides²⁰. In a preliminary sets of experiments, these authors established a strong correlation between the stimulation of gastric somatostatin secretion and the inhibition of food intake following intravenous bombesin administration in gastric fistulated cats ($r = 0.997$). Moreover, pre-treatment with the BN receptor antagonist [Leu¹³-Ψ(CH₂NH)Leu¹⁴]BN dose-dependently reversed both the BN-induced satiety and gastric somatostatin release in cats, suggesting the potential mediation of some of the BN effects on ingestion by somatostatin release. In light of the reported transient effects of somatostatin-14 on food intake (i.e. inhibition is only observed in the first 30 min post-injection) when intragastrically infused at doses comparable to the ones induced by BN administration, this peptide perhaps contributes to the short-term effects of BN-induced satiety ²⁰. Merali and Banks's recent data further suggest that the activation of the histaminergic system by BN may also be involved in the mediation of BN's effects on feeding ²²⁹. Thus, their results demonstrated successful

blockade of BN-induced satiety in rats pretreated with peripheral administration of selective H₃-receptor agonists (R- α -methylhistamine and Imetit) known to inhibit the synthesis and release of histamine. This blockade of BN-induced satiety was dose-dependent and appeared specific as pretreatment with R- α -methylhistamine failed to attenuate the satiety effects of systemically administered CCK. Moreover, the fact that the effects of Imetit on BN-induced satiety were successfully reversed by pretreatment with the H₃-receptor antagonist, thioperamide, suggests that the effects of BN might specifically be mediated through histamine release. In light of the presence of H₃ receptors on nonhistaminergic neurons as well, functioning as presynaptic inhibitory heteroautoreceptors affecting the release of other neurotransmitters such as norepinephrine, 5-HT and acetylcholine, the antagonism of BN effects by the H₃ agonists may involve one or more of those neurotransmitters.

Thus, while several neurotransmitter systems have been shown to affect BN-induced locomotion or grooming^{230,232,288,374}, there is a lack of conclusive evidence supporting the role of any of these neurotransmitters on BN-induced satiety. Finally, despite the BN's stimulatory action on the secretion of other intestinal hormones including enteroglucagon, gastric inhibitory polypeptide, neurotensin and motilin^{310,333}, the mechanisms by which these hormones might participate in the ingestive effects of BN still remain unknown.

Thesis research objectives

As can be appreciated from the preceding review of the literature, the mechanisms by which BN-like peptides inhibit feeding remain largely unknown. However, the demonstration of regional meal-related peptide level fluctuations as well as the enhancement of feeding observed following the blockade of BN receptors suggest a role for BN-like peptides as putative satiety agents. This contention is further supported by the observed evolutionary conservation of pharmacological action of BN on feeding across several species^{21,133,186,264,349,393}. It is of interest to note that the suppression of food intake by BN appears behaviorally specific; low to medium doses of BN that suppress food intake do not suppress water intake¹²³ nor do they induce taste aversion^{181,264,375}. Finally, unlike malaise-inducing agents, BN does not affect the initial rate of feeding; indeed, the animal display normal postprandial behavioral sequence³⁵⁰.

Thus, the principal objective of the present research was to investigate, using a multidisciplinary approach, some of the behavioral, pharmacological and physiological mechanisms underlying the action(s) of BN-like peptides on ingestive behavior.

In an attempt to attain this overall objective, we have proposed the following set of five specific objectives, addressing particular questions or problems:

- 1) When during early development, do rats become sensitive to the effects of BN? Furthermore, how do these effects compare to those of another satiety peptide, namely oxytocin? In these experiments, we also compared the potency and specificity of these peptides in ontogeny.

- 2) Recently, reported fluctuations of BN-like immunoreactivity (BLI) were observed in 12 h food deprived rats compared to rats refed for 10 or 30 min consequent to food deprivation. We wanted to determine whether a) similar changes in BLI would also be observed following a *spontaneous* (non imposed) meal intake of *ad libitum* fed (non deprived) animals, b) changes in BLI previously observed in entire brain regions (e.g. the hypothalamus and hippocampus) could be localized more precisely to specific brain nuclei?
- 3) Although the preceding postmortem study revealed meal-related changes in BLI at specific brain nuclei, an important question remained unresolved: Do peptidergic fluctuations associated with the different feeding states represent fluctuations in peptide release, synthesis or metabolism? In light of interesting changes observed in BLI at the PVN during the pre-, prandial and postprandial states of ingestion, we decide to use push-pull perfusion combined with RIA to determine the dynamics of BN-like peptide release at this site in response to the physiological stimulus, the meal.
- 4) The above experiment provided direct support for the physiological release of BN-like peptides during satiety. Thus, the next study examined whether chronic perturbation of this peptidergic system through sustained central exposure to BN affected a) spontaneous feeding, b) behavioral profile, c) ingestive response to acute BN, and d) changes in BN receptor density within the CNS (using receptor

autoradiography)?

- 5) Finally, does BN interact with other satiety peptides or hormones such as corticotropin-releasing factor (CRF) and oxytocin in the induction of satiety and/or related behaviors?

These five main “paths” of inquiry that constitute this thesis work, will initially be presented in the form of published or submitted scientific papers. The experiments presented (in the various chapters) are not necessarily presented in the chronological order they were conducted in, but rather in an order that logically flow better in terms of results obtained. An attempt will then be made to merge these findings into a general discussion section at the end, focusing on the global understanding of the physiological mechanisms underlying effects of BN-like peptides on ingestive behavior.

Chapter One

Pharmaco-ontogenic modulation of feeding by oxytocin, bombesin and their antagonists

The majority of studies on bombesin have been performed in adult rats and, little or no information is available on the ontogeny of bombesin-induced satiety. This study therefore aimed to determine when during early development do rats become sensitive to the behavioral and ingestive effects of BN. The potency and efficacy of BN's effects were then compared to that of oxytocin. Finally, the physiological role(s) of these peptides were assessed through the use of specific antagonists.

Abstract

Oxytocin (OX) and bombesin (BN) can both suppress food intake in adult rats. In light of important transient fluctuations in peptide and/or receptor expression early after birth, this study characterized the satiating effects of OX and BN during early development and elucidated their physiological relevance, by exploring the effects of BN or OX antagonists, [D-Phe⁶, DesMet⁶⁻¹⁴]-BN(6-14), Ethyl amide (DesMet) and [Des-Glycinamide⁹, d(CH₂)₅¹, O-Me-Tyr², Thr⁴, Orn⁸]-Vasotocin (Vasotocin), respectively. On postnatal days (PD) 1, 5, 10 and 15, groups of food deprived Sprague-Dawley rat pups (n = 8-11) were injected s.c. with saline, BN (0.6, 0.06 or 0.006 mg/kg), DesMet (10 mg/kg), OX (1.2, 0.6, 0.3, 0.15 mg/kg) or vasotocin (1.0 mg/kg), and their ingestive behavior monitored. Results revealed that BN and OX dose-dependently suppressed milk intake from PD 1 to PD 15, although the doses BN (but not OX), required were higher than those needed in adults. OX and BN antagonists by themselves did not affect food intake except at PD 15 for DesMet and PD 1 and 10 for vasotocin, where they enhanced feeding. These results suggest that pharmacological effects to OX and BN are apparent from PD 1 and that these peptides may play a role in the regulation of ingestive behavior very early in ontogeny.

Introduction

In adult rats, central or systemic administration of the amphibian tetradecapeptide bombesin (BN) and its mammalian counterparts have been shown to suppress food intake^{21,133,186,264,349,393}. BN-like peptides also elicit intense grooming following central administration^{128,180}. While the ontogeny of BN-induced grooming has been explored^{289,290}, little or no information on the development of BN-induced satiety is presently available. Biochemical studies have revealed substantial changes in the distribution pattern of BN-like peptides and their receptors early in life^{127,345}. Thus, Battey et al. have demonstrated spatiotemporal changes in BN receptor mRNA expression in the central nervous system (CNS) and peripheral tissues of rat embryos which appeared to gradually evolve into the adult distribution pattern²⁷. Postnatally, dramatic increases in BN/GRP binding site density have been reported around birth, which remained constant during the first postnatal week to then increase again starting at postnatal day (PD) 7 and reached adult density at PD 10¹²⁷. The function of BN receptors early in development remains undetermined, but their presence suggests potential BN receptor-ligand interactions during development. Behaviorally, scratching and grooming have been demonstrated following central or systemic administration of BN within the first three weeks of the neonatal life^{289,290} suggesting that BN binding sites are indeed functional early in ontogeny. One short study attempted to quantify BN satiation effects, but that study was limited to 15 day-old rat pups¹⁵⁶.

Although important fluctuations of BN receptors are characterized in early ontogeny, the endogenous levels of BN-like peptides remain minimal in the hypothalamus, midbrain and hindbrain prior to PD 14. It is only in the third week after birth that notable increases in the peptide concentration are quantified and BN-like immunoreactivity was found to reach adult levels by 21-23 days of age ¹²⁷. The differences in the sequential expression and development of BN-like peptides and receptors are intriguing.

Peripheral or central administration of the nonapeptide oxytocin (OX) has also been shown to produce satiety in the adult rats ^{14,274}. Specific OX binding sites were initially detected at embryonic day 14 using *in vitro* light microscopic autoradiography, in the dorsal motor nucleus of the vagus. Receptor sites are progressively established in many other central and peripheral nervous system regions and a stable distribution of binding sites is present from PD 5 to PD 16. Interestingly, in the neonate, central OX receptor distribution differed considerably from that of the adult rat and, an adult pattern of receptor distribution appeared to be definitive only after puberty (i.e. after PD 35) ^{116,368}. Immunohistochemical studies revealed that OX is detectable in its entire molecular form from the day of birth in the rat brain, whereas OX precursor and CH-terminal are present from embryonic day 16 onwards ⁸. Furthermore, the observation of electrophysiological activity elicited by iontophoretic application of OX to vagal neurons in slices from animals sacrificed at ages PD 1 to PD 12 suggest that the OX receptors present at birth are functional ³⁶⁸. However, to our knowledge, there are no reports demonstrating behavioral effects of exogenous OX in the neonatal rats. Administration of OX during the rat infancy has solely been used to demonstrate its long term effect on brain

development and anxiety⁴⁴ or its potentiation of OX novelty-induced grooming²⁶⁸, in the adult rat.

Thus, in light of the important transient spatio-temporal changes in peptide and/or receptor expression early after birth, the objectives of the present study were three fold: 1) to determine when during development do rats become sensitive to BN and OX satiety effects, 2) to compare the potency and efficacy of BN and OX to induce satiety-like state in the neonatal rats and, 3) to establish whether the blockade of endogenous peptide action with BN antagonist, ([D-Phe⁶, DesMet⁶⁻¹⁴]-BN(6-14) Ethyl amide (DesMet)) or the OX antagonist, ([Des-Glycinamide⁹, d(CH₂)₅¹, O-Me-Tyr², Thr⁴, Orn⁸]-Vasotocin (Vasotocin), affect food intake during development.

Material and Methods

Animals

All experiments were performed using male Sprague-Dawley rat pups bred and reared in our laboratory colony. Litters were standardized to 8 or 9 pups per dam on the day of birth (PD 0) and the pups were kept with the mother until the day of testing (PD 1, 5, 10 and 15). The colony room environment was maintained at 20° C with a relative humidity of 60% and a 12 h light-dark cycle (lights on at 7:00 h AM).

Experimental procedures

Before testing, the pups were separated from their mother and food deprived for a period of 5-7 h by placing them in a moist, warm incubator maintained at 32° C. The pup's bladder was emptied by manual stroking of the anogenital region with a cotton swab. All experiments were performed between 14:00 and 17:00 h. Rat pups were tested at PD 1, 5, 10 and 15. All drugs were freshly dissolved in saline and drug treatments divided across litters in a randomized order. Each pup received a subcutaneous (s.c.) injection of either vehicle (saline), one of the doses of BN (0.006, 0.06, 0.6 mg/kg) or OX (0.15, 0.3, 0.6, 1.2 mg/kg), or either a dose of BN antagonist (10 mg/kg) in combination with the BN (0.6 mg/kg) or a dose of OX antagonist (1 mg/kg) combined with OX (1.2 mg/kg). DesMet and vasotocin were also tested on their own to verify if they demonstrated any intrinsic activity. All drugs were obtained from Bachem CA.

After injection of either saline or one of the drug solutions, 1 and 5-day-old pups were placed back in the incubator in individual compartments (10 x 10 x 5 cm) containing a piece of absorbent paper saturated with warm milk, while 10 and 15-day-old pups were placed in bigger observation compartments (15 x 13.5 x 21.5 cm) with the base maintained to approximately 30° C by an electric water blanket (American Medical Supplies, model K-20-C). The testing room was maintained at 20° C with a relative humidity of 60%. Following drug administration, the behavioral activity of rat pups was monitored for 30 min, by recording the frequency of mouthing, grooming, exploring, yawning and resting behaviors. The frequency of tumbling was also monitored but only until PD 10, when this

behavior was no longer elicited (see Table 1 for operational definitions of behaviors recorded). The amount of milk consumed was estimated from the within session change in body weight.

TABLE 1

Behaviors measured following drug administration

<i>Behavior</i>	<i>Definition</i>
Mouthing	Opening or closing of the jaws, movements of the tongue, or both.
Grooming (<i>Scratching and/or Washing</i>)	<i>Scratching</i> - Contact of the hindpaw with the side of the face/head or body/flank followed by a scratching action. <i>Washing</i> - Wiping of the face with circular movements of the forelimbs or active licking of the abdomen and thorax.
Exploring	The animal is moving around, actively examining an area of its testing compartment
Resting	The animal is inactive with eyes open or lying in curled position with eyes closed
Tumbling	The animal rolls onto its back and makes a paddling motion with its limbs to get back on its feet.

Statistics

Results are expressed as mean $\pm$ S.E.M. Food intake was estimated from within session change in body weight and expressed as percent body weight gain (% BWG).

Data obtained using BN and OX analogues were analyzed separately for each age group

using one-way analysis of variance (ANOVA) followed by Tukey's post hoc comparisons. The visually monitored behavioral data were analyzed by individual behavior using one-way ANOVA. Post hoc comparisons were performed using Tukey's test. Differences were considered significant when $P \leq 0.05$.

RESULTS

Feeding

Postnatal day 1

There was a significant effect of treatment with BN and/or DesMet on milk intake ($F_{5,47} = 9.55$; $P < 0.0001$). Post hoc analyses revealed that BN (0.006, 0.06 and 0.6 mg/kg; s.c.) dose-dependently suppressed food intake in 1-day-old rat pups. Pretreatment with DesMet (10 mg/kg; s.c.) reversed the feeding suppression induced by BN (0.6 mg/kg; s.c.). When administered alone, this dose of DesMet was without effect on milk ingestion (see Fig. 1).

Statistical analysis also revealed a significant effect of treatment on % BWG for rat pups treated with OX analogues ($F_{7,59} = 13.66$; $P < 0.0001$). Post hoc analyses revealed that OX (0.15, 0.3, 0.6 mg/kg; s.c.) induced a dose-related suppression of food intake at this age. However, the highest dose tested (1.2 mg/kg; s.c.) failed to further suppress the pups milk consumption. Pretreatment with vasotocin (1 mg/kg; s.c.), completely blocked oxytocin (1.2 mg/kg)-induced satiety. Vasotocin slightly enhanced food intake when administered on its own (see Fig. 2).

Postnatal day 5

ANOVA revealed a significant effect of drug BN and/or antagonist treatment on milk intake ($F_{3,42} = 8.16$; $P < 0.0001$). Post hoc analysis revealed significant inhibition of milk intake with the higher doses of BN (0.06 or 0.6 mg/kg; s.c.). Administration of DesMet alone failed to alter food ingestion while pretreatment with this antagonist (10 mg/kg; s.c.) completely blocked BN (0.6 mg/kg)-induced suppression of food intake. (see Fig.1).

Similarly, there was a significant effect of treatment on milk intake in rat pups tested with OX analogues ($F_{7,58} = 7.75$; $P < 0.0001$). Tukey's analyses revealed that OX administration significantly suppressed milk ingestion at all doses tested. However, at this age, pretreatment with vasotocin only partially blocked OX-induced satiety. When administered alone, the same dose of vasotocin (1 mg/kg; s.c.) failed to affect the pups' milk ingestion (see Fig.2).

Postnatal day 10

ANOVA revealed a significant effect of BN and/or antagonist treatment on % BWG ($F_{3,42} = 9.67$; $P < 0.0001$). Tukey's post hoc comparisons revealed that the medium and high doses of BN (0.06 and 0.6 mg/kg; s.c.) were equally effective in suppressing food intake. However, at the lowest dose (0.006 mg/kg; s.c.) BN, failed to alter milk ingestion. Pretreatment with DesMet consistently blocked BN (0.6 mg/kg)-induced satiety. Again, administration of the BN antagonist DesMet (10 mg/kg) was without significant effect (see Fig. 1).

There was also a significant effect of treatment on milk intake following testing with OX analogues ($F_{7,63} = 22.53$; $P < 0.0001$). Tukey's post hoc comparisons revealed that administration of OX (0.3, 0.6 and 1.2 mg/kg; s.c.) suppressed food intake in a dose-related manner while the lowest OX dose tested (0.1 mg/kg; s.c.) slightly enhanced milk intake at that age. Pretreatment with vasotocin blocked OX (1.2 mg/kg)-induced satiety. On its own, administration of vasotocin (1 mg/kg; s.c.) significantly enhanced milk ingestion at that age (see Fig. 2).

Postnatal day 15

ANOVA revealed a significant effect of the drug treatment on milk intake ($F_{5,42} = 25.77$; $P < 0.0001$). Post hoc analyses revealed that BN administration significantly suppressed milk consumption. Similar to the dose-related trends observed at PD 10, the higher doses of BN (0.06 and 0.6 mg/kg; s.c.) were equally efficacious at inhibiting food intake. Pretreatment with DesMet again attenuated BN-induced food intake suppression. When administered alone, DesMet slightly enhanced the pups' milk intake (see Fig. 1).

There was also a significant effect of the drug treatment on milk intake in pups treated with OX analogues ($F_{7,56} = 13.08$; $P < 0.0001$). At this age, administration of OX produced a dose-related suppression of food intake. Pretreatment with vasotocin reversed OX (1.2 mg/kg)-induced suppression of food intake, while administration of vasotocin alone was without effect (see Fig. 2).

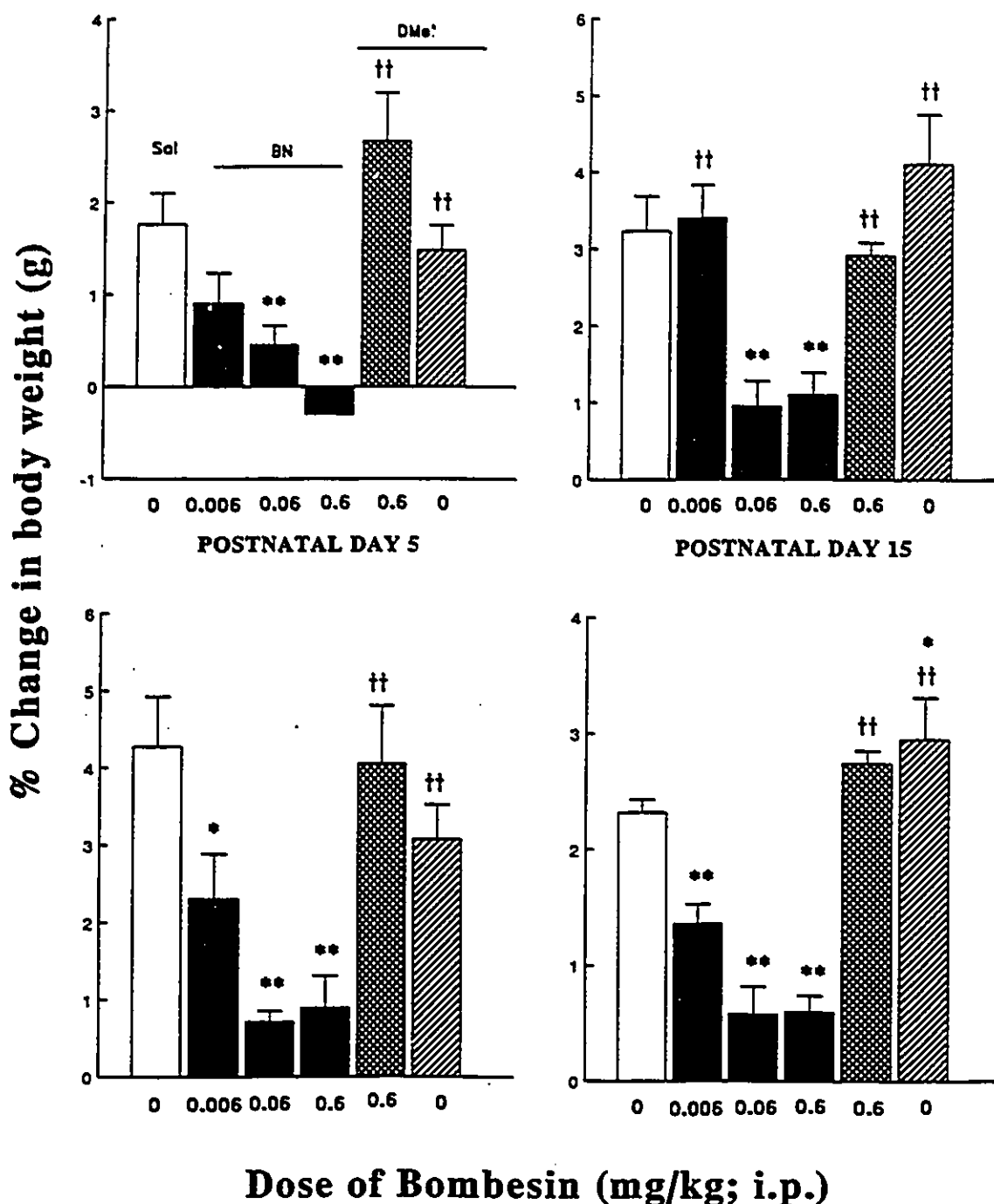


Fig.1 Effect of BN and DesMet (DMet) on milk intake in neonatal rats from postnatal day 1 to 15. Each column represents the mean $\pm$ S.E.M. of percent body weight gain. Open columns represent saline treated group whereas the solid columns represent BN-treated groups. Hatched column represents antagonist alone group and the cross-hatched column represents animals treated with both the antagonist and BN. *,** significantly different from saline at $P < 0.05$ and $P < 0.01$, respectively. †† significantly different from BN (0.6 mg/kg) group at $P < 0.01$ (Tukey's test).

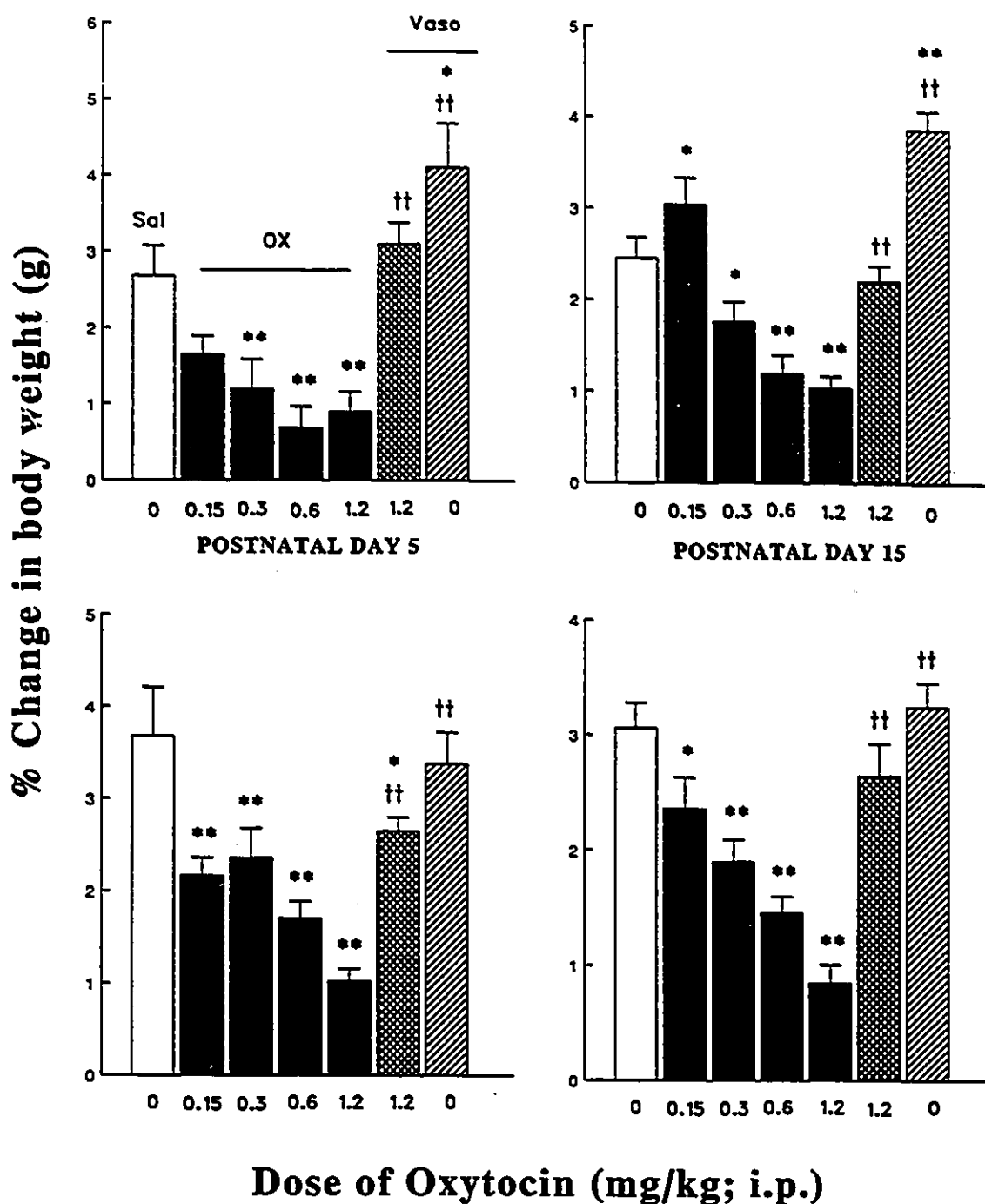


Fig.2 Effect of OX and Vasotocin (Vaso) on milk intake in neonatal rats from postnatal day 1 to 15. Each column represents the mean $\pm$ S.E.M. of percent body weight gain. Open columns represent saline treated group whereas the solid columns represent OX-treated groups. Hatched column represents antagonist alone group and the cross-hatched column represents animals treated with both the antagonist and OX.

*,** significantly different from saline at $P < 0.05$ and $P < 0.01$, respectively.

†† significantly different from OX (1.2 mg/kg) group at $P < 0.01$ (Tukey's test).

Behavioral monitoring

Notwithstanding the treatment received, rats pups of all age groups displayed a relatively high frequency of exploratory activity. However, at PD 5, there was a significant effect of drug treatment on exploratory activity ($F_{5,48} = 3.47$; $P < 0.009$) and, low and medium (0.006 and 0.06 mg/kg) doses of BN further enhanced exploratory activity as compared to saline- but not DesMet-pretreated pups receiving BN (see Table 2). At PD 1, there was a significant effect of drug treatment on exploratory activity of rat pups treated with OX analogues ($F_{6,47} = 2.43$; $P < 0.04$) and, the frequency of this behavior appeared to be decreased following high dose of OX (1.2 mg/kg) or vasotocin (1 mg/kg), as compared to saline-injected pups at PD 1. In contrast, at PD 10, exploratory behavior appeared slightly enhanced by the low and high doses of OX ($F_{6,55} = 2.28$; $P < 0.04$) (see Table 3).

Rat pups spent a significant portion of their time resting, and the incidence of this behavior decreased gradually as the pups aged. ANOVA revealed a significant effect of the drug treatment on the resting frequency at PD 1 ($F_{5,41} = 4.19$; $P < 0.004$). At this age, the resting frequency was enhanced by BN at the 0.06 and 0.6 mg/kg doses. There was also a significant effect of treatment on the frequency of resting at PD 5 ($F_{5,43} = 2.96$; $P < 0.02$). At this age, the frequency of resting also appeared significantly elevated for BN-treated animals as compared to saline-treated animals, independently of the dose received. Similarly, at PD 10, both the 0.06 and 0.6 mg/kg doses of BN enhanced resting as compared to saline ($F_{5,42} = 2.36$; $P < 0.05$). At PD 15, there was also a significant effect of the treatment on resting frequency ($F_{5,42} = 4.09$; $P < 0.004$); BN at the lowest dose

decreased resting. However, at the higher doses, this effect disappeared. Animals receiving DesMet with or without BN, also appeared to rest less (see Table 2). Oxytocin did not significantly affect the resting frequency except at PD 15 when a significant effect of treatment was observed ($F_{6,49} = 2.54$; $P < 0.03$). At that age, the frequency of resting appeared enhanced in rats treated with OX (0.3 mg/kg) and/or vasotocin (1 mg/kg) (see Table 3).

At PD 1, the frequency of mouthing was suppressed with BN as well as OX, reflecting suppression of food intake ($F_{5,41} = 9.69$; $P < 0.0001$ and $F_{6,47} = 31.55$; $P < 0.0001$, for BN and OX, respectively). At PD 5, there was a significant effect of treatment for rats receiving BN or OX analogues ($F_{5,43} = 6.21$; $P < 0.0002$ and $F_{6,49} = 9.06$; $P < 0.0001$, respectively). The frequency of mouthing was suppressed in rats receiving BN as compared to saline- and DesMet + BN treated groups. As for OX, the frequency of mouthing was significantly suppressed at PD 5 in rats receiving 0.6 and 1.2 mg/kg doses of OX as compared to saline and vasotocin (1mg/kg)-treated animals. At PD 10, there was also a significant effect of drug treatment for rats treated with BN or OX analogues ($F_{5,43} = 4.99$; $P < 0.001$ and $F_{6,53} = 2.40$; $P < 0.03$, respectively). The mouthing behavior was suppressed in a dose-related manner, in both BN and OX-treated rat pups, with the suppression being most apparent in pups receiving the high doses of BN and OX, as compared to saline-treated rats. Finally, at PD 15, there was a significant effect of the drug treatment on the frequency of mouthing for pups treated with the BN or OX analogues ($F_{5,42} = 8.65$; $P < 0.0001$ and $F_{6,49} = 9.67$; $P < 0.0001$, respectively). At this age, the frequency of mouthing reflected changes in BWG and was significantly reduced

with BN at the 0.6 mg/kg dose. Mouthing also appeared enhanced in rats pretreated with DesMet before BN administration as compared to saline-treated pups. As for OX, the mouthing frequency appeared reduced by all doses of OX tested (see Tables 2 and 3).

At the doses tested, the frequency of grooming was relatively low and was not enhanced by BN at any of the ages tested (see Table 2). In contrast to BN, high dose of OX significantly enhanced grooming at PD 1 ($F_{6,47} = 6.53$; $p < 0.0001$), PD 5 ($F_{6,50} = 19.8$; $p < 0.0001$) and PD10 ($F_{6,55} = 4.34$; $p < 0.0012$), but suppressed this behavior at PD 15 ($F_{6,49} = 4.88$; $p < 0.0006$). At PD 1, OX (0.6 and 1.2 mg/kg) significantly enhanced the frequency of grooming as compared to saline. At PD 5, OX dose dependently increased the frequency of grooming as compared to saline, with all doses being effective (see Table 3).

The effects of the different drug treatments on yawning and tumbling behaviors was also assessed. The incidence of yawning was rarely observed and no drug-related changes were apparent. However, at PD 1, tumbling frequencies were elevated in rats treated with OX at the 0.1, 0.3 and 1.2 mg/kg doses ($F_{6,47} = 3.25$; $P < 0.009$). At PD 5, the frequency of tumbling was significantly suppressed as compared to PD 1 and no differences were apparent between treatment groups. By PD 10, tumbling had completely disappeared (data not shown).

Table 2. Effect of BN and DesMet administration on behavior in neonatal rats.

Treatment (mg/kg; s.c.)	Mouthing	Grooming	Exploring	Resting
PD 1				
Saline	4 ± 1.2	3.5 ± 1.3	9 ± 1.2	13 ± 3.4
BN 0.006	2.2 ± 0.6	2.3 ± 0.5	13.5 ± 2.2	18.7 ± 2.9
BN 0.06	1.4 ± 0.3	4.3 ± 0.9	9.3 ± 1.9	25.6 ± 1.5**
BN 0.6	1.2 ± 0.4*	2.1 ± 0.6	10.7 ± 0.6	22.3 ± 1.5**
DesMet 10 + BN 0.6	6.6 ± 1.4*	5.0 ± 0.8	13.4 ± 2.3	16.6 ± 1.6
DesMet 10	3.1 ± 1.1	2.3 ± 0.5	12.9 ± 1.1	19.8 ± 1.4*
PD 5				
Saline	10.3 ± 2.1	1.2 ± 0.2	7 ± 1.0	14.1 ± 1.2
BN 0.006	5.4 ± 1.3*	2.3 ± 0.8	14.1 ± 2.3**	20.1 ± 2.6*
BN 0.06	2.5 ± 1.1**	2.5 ± 0.5	12.5 ± 1.9*	27.1 ± 0.9**
BN 0.6	3.9 ± 1.5**	1.8 ± 0.4	11.9 ± 1.2	21.5 ± 1.5**
DesMet 10 + BN 0.6	11.1 ± 0.9	1.0 ± 0.4	14.8 ± 2.1**	19.6 ± 1.6*
DesMet 10	8.9 ± 0.7	1.4 ± 0.7	11.6 ± 1.2	21.1 ± 1.6**
PD 10				
Saline	11.4 ± 2.2	3.1 ± 0.6	14.3 ± 1.8	11.0 ± 1.7
BN 0.006	8.4 ± 1.4	3.5 ± 0.8	13.4 ± 2.0	15.5 ± 2.1
BN 0.06	6.9 ± 1.5	2.3 ± 0.5	12.4 ± 1.2	18.4 ± 1.8*
BN 0.6	6.1 ± 0.8*	2.8 ± 0.6	13.9 ± 2.2	19.1 ± 1.9*
DesMet 10 + BN 0.6	13.8 ± 0.8	0.6 ± 0.3*	13.1 ± 2.2	15.4 ± 1.8
DesMet 10	9.9 ± 1.7	3.6 ± 1.0	13.6 ± 2.1	15.1 ± 2.4
PD 15				
Saline	12.9 ± 1.3	2.0 ± 0.8	18.0 ± 2.6	12.4 ± 2.9
BN 0.006	15.6 ± 1.5	4.0 ± 0.4*	20.5 ± 2.0	2.3 ± 0.9**
BN 0.06	9.1 ± 2.4	4.1 ± 0.6*	22.1 ± 3.1	8.8 ± 1.6
BN 0.6	7.5 ± 1.3*	1.9 ± 0.4	21.6 ± 3.6	11.0 ± 2.8
DesMet 10 + BN 0.6	18.2 ± 1.4*	2.6 ± 0.7	17.5 ± 2.4	5.0 ± 2.1*
DesMet 10	15.0 ± 2.3	3.6 ± 0.7	21.8 ± 2.7	3.1 ± 1.7**

Note. Each cell represents the mean ± S.E.M. of frequency of mouthing, grooming, exploring and resting during the 30-min test interval. *, ** significantly different from saline condition at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

Table 3. Effect of OX and Vasotocin administration on behavior in neonatal rats.

Treatment (mg/kg; s.c.)	Mouthing	Grooming	Exploring	Resting
PD 1				
Saline	5.5 ± 0.8	0.9 ± 0.2	19.4 ± 2.4	20.4 ± 1.8
OX 0.15	3.4 ± 0.1	3.5 ± 0.8	16.9 ± 1.6	17.5 ± 1.6
OX 0.3	1.8 ± 0.5*	2.5 ± 0.9	15.8 ± 1.9	19.3 ± 1.6
OX 0.6	2.0 ± 0.7*	4.0 ± 1.2*	19.5 ± 2.3	15.1 ± 1.9
OX 1.2	0.9 ± 0.3**	9.0 ± 1.9**	10.8 ± 2.4**	18.3 ± 1.7
Vaso 1.0 + OX 1.2	10.0 ± 0.8*	2.9 ± 0.9	15.1 ± 2.0	16.3 ± 1.4
Vaso 1.0	8.5 ± 1.0	1.9 ± 0.8	13.0 ± 1.5*	19.3 ± 2.1
PD 5				
Saline	7.9 ± 1.1	1.3 ± 0.7	17.4 ± 2.3	16.9 ± 1.7
OX 0.15	8.1 ± 1.7	5.8 ± 1.4**	12.3 ± 1.3	19.0 ± 0.8
OX 0.3	6.1 ± 0.5	9.1 ± 1.7**	15.3 ± 1.8	16.4 ± 1.4
OX 0.6	4.3 ± 0.9*	8.1 ± 1.3**	12.0 ± 1.3	19.3 ± 1.6
OX 1.2	2.5 ± 1.2**	13.5 ± 1.4**	13.5 ± 1.5	16.4 ± 1.6
Vaso 1.0 + OX 1.2	6.3 ± 0.5	0.9 ± 0.3	16.1 ± 1.4	21.0 ± 1.2
Vaso 1.0	9.5 ± 1.3	0.5 ± 0.3	13.0 ± 1.7	18.6 ± 1.4
PD 10				
Saline	13.9 ± 1.1	0.8 ± 0.3	16.1 ± 1.3	15.5 ± 1.7
OX 0.15	13.2 ± 2.4	1.2 ± 0.4	18.1 ± 1.8	13.8 ± 1.1
OX 0.3	12.0 ± 2.4	0.6 ± 0.3	21.3 ± 2.5*	10.8 ± 3.3
OX 0.6	9.8 ± 1.5	1.6 ± 0.4	18.3 ± 2.1	14.9 ± 2.9
OX 1.2	8.6 ± 1.0*	3.0 ± 0.8**	21.3 ± 2.4*	12.8 ± 2.2
Vaso 1.0 + OX 1.2	17.4 ± 1.9	0.5 ± 0.2	13.0 ± 1.4	16.0 ± 2.1
Vaso 1.0	14.6 ± 1.7	0.8 ± 0.3	18.0 ± 1.4	11.9 ± 1.4
PD 15				
Saline	17.8 ± 0.7	4.8 ± 0.8	22.9 ± 0.8	1.1 ± 0.4
OX 0.15	13.9 ± 0.9*	5.1 ± 1.0	23.3 ± 1.4	2.0 ± 0.8
OX 0.3	11.8 ± 0.8**	0.9 ± 0.4**	21.3 ± 1.5	7.4 ± 1.2**
OX 0.6	13.0 ± 1.0**	3.1 ± 1.1	19.8 ± 2.2	4.0 ± 0.9
OX 1.2	13.3 ± 1.8*	1.5 ± 0.8**	20.8 ± 1.7	4.6 ± 1.2
Vaso 1.0 + OX 1.2	20.4 ± 1.8	1.6 ± 0.3**	17.8 ± 1.8	5.0 ± 1.8*
Vaso 1.0	18.3 ± 1.1	2.4 ± 0.4*	23.0 ± 2.2	5.3 ± 2.1*

Note *,** significantly different from saline (see Table 2 for additional details).

DISCUSSION

In the present study, we present the first evidence that systemic administration of BN and OX dose-dependently suppress food intake in neonatal rats from PD 1 through PD 15. Pretreatment with both BN and OX antagonists (DesMet and vasotocin, respectively) blocked BN- and OX-induced satiety at all ages tested except for OX-induced satiety at PD 5, which was only partially reversed by vasotocin pretreatment. In general, DesMet and vasotocin failed to alter milk intake when administered alone. However, at specific time points (PD 15 for DesMet and PD 1 and 10 for vasotocin) administration of these antagonists slightly enhanced food intake on their own. Together, these findings suggest that some physiological mechanism(s) potentially involving the two peptides may be participating in appetite control from the first hours following birth.

These findings are also interesting in light of the presence of relatively high concentrations of BN-like peptides in human ³⁴ and bovine ¹⁵⁷ milk. The role of these peptides in maternal milk remains unknown. However, if the BN gets absorbed from the gastrointestinal tract, then it might interact with the BN-binding sites that are expressed before BN-like immunoreactivity is endogenously available ¹²⁷. Interestingly, it is during the first postpartum weeks that human milk concentrations of BN are most elevated ³⁴, a neonatal period characterized by low levels of endogenous BN-like peptides ¹²⁷. Moreover, the similarities between BN bioactivity and the peptide extracted from milk suggest that it contains the C-terminal heptapeptide sequence required for its full biological response ^{35,105}. Thus, since milk constitutes the main form of nutrition for the

neonates, it is possible that BN-like peptides may act as one of the signals regulating food ingestion by the neonates; a signal first exogenously provided by the mother's milk but which is gradually replaced by endogenously expressed peptide.

In terms of effective doses, our findings revealed that while the 0.006 mg/kg dose of BN failed to consistently alter feeding at all ages, both the higher doses (0.06 and 0.6 mg/kg) tested reliably induced satiety in pups of all ages. Moreover, the observation that BWG was not further reduced with the highest dose of BN as compared to the 0.06 mg/kg dose suggests that maximal feeding suppression is obtained with doses > 0.006 but ≤ 0.06 mg/kg.

Interestingly, at PD 15, DesMet slightly potentiated feeding when administered on its own, suggesting that BN may play a physiological role in the control of feeding early during development. This observation is consistent with reports from adult rats, where BN antagonists have been shown to enhance food intake^{115,236}. Furthermore, the blockade of BN-induced satiety in rat pups pretreated with DesMet suggest that BN's satiety effects are mediated through specific activation of BN receptors.

In the present study, BN failed to affect grooming at any of the doses tested. Although this finding is in contrast with previous studies reporting enhanced grooming following peripheral administration of BN, the discrepancy can be reconciled based on the fact that the doses of BN used in those studies were considerably higher than the ones used in the present study^{156,289,290}. Indeed, at equivalent doses, BN failed to alter

grooming in 5 to 20-day-old pups¹⁵⁶. Jackson and Kitchen's findings while limited to 15-day-old rat pups also suggest a dissociation between BN-induced satiety and grooming at that age¹⁵⁶. A plausible explanation for the observed findings could be that at the doses tested, the feeding suppressant effects of BN may have been attributable to a direct action of the peptide at receptor sites located on peripheral organs while at higher doses, BN may also reach brain sites thus altering grooming behavior via its CNS action. In the adult rat, BN-induced grooming is only observed following central administration of the peptide^{162,231,266}. Moreover, increasing doses of BN are required to elicit grooming as the pups mature (i.e. the lowest effective dose is 1 mg/kg at PD 1 while 10 mg/kg are required to induce grooming at PD 5 and PD 10) possibly reflecting the maturation of the blood brain barrier in older pups²⁹⁰. Alternately, studies using adult rats have also reported dissociation between the effects of BN on grooming and feeding following central administration of BN. Thus, low dose BN injected into the NTS and/or fourth ventricle have been shown to suppress feeding while leaving grooming unaltered^{162,191}. Thus, it remains possible that in the present study, BN may have been acting centrally to induce its effects and, that the observed dissociation of grooming and feeding may be attributable to similar mechanisms as the ones implicated in adults.

Except for PD 5 where enhanced locomotor activity was observed in BN-treated rats, BN failed to significantly alter exploratory activity at any other time point during development. This finding is consistent with findings with higher doses of BN which also failed to increase exploration in neonates²⁹⁰.

Analysis of behavioral data also revealed that the frequency of resting was elevated in BN-treated rat pups as compared to saline-treated animals. Thus, as the BN dose increased, so did the frequency of resting. Such finding is in agreement with the decreased frequency of mouthing and consequent reduced BWG recorded from BN-treated pups. Although this observation is in contrast to enhanced locomotor and exploratory activities noted in adult rats ^{162,286}, our observations are consistent with the lack of BN effects reported on exploratory activity in neonatal rats ²⁹⁰.

While different studies have suggested the participation of OX in feeding related processes ^{38,62,376}, only a few microinjection studies are presently available demonstrating the role of this peptide in the control of food intake in adult rats ^{14,274}. In these studies, the lowest peripheral dose shown to induce satiety in adult rats was 0.375 mg/kg, a dose which led to approximately 36 % suppression of food intake ¹⁴. In the present study, a similar dose of OX (0.3 mg/kg) successfully inhibited feeding in neonates at all ages tested. Decreased BWG was also observed at PD 5 and PD 15 using a lower OX dose (0.15 mg/kg). However, this dose failed to significantly alter BWG at PD1 and slightly enhanced BWG at PD 10. When comparing doses of BN and OX that consistently inhibited milk intake at all ages (0.06 and 0.3 mg/kg, respectively), BN appeared more potent and efficacious to suppress milk intake in neonates. Such efficiency comparisons are maintained in adulthood as studies have shown that OX doses as high as 0.375 mg/kg are required to induce approximately 40 % suppression in feeding in adult rats while BN doses as low as 6 µg/kg produced similar reduction of food intake ^{14,124}. In general,

administration of vasotocin prior to OX administration blocked OX-induced suppression of milk intake suggesting that this peptide's effects are mediated by OX receptors. In contrast to BN antagonist, administration of vasotocin on its own significantly enhanced feeding as early as PD 1. Milk ingestion also appeared enhanced following administration of vasotocin at PD 10. This suggests that physiological participation of OX in early ontogeny is compatible with endogenous expression of the peptide from the day of birth in the rat CNS⁸. In contrast, BN-like peptides are only detectable in the rat brain in the second week and reach adult levels in the third week postpartum¹²⁷, a period at which DesMet slightly enhanced feeding.

The exact physiological mechanisms underlying the effects of OX on milk intake during development are not known. However, it appears possible that OX effects observed early during ontogenic development may partly be attributable to activation of oxycitocineric pathways in the CNS. Indeed, the increased grooming recorded at PD 1 and PD 5 is likely to be related to OX activation of neuronal populations as grooming is potentiated by central, but not peripheral, administration of OX in adult rats¹⁴. However, it is unlikely that the ingestive effects of OX are exclusively dependent on centrally mediated processes as OX suppressed BWG but failed to alter grooming in pups of 10 and 15 days of age perhaps suggesting the decreased permeability of the blood-brain-barrier. These findings together with the observation of no major alterations in exploratory activity following OX administration in neonates and of elevated frequencies of tumbling exclusively at PD 1 following OX administration suggest the dissociation of the effects of

OX on ingestion from its effects on other behaviors, such as grooming, tumbling or exploring.

In conclusion, the present study indicates that BN and OX receptors are pharmacologically functional and participate in the regulation of feeding related behaviors as early as 1 day after birth. We also demonstrated the ability of exogenous BN to induce satiety prior to measurable expression of BN-like peptides, in neonatal rats ¹²⁷. One could speculate from our observations that BN-like peptides in mammalian maternal milk may provide exogenous ligand for those receptors during ontogenic development, regulating the amount of food the neonate consumes. More research is clearly needed to determine the exact mechanism(s) and physiological role(s) of these peptides during ontogenic development.

Chapter Two

Spontaneous meal is associated with distinct changes in the endogenous levels of bombesin-like peptides and corticotropin-releasing factor in specific brain nuclei.

The first set of experiments demonstrated that like the adult animals, the neonatal rats are also responsive to the feeding suppressant effects of BN, and that the BN/GRP receptors are functional as early as day 1 of life. These findings together with the reported evolutionary conservation of this response suggest rudimentary physiological importance of this family of peptides in the regulation of food intake. Thus, the objective of the subsequent experiment was to directly determine the physiological participation of BN-like peptides in regulation of ingestive process. It was hypothesized that if these peptides are indeed involved in the modulation of ingestion, their levels should fluctuate in a meal-dependent manner at relevant nuclei in the brain. In this experiment, fifteen specific brain nuclei were micropunched for peptide level determination in rats at various phases of spontaneous ingestion. These experiments were carried out in adult rats due to technical difficulties in attaining adequate control and neuroanatomical resolution required for such studies.

Abstract

Bombesin (BN) and corticotropin-releasing factor (CRF) have both been shown to induce satiety in rats, when injected centrally. To explore the physiological involvement of these peptides in the regulation of food intake, we investigated whether utilization of these peptides changed in a meal-dependent manner. Alterations in the endogenous levels of CRF and BN-like peptides associated with the initial spontaneous meal of the nocturnal cycle were determined in fifteen hypothalamic and extrahypothalamic brain nuclei, in the following three groups of rats: 1) a preprandial group consisting of rats sacrificed before feeding, 2) a prandial group consisting of rats sacrificed during the meal and, 3) a postprandial group consisting of rats sacrificed 8-12 min following the meal. Findings revealed site-specific changes in BN and CRF content during the course of a meal. Alterations in BN levels were observed at the paraventricular, arcuate and dorsomedial nuclei of the hypothalamus and at the nucleus accumbens. At all three hypothalamic nuclei, BN content was significantly elevated during ingestion (by about two fold) as compared to before or after the meal while at the accumbens, meal-related fluctuations in BLI were characterized by increased preprandial levels accompanied by reduced BN content both during and after meal intake. In contrast, feeding-related alterations in CRF levels were observed at the lateral (LH) and ventromedial (VMH) hypothalamic nuclei and at the central nucleus of the amygdala (Ce). At the LH, CRF content dropped following feeding as compared to preprandial levels. At the VMH, CRF concentrations were significantly elevated both before and after food intake when compared to prandial levels. In contrast, at the Ce marked increases in CRF concentrations were observed during

ingestion as compared to before or after meal intake. These data further support the physiological participation of BN-like peptides in the regulation of spontaneous food ingestion. Furthermore, these data also demonstrate for the first time, site-specific fluctuations of CRF in relationship to the animal's feeding status, implicating physiological involvement of this peptide as well, in regulation of food intake.

Introduction

The tetradecapeptide bombesin (BN) was originally isolated from the skin of the European frog *Bombina bombina*⁹. Since then, BN-like immunoreactivity and receptor sites have been shown to be widely distributed in mammalian brain and gastrointestinal tissues^{92,245,249,281}. Central or systemic administration of BN has been shown to induce satiety in a variety of species, including man^{21,133,264,349,393}. The physiological mechanisms underlying BN ingestive effects are not fully understood. Stuckey et al.³⁵⁰ found that total neural disconnection of the gut from brain completely abolished the feeding suppressant effects of systemically-administered BN, suggesting the participation of the brain in BN-induced satiety. This assumption was further supported by attenuation of satiety elicited by systemically- or centrally-administered BN in rats following immunoneutralization of central BN with BN antiserum²³⁵. The observation that discrete forebrain and hindbrain structures, such as the PVN and NTS, are more sensitive to the feeding suppressant effects of BN suggest the involvement of distinct neuronal populations in BN's effects^{162,186,193}. Moreover, endogenous levels of hippocampal and hypothalamic BN-like immunoreactivity have been shown to increase progressively upon re-feeding in food deprived rats²³⁴. Recently, we also demonstrated *in vivo* fluctuations of BN-like peptides release at the PVN in relationship to spontaneous meal ingestion and termination of *ad libitum* fed animals²⁹³. Together with studies demonstrating enhancement of feeding following central administration of BN antagonists^{115,236}, these findings support a physiological role for BN-related peptides in the control of ingestion.

Central administration of the 41-amino acid polypeptide corticotropin-releasing factor (CRF) has also been shown to inhibit food intake ^{48,255}. Like BN, the physiological mechanism(s) of action underlying CRF's effect on feeding remains unknown. However, the ability of centrally administered CRF to suppress food intake in hypophysectomized rats suggest that these effects are independent of CRF's stimulation of ACTH secretion ²⁵⁵. Interestingly, adrenalectomy has been reported to markedly attenuate CRF-induced satiety, an effect principally attributable to disruptions of adrenal medullary catecholaminergic secretions ¹³¹. The failure of intravenous administration of CRF antiserum to alter CRF-induced catecholamine release suggests that this response mainly depends on direct action of CRF within the central nervous system ⁵⁴. Microinjection of CRF into distinct brain nuclei revealed that CRF potently inhibited food intake (by approximately 50% after 1 hr) following injection into the PVN, but not the globus pallidus, striatum or the hypothalamic lateral and ventromedial nuclei ¹⁷⁶. Krahn et al. also demonstrated partial reversal of CRF-induced satiety following central administration of the CRF antagonist, alpha-helical CRF₉₋₄₁ (α -CRF) ¹⁷⁴. While this antagonist failed to alter feeding in 24 h fasted rats, it blocked stress-induced anorexia. At present, evidence supporting the physiological role of CRF in the control of food intake remains limited, as most investigations have been pharmacological in nature.

Based on the similarities between the biological actions of BN and CRF, we have proposed that BN may mediate some of its effects by facilitating the release of CRF. This

view is supported by the observation that CRF antagonist, α -CRF, can block some of the effects of exogenous BN^{237,294}.

Thus, the major objective of the present study was to determine whether endogenous levels of BN and/or CRF change in a meal-dependent manner in various brain nuclei, in spontaneously feeding rats. In light of previously reported changes in BN-like immunoreactivity (BLI) in grossly dissected hypothalamic and hippocampal regions²³⁴, the present study focused on changes occurring in 15 distinct hypothalamic and extrahypothalamic brain nuclei. In further contrast to the earlier study, the current experiment focused on the first spontaneous meal of the dark phase, in non-deprived free feeding animals.

Material and methods

Animals

Male Sprague-Dawley rats, weighing 300-350 g ($n = 36$) and obtained from Charles River laboratories (St-Constant, Québec) were used in this experiment. The animals were housed individually in sound-attenuated chambers with a 12:12-h reversed light-dark cycle (lights off 9:15 h) and temperature maintained at 23-24° C with 60 % relative humidity. All animals had *ad libitum* access (through short tunnels 6.5 x 6.5 x 10 cm with grid floors) to powdered Purina rat chow contained in a bin placed atop an electronic balance (accurate to 0.1 g, Omnitech Instrument, OH). The balances were

connected to a microprocessor that measured cumulative food intake every minute. In order to minimize disturbance around testing time, food bins were replenished every day at 18:00 h and food intake electronically recorded from 18:00 h to 13:00 h daily.

Experimental protocol

All experimental sessions began 30 min prior to dark onset (at 8:45 h) and extended into the dark cycle until 12:00 h. Three separate groups of rats were included in the present study: 1) a *preprandial group* consisting of rats that had not consumed any food for a minimum period of 30 min preceding dark onset and at least 5 min after dark onset. The selected rats were usually resting and had not approached the food during the observation period. 2) A *prandial group* consisting of rats that had eaten at least 1 g of food during their first meal of the dark phase. These rats were removed from the cage during ingestion and sacrificed immediately. 3) Finally, a *postprandial group* consisting of rats sacrificed between 8 to 12 min after completion of their first meal. Like for the prandial condition, rats in the postprandial group had to have consumed at least 1 g of food during this first meal. During each experimental day, an equal number of animals from each of the three feeding conditions were sacrificed.

Tissue processing

Rats were sacrificed by decapitation and the brains quickly removed, fresh frozen on dry ice and stored at -80° C. Serial coronal sections were then obtained using a cryostat and a total of 16 discrete brain nuclei were micropunched using a modification of the method of Palkovits and Brownstein ²⁷⁷. The selected brain nuclei included six hypothalamic areas, namely the paraventricular nucleus (PVN), lateral (LH), anterior (AH), dorsomedial (DM) and ventromedial (VMH) hypothalamic nuclei and arcuate nucleus-median eminence (Arc-ME) and several extrahypothalamic nuclei including the frontal cortex (Fr), olfactory tubercle (Tu), nucleus accumbens (Acb), caudate putamen (CPu), dentate gyrus (DG), CA2 field of the Ammon's horn (CA2), central nucleus of the amygdala (Ce), posteromedial cortical amygdaloid nucleus (PMCo) and nucleus tractus solitarius (NTS). All nuclei were bilaterally micropunched (Micro Punch MP-600, ASI instruments, USA) except for the Arc-ME which was obtained using a single 1.0 mm micropunch. Additionally, in order to capture its anterior and posterior portions, this region was micropunched from 2 consecutive coronal sections which were analyzed individually. The punched tissue was boiled in tubes containing 200 µl of 0.02 M acetic acid solution then sonicated (Kontes Micro-ultrasonic Cell Disruptor, setting 5 - Kontes, Vineland, NJ). Portion of the homogenate (40 µl) was then withdrawn for protein assay and the remainder of the homogenate was centrifuged for 4 min at room temperature. The supernatant was extracted, freeze dried and kept at -80° C for subsequent peptide level determination, using radioimmunoassays. Protein assays were performed using

bicinchoninic acid (BCA) with a Protein Analysis Kit (Pierce Scientific, Brockville, Ontario) and a spectrophotometer (Brinkmann, PC 800 Colorimeter, USA).

Radioimmunoassay (RIA)

Tyr⁴-BN was iodinated using a modification of the technique of Salacinski et al.³¹⁷ and purified using a Sephadex column (Sephadex LH-20, 0.6 x 20 cm). The peak fractions were pooled, heated at 80° C in the presence of 1 M 1,4-Dithiothreitol (DTT), and further purified through Sep-Pak column separation. The specific activity of the peptide was calculated to be 2000 Ci/mmol, using the method of Chiang et al.⁶⁷.

Radiolabelled [¹²⁵I-Tyr⁴]-rCRF (Amersham Life Science, St. Catharines, Ont.) with specific activity of 2000 Ci/mmol, was used in these RIAs. Aliquots of the labelled peptides were stored at -25°C until use.

The solid phase RIA procedure used for the detection and quantification of CRF and BN was a modification of the procedure described by Maidment's original protocol²¹⁷ and carried out at 0-4° C, in duplicate. Briefly, the RIA procedure was conducted using well plates coated with protein A/G (a genetically engineered protein that combines IgG binding profiles of both Protein A and G; 0.1 µg/100 µl/well) at least 24 h before use. Following this incubation, the plates were rinsed with wash buffer (0.15 M phosphate buffered saline, pH 7.4) and, 50 µl of BN or CRF antibodies (final dilution 1:300,000) was

pipetted into the designated wells. The plates were then stored at 4° C overnight. Following this incubation, the antibody was discarded and the reconstituted lyophilised brain extracts and standards (in femtomolar units ranging from 0.5 to 512 for BN and in picograms from 0.31 to 625 for CRF) were pipetted into the designated wells. Twenty-four hours later, a fixed concentration of ^{125}I -labelled BN or CRF (approximately 5000 counts per min) was added to each well. Reference and blank samples were also run to determine total and non specific binding of the radiolabelled ligands. This final incubation with the radioligands lasted 24 h after which the content of the wells was dumped into the hot waste, the plates rinsed with wash buffer, blotted dry on paper and the wells broken apart into 12 x 75 mm culture tubes for gamma counting (Packard Cobra II Autogamma, model D5002).

The BN antibody used in the RIAs (α -BN2, kindly provided by Dr. T.W. Moody) recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with the mammalian peptide GRP (110%), BN (100%) and NMC (82%), but only weakly with NMB-10 or substance P ($\leq 0.1\%$)^{245,247}. We have shown in the past that the major source of BN-like immunoreactivity from the brain is attributable to GRP₁₈₋₂₇²³⁴. The specific anti-CRF serum (rC70 kindly provided by W. Vale) recognized CRF₁₋₄₁ and cross-reacted very poorly with other peptides³⁷¹.

Statistical Analysis

Results are presented as mean $\pm$ S.E.M. BN or CRF concentrations. Data were analyzed by two factor (brain region and feeding state) analysis of variance (ANOVA) followed by Tukey post-hoc comparisons. A probability of less than 5% was considered statistically significant.

RESULTS

Globally, the tissue levels of CRF were considerably higher than those of BN, in all brain nuclei examined. Furthermore, the changes associated with feeding state also appeared distinct for the two peptides.

In agreement with previously published immunohistochemical data^{247,281}, moderate to high levels of BN-like immunoreactivity were observed in amygdaloid and hypothalamic nuclei as well as in the NTS. Lower levels of BN were observed in the remaining extrahypothalamic nuclei. In general, the distribution pattern of CRF-like immunoreactivity was similar and proportional to that of BN, with moderate to high concentrations of CRF detected in the central and posteromedial cortical amygdaloid nuclei, NTS, and hypothalamic nuclei. However, in contrast to BN, very high concentrations of CRF were observed at the arcuate/median eminence nucleus, a finding concordant with immunohistochemical studies^{39,83} and the role of CRF as the principal

regulator of the HPA axis. Moderate CRF levels were present in the olfactory tubercle, a site where low concentrations of BN-like peptides were observed. Finally, low levels of CRF were found in all other sampled nuclei.

Rats sacrificed during the prandial condition had eaten an average of 1.45 ± 0.07 g and were still eating when sacrificed. The average amount consumed during the initial meal of the dark phase (postprandial condition) was 2.34 ± 0.21 g. Hypothalamic and extrahypothalamic concentrations of BN and CRF corresponding to the animal's feeding status are presented in Fig 1 and 2, respectively. As depicted in Fig 1 (upper panel), significant feeding-related alterations of BN-like immunoreactivity were observed at the PVN ($F_{2,28} = 8.79$; $P < 0.001$), Arc-ME 1 ($F_{2,27} = 5.58$; $P < 0.009$), Arc-ME 2 ($F_{2,20} = 4.77$; $P < 0.02$) and DM ($F_{2,27} = 8.09$; $P < 0.001$). In these hypothalamic nuclei, BN-like immunoreactivity is significantly elevated during ingestion as compared to the pre- and postprandial states. These fluctuations appear specific as no significant differences in the AH, LH and VMH was observed in relationship to feeding status. Outside the hypothalamus, meal-related alterations in BN concentrations were only observed at the Acb where concentrations of BN-like peptides were found to be significantly elevated before feeding as compared to during and after meal intake ($F_{2,25} = 4.56$; $P < 0.02$)(see Fig 1, lower panel).

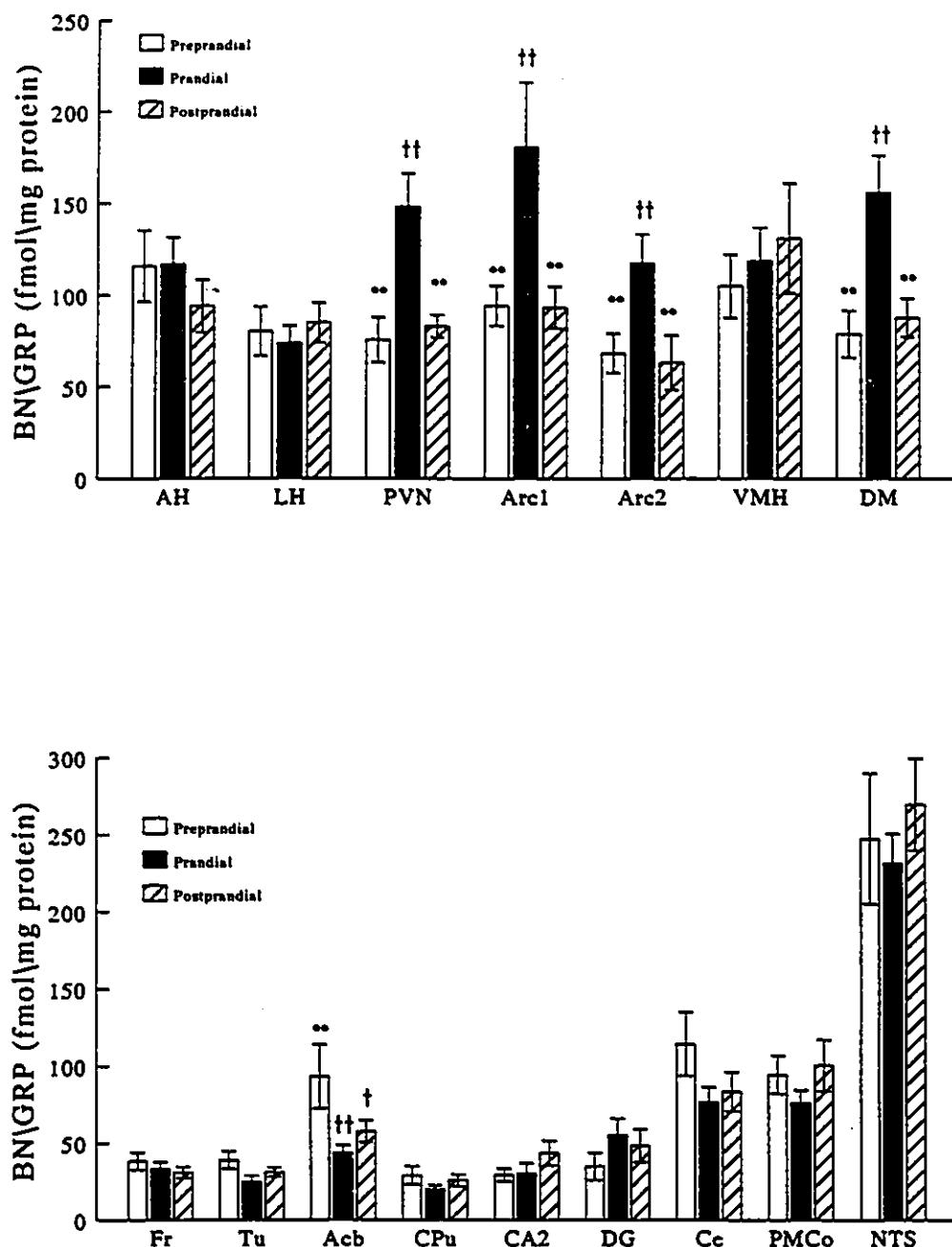


Fig.1 Prandial changes in BN/GRP concentrations associated with the first spontaneous meal of the dark phase. *Upper panel* - Hypothalamic BN-like immunoreactivity (mean $\pm$ S.E.M.) is displayed for the AH, anterior hypothalamus; LH, lateral hypothalamus; PVN, paraventricular nucleus; Arc, arcuate nucleus-median eminence; VMH, ventromedial hypothalamus; DM, dorsomedial hypothalamus. ^{††} Significant differences from Pre- and postprandial conditions (Tukey's test) at $P < 0.01$; ^{**} Significant differences from prandial condition, at $P < 0.01$. *Lower Panel* - Extrahypothalamic BN concentrations in the Fr, Frontal cortex; Tu, olfactory tubercles; Acb, nucleus accumbens; Cpu, caudate putamen; CA2, CA2 field of the Ammon's horn; DG, dentate gyrus; Ce, central nucleus of the amygdala; PMCo, posteromedial cortical amygdaloid nucleus; NTS, nucleus tractus solitarius. ^{††} Significant differences from preprandial condition at $P < 0.05$, $P < 0.01$, respectively. ^{**} Significantly different from prandial condition at $P < 0.01$.

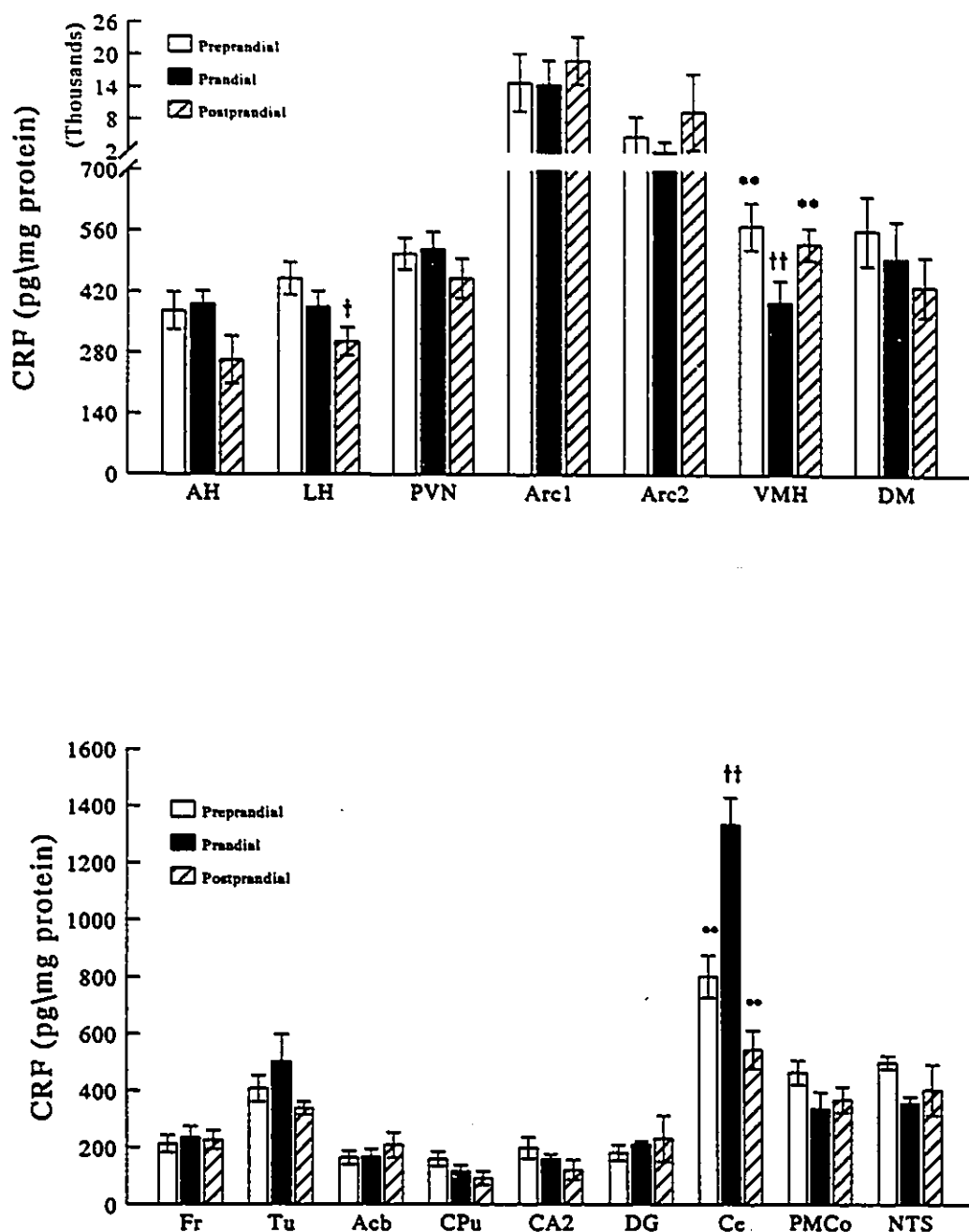


Fig.2 Prandial changes in CRF concentrations associated with the first spontaneous meal of the dark phase. *Upper and lower panels* - Hypothalamic and extrahypothalamic CRF-like immunoreactivity (mean $\pm$ S.E.M.), respectively. ** Significant differences from prandial condition (Tukey's test) at $P < 0.01$. [†], ^{††} Significant differences from preprandial condition, at $P < 0.05$ and $P < 0.01$, respectively. See the legend for Fig 1, for abbreviations of the brain nuclei.

As depicted in Fig. 2 (upper panel), hypothalamic alterations of CRF levels in relationship to feeding status were also site-specific and observed at the LH and VMH. At the LH, CRF concentrations were significantly elevated in the preprandial as compared to the postprandial state ($F_{2,18} = 4.20$; $P < 0.03$). In contrast, at the VMH the CRF levels were reduced during food intake, as compared to before and after spontaneous meal ingestion ($F_{2,16} = 3.82$; $P < 0.04$). An inverse pattern of CRF change was observed at the Ce, where a marked increase in CRF content was observed during food intake per se, while CRF content was diminished during the pre- or postprandial states of ingestion ($F_{2,18} = 25.8$; $P < 0.0001$) (see Fig. 2, lower panel).

DISCUSSION

In this study, we determined endogenous fluctuations in BN and CRF concentrations in relation to feeding status. Changes were characterized in fifteen distinct hypothalamic and extrahypothalamic nuclei of *ad libitum* fed rats and, represent peptidergic alterations associated with the first spontaneous meal of the dark phase. To our knowledge, this is the first study to report meal-induced fluctuations of CRF content and, the first demonstration of changes in BN concentrations in discrete brain nuclei in relationship to spontaneous feeding. Previously, feeding-related alterations in BN-like immunoreactivity have been demonstrated in response to food ingestion, however, these changes were restricted to the whole hypothalamus and hippocampus and conducted in food deprived animals²³⁴. In a recent study, we reported *in vivo* pre- and postprandial

increases in the release of BN-like peptides at the PVN accompanied by inhibition of the release of BN during spontaneous meal ingestion in the dark phase²⁹³. In the present study, feeding-related alterations in peptide content appeared distinct for BN and CRF and were observed in different brain nuclei. Significant meal-related fluctuation in BN immunoreactivity were observed in the hypothalamic PVN, DM and Arc-ME nuclei and at the Acb. In contrast, meal-related changes in CRF content were restricted to the LH, VMH and Ce. Furthermore, it is of interest to note that while the pattern of BN content fluctuation was similar in the three hypothalamic nuclei, feeding-related alterations in CRF content were different at LH and VMH.

Our findings revealed that concentrations of BN-like peptides at the hypothalamic PVN, DM and Arc-ME were reduced during the pre- and postprandial ingestive states as compared to levels during ingestion. While these findings support the physiological participation of BN-like peptides in the control of feeding, they do not reveal their mechanism(s) of action. Increased tissue levels could reflect increased synthesis and release of the peptide or may indicate reduced utilization (release) of the peptide. However, based on our recent observation of increased release of BN-like peptides at the PVN during the pre- and postprandial states of ingestion and an inhibition of BN release during the meal²⁹³, we can postulate that increased BN content at the PVN reflects reduced release of the peptide whereas, reduced peptide content reflects increased utilization of BN at that site. This profile may also suggest the existence of a time delay for the synthesis and replenishment of the tissue stores of BN-like peptides. It is thus

possible that at the other brain sites, decreased tissue levels of BN may also indicate increased release and vice-versa. However, as this has not been established empirically at the Arc-ME and DM, this remains a conjecture.

Meal-related fluctuations in the content of BN-like peptides at the Acb are characterized by increased preprandial levels accompanied by reduced BN content both during and after meal intake. While the exact participation of the Acb in feeding behavior has not clearly been established, its role in behavioral reinforcement is well documented. For example, pharmacological manipulations that increase dopamine release at the Acb have been shown to facilitate locomotion²⁴³, self-stimulation^{47,70} and feeding^{146,388}. Moreover, feeding and operant responding for food reward have been shown to stimulate dopamine release at the Acb¹⁴⁰. Interestingly, microinjection of BN into the Acb is also accompanied by accumbal DA release (unpublished observation). Thus, the attenuated BN concentrations during feeding may correspond to an increase in the release of BN at that site during feeding associated with the reinforcing properties of food. Postprandially, reduced BN concentrations may represent an increase in the release of accumbal BN-like peptides attributable to the increased locomotor and grooming activity observed following feeding. This hypothesis is supported by findings demonstrating enhanced locomotor activity following administration of BN in rats^{162,180}. Interestingly, like BN, meal-induced increase in dopamine release at the Acb has also been shown to outlast the duration of the feeding episode¹⁴¹. As for the increased BN content observed at the Acb before feeding, it may represent low levels of BN release at this feeding state which is characterized by

low activity levels and absence of feeding. Alternative explanations such as the presence of circadian rhythms associated with the onset of the dark phase could be proposed for the observed peptidergic fluctuations. Indeed, transient increase in neurotransmitter release has previously been demonstrated during the hour following dark onset ³⁴⁴ which correlated with the burst of feeding that rats exhibited at that time. Microinjection of BN in the suprachiasmatic nucleus (SCN) has been shown to modulate the firing pattern of SCN in vitro ^{291,359}. However, this SCN-dependent or light associated circadian rhythm has been shown independent of the feeding-associated circadian rhythm which is detectable even after the destruction of the SCN and, therefore referred to as SCN-independent rhythm ¹⁴⁹.

In contrast to BN, distinct patterns of changes in CRF content were observed in relationship to the animal's feeding status. Due to the absence of studies demonstrating release of CRF in the context of food ingestion, the interpretation of these results remains tentative. For example, if we suppose that similar physiological mechanisms may be underlying accumulation of tissue levels of BN and CRF, then meal-related alterations of CRF content at the Ce may represent an increase in the release of CRF both before and after meal intake accompanied by a reduction in the release of CRF during feeding. Interestingly, increase in the release of CRF at the amygdala has recently been demonstrated in rats exposed to aversive stimuli ²⁸⁷. Thus, fluctuations in CRF levels observed at the Ce might similarly be associated with the affective and/or emotional aspects of the feeding process. In this context, the increased CRF content observed at

the Ce during meal intake perhaps reflects an inhibition of CRF release, which might correspond to the positive reinforcement and/or emotions associated with food intake. Conversely, the decreased peptide content observed both pre- and postprandially at that site may correspond to increased release of CRF in feeding states where the animal is satiated and does not perceive food as reinforcing.

At the LH, CRF levels gradually decreased from the pre- to the postprandial states with postprandial levels being significantly suppressed as compared to preprandial values. Again, if we interpret changes in CRF content at the LH based on observations with BN release at the PVN, the pattern of CRF changes at the LH may correspond to gradually increasing CRF release at that brain site over the course of the different ingestive states. This gradual progression in CRF release is consistent with the role of CRF as a satiety peptide. Indeed, enhanced CRF levels observed postprandially might serve to maintain a satiety-like state between meals. Similarly, low CRF release at the preprandial phase might be linked to state of hunger and/or anticipation of the ensuing meal. Such a physiological profile has been shown to characterize the endogenous release of CCK from primate lateral hypothalamus in response to intragastric meal infusion³²². However, from such meal-related fluctuations, one would assume that microinjection of CRF into the LH would induce a satiety-like state. Surprisingly, however, exogenous administration of CRF at the LH failed to significantly alter feeding in 24 h food deprived rats¹⁷⁶. It is possible that due to extended food deprivation, effects of CRF may be small to negligible. In order to reconcile physiological and pharmacological findings, an alternative

explanation might also be proposed based on the role of the LH as the "hunger center". In this context, the observed elevation of CRF concentration at the preprandial state may correspond to an increased release of CRF, reflecting the animal's hunger and/or anticipation of the coming meal. Then, as the animal initiates a meal and gradually becomes satiated, the release of CRF diminishes to parallel the animal's decreasing hunger.

Finally, meal-related fluctuations in CRF levels at the VMH were characterized by increased CRF content at both pre- and postprandial ingestive states as compared to levels during ingestion. Concordant with the participation of this nucleus in feeding regulation³⁸⁷ and a proposed role for CRF as a satiety peptide, the increased CRF concentrations observed before and after meal intake may represent increased release of CRF before and after spontaneous meal intake while inhibition of CRF release occurs during feeding. Interestingly, while such fluctuations in the endogenous release of CRF would parallel the ones observed with the release of BN at the PVN, feeding-related changes in the concentrations of these peptides take opposite directions. Such findings may be attributable to faster turnover of CRF as compared to that of BN-like peptides. Indeed, transient endogenous changes in CRF occur very rapidly following stress. Thus, it is possible that similar to the observations following stress, very rapid changes may also occur during feeding. This could explain the differential pattern of endogenous fluctuations observed with CRF content at the VMH versus meal-induced fluctuations in BN content at the PVN, Arc-ME and DM sites.

The absence of significant alterations at the PVN and Arc-ME in relationship to feeding status might appear surprising. Indeed, high concentrations of the CRF immunoreactivity and receptors sites are present in both these nuclei and, the contribution of these brain sites in feeding processes is well documented^{197,199,387}. Moreover, CRF potently induces satiety when microinjected at the PVN in 24 h food deprived rats¹⁷⁶. The role of these nuclei in controlling CRF endocrine activities has also been demonstrated³⁸. However, as mentioned earlier CRF-induced satiety appear independent of its ability to release ACTH²⁵⁵. Nonetheless, the participation of these nuclei in the control of endocrine secretions together with reported fluctuations in CRF content at these brain loci during stress-related situations, may suggest their participation in stress-induced anorexia. Interestingly, while stress-induced anorexia is reversed by alpha-helical CRF₉₋₄₁, this antagonist failed to alter feeding when administered in non stressed fasted rats¹⁷⁴. These differential findings suggest that the ability of alpha-helical CRF₉₋₄₁ to enhance feeding on its own may depend on the state of the CRF system in the rats tested. Similarly, it is also possible that some of the structures physiologically participating in CRF regulation of feeding might operate under normal “spontaneously occurring” feeding conditions while other nuclei might actively participate to stress-induced satiety.

In conclusion, the present study demonstrated site-specific alterations in endogenous BN and CRF concentrations in relationship to feeding status, in non deprived animals. The reported findings suggest the existence of distinct feeding-related fluctuations for the two peptides. Fluctuations in BN contents have been interpreted in

relationship to dynamic fluctuations observed in the release of this peptide at the PVN. However, at present, we can only postulate on the exact physiological mechanisms underlying meal-related fluctuations in CRF content. Despite these limitations, the data presented here clearly indicate locus-specific endogenous fluctuations in BN and CRF concentrations over the course of the first spontaneous meal of the dark phase.

Chapter Three

Push-pull perfusion reveals meal-dependent changes in the release of Bombesin-like peptides in the rat paraventricular nucleus.

The results from the previous set of experiments revealed that endogenous tissue levels of BN-like peptides changed in relationship to the feeding status of the animal, at distinct hypothalamic sites. During ingestion, the levels of BN-like peptides were elevated at Arc, PVN and DM. In order to determine whether the observed prandial elevation of the peptide levels reflected actual increase in the turnover rate of the peptide or reduced release or utilization of the peptide, we set out to directly measure the dynamics of peptide release at the PVN, during various phase of the ingestive cycle.

Abstract

Bombesin (BN)-like peptides have been implicated in the regulation of ingestive behavior. The main objective of the present study was to monitor the dynamics of central BN-like peptide release in relationship to spontaneous meal ingestion and termination. Peptide level fluctuations were determined using *in vivo* push-pull perfusion of the hypothalamic paraventricular nucleus (PVN) and off PVN sites, combined with *ex vivo* radioimmunoassay. Analysis across all meals revealed significant differences between preprandial, prandial and postprandial extracellular BN-like immunoreactivity (BLI) at the anterior aspect of the PVN, with about a 3-fold diminution during a meal as compared to before or after a meal. Meal-related fluctuations were not detected at more distal hypothalamic sites or at sites within the caudate nucleus. When the analysis was restricted exclusively to the first meal after dark onset, a similar pattern of change in the interstitial levels of PVN BLI was generally observed; levels being higher preprandially as compared to the prandially (albeit by a smaller magnitude), and the termination of the first meal being accompanied by a robust (about 3-fold) increase in BLI. This is the first demonstration of site specific *in vivo* release of BN-like peptides in relation to feeding status and it further supports the physiological role of this family of peptides in the regulation of food intake.

Introduction

Central and systemic administration of the tetradecapeptide Bombesin (BN) and BN-like peptides have been shown to induce a satiety-like state in many species, including man ^{21,133,186,264,349,393}. A number of studies also suggest that these peptides may play a physiological role in the regulation of food intake ^{123,179,236}. However, the mechanism through which BN elicits its satiety effect remains largely unknown. Central mechanisms are required for complete expression of BN satiety response as neural disconnection of the afferent fibers from the gastrointestinal tract to the brain by subdiaphragmatic vagal deafferentation, cordotomy and dorsal rhizotomy ³⁵⁰, as well as central pre-treatment with bombesin antiserum ²³⁵ significantly attenuated the satiety effect of systemically injected bombesin. Furthermore, central blockade of BN/GRP receptors has been shown to enhance feeding in rats ^{115,236} as well as to attenuate the effects of central or systemic administration of BN ^{236,259}.

Recently, data from our laboratory revealed increased levels of BN-like immunoreactivity (BLI) in postmortem rat hypothalamus, following feeding ^{168,233}. These results implied a physiological role of this family of peptides in the mediation of satiety. However, alterations in the tissue levels of BLI are difficult to interpret since increased tissue peptide levels could be due to many factors including increased release of the peptide accompanied by increased synthesis, or decreased release accompanied by increased or unaltered synthesis rate.

Several studies have identified the paraventricular nucleus of the hypothalamus (PVN) as being one of the more sensitive hypothalamic sites associated with peptidergic or aminergic modulation of food ingestion^{186,197,199,387}. Moreover, hypothalamic lesions that included the PVN were found to be particularly effective in producing hyperphagia in the rat¹²⁹. Additionally, biochemical and histochemical localization of BN-like peptides within discrete regions of the rat brain have revealed a rich innervation of BN/GRP terminals as well as considerable levels of BLI at this brain site^{68,77,282}. Thus, in the present study, we adopted push-pull perfusion technique to monitor the dynamics of *in vivo* BN-like peptide release from the PVN in response to spontaneous meal ingestion and termination, in freely moving *ad libitum* fed animals.

Materials and methods

Animals

Male Sprague-Dawley rats (300-350g) obtained from Charles River Laboratories (St-Constant, Québec) were used in all experiments. The animals were housed individually in a temperature (23-24°C), humidity (60%) and light (12 h light/dark cycle; lights on at 7:00 h) controlled environment. All animals had *ad libitum* access to food (powdered Purina rat chow) and water.

Surgery

Rats were anesthetized with pentobarbital (65 mg/kg; i.p.) and placed in a stereotaxic instrument with the skull level. Stainless steel, 22 gauge guide cannulae aimed at the paraventricular nucleus of the hypothalamus (1.8 mm posterior to bregma, 0.4 mm lateral to the midline and 8.1 mm ventral to the skull surface) or at the caudate putamen (0.2 mm posterior to bregma, 2.5 mm lateral to the midline and 5.5 mm ventral to the skull surface) were implanted according to coordinates from Paxinos & Watson²⁸⁴. The cannulae were anchored by three stainless steel screws and acrylic dental cement, and plugged by a stainless steel wire stylet. The rats were allowed one week of postsurgical recovery and acclimatation to reversed 12 h light-dark cycle (dark onset at 11:00 h).

Experimental protocol

All experiments were conducted mainly during the dark cycle between 10:00 and 15:00 h. On the day of experiment, the stylet within the guide cannula was replaced with a 29 ga stainless steel inner (or push) cannula, that protruded 0.3 mm beyond the guide cannula tip. The perfusion assembly was connected by polyethylene tubing (PE20 and PE50) to two 2.5 ml Teflon-tipped Hamilton gas-tight syringes mounted on the infusion-withdrawal decks of a Harvard pump (Harvard Apparatus model 954, Millis, Mass.), via a two-channel swivel (Instech Laboratories, Horsham, PA). This configuration prevented tangling of the tubing and insured mobility for the animal. Ringer's solution [Na^+ 145 mM,

K⁺ 2.7 mM, Mg⁺⁺ 1.0 mM, Ca⁺⁺ 1.2 mM, Cl⁻ 150.0 mM; Moghaddam & Bunney (1989)²⁴⁴] was infused at a rate of 5.0 µl/min and pooled samples (150 µl) harvested every 30 min. Individual samples were collected in a tube containing 10 µl of 0.2 M acetic acid. All samples were freeze dried using a lyophilizer (Labconco 4451F) for subsequent peptide level analysis using radioimmunoassay (RIA). The amount of powdered Purina rat chow consumed was recorded every 30 min using a digital scale, to match with the corresponding samples collected. Each rat was tested for two consecutive days. As indicated, push-pull perfusates were collected every 30 min. For each meal, samples corresponding to the interval(s) during which some food was consumed (0.5 to 2.2 g) was (were) considered to represent the prandial state, whereas the sample immediately preceding or following the meal were operationally defined as representing preprandial and postprandial states, respectively. On rare occasions (n = 2), where a single 30 min interval separated two meals, this sample was considered as postprandial for the preceding meal and as preprandial for the subsequent meal.

Histology

At the end of the experiment, the rats received an overdose of pentobarbital and were intracardially perfused with saline followed by 10% formaldehyde solution. The fixed brains were sectioned and stained with thionine for the verification of the perfusion sites, using the atlas of Paxinos and Watson²⁸⁴ and Swanson³⁵⁵.

Radioimmunoassay (RIA)

Tyr⁴-BN was iodinated using a modification of the technique of Salacinski et al.³¹⁷ and purified using a Sephadex column (Sephadex LH-20, 0.6 X 20 cm). The peak fractions were pooled, heated at 80°C in the presence of 1 M 1,4-Dithiothreitol (DTT), and further purified through Sep-Pak column separation. The specific activity of the peptide was calculated to be approximately 2000 Ci/mmol, using the method of Chiang et al.⁶⁷. The aliquots were stored at -25° C. On the day of the assay, the freeze dried brain perfusates were reconstituted with RIA buffer (0.05 nM phosphate buffered saline, pH 7.4, with 0.25% Bovine Serum Albumin). The RIA was carried out at 0-4° C, in duplicate, as described previously¹⁶⁸. Briefly, to the sample and/or standard tubes, 200 µl of BN antibody (final dilution of 1:300,000) was added, followed 1 hr later by 100 µl (approx. 5000 cpm) of ¹²⁵I-Tyr⁴-BN. The ligand-antibody complex was then isolated 16 hr later, using 100 µl of Amerlex-M donkey anti-rabbit antibody coated onto magnetizable polymer beads (Amersham Canada, St. Catherines, Ontario). The precipitates were counted in a gamma counter (Packard Cobra II Autogamma, model D5002).

The antibody used in the RIAs recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with the mammalian peptide GRP (110%), BN (100%) and NMC (82%), but not with NMB-10 (<0.1%) or substance P (0.1%)^{245,247}.

Extraction and Chromatography of hypothalamic extracts

The hypothalami of rats ($n = 3$) were pooled, and subjected to homogenization and sonication (Kontes Micro-ultrasonic Cell Disrupter, setting 5 - Kontes, Vineland, NJ). The homogenate was centrifuged at $10,000 \times g$ for 10 min. and the supernatant frozen at $-70^{\circ}C$ and lyophilized. The desiccated samples were then stored at $-70^{\circ}C$ until analysis. Prior to centrifugation, $100 \mu l$ of each homogenate was collected and assayed for protein content using bicinchonimic acid with a BCA Protein Analysis Kit (Pierce Scientific) and a spectrophotometer (Cary 2200 - Varian, Springvale, Australia).

High-pressure liquid chromatography (HPLC) was then used to resolve the components of acid-extracted rat hypothalami and to determine the specificity of the first antibody to mammalian BN-like peptides¹⁶⁸. Hypothalamic samples were loaded on a primed Sep-Pak cartridges (Waters Associates, Milford, MA), rinsed with 0.1% trifluoroacetic acid (TFA), and eluted with 40% acetonitrile (ACN) in 0.1% TFA. The samples were then lyophilized and reconstituted in 0.1% TFA before reverse phase HPLC analysis using a μ -Bondapak C_{18} column (Waters Associates, Milford, MA). The HPLC column was equilibrated with 18% ACN in 0.1% TFA at 1 ml/min. Once injected, the material was eluted with a linear gradient of ACN, starting at 10 min to reach 30% ACN at 30 min. This concentration of ACN was maintained up to 50 min of elution²⁰⁷. BLI was measured in collected HPLC fractions using the RIA procedure. Then, $50 \mu l$ of five synthetic standards (amphibian BN, porcine GRP₁₋₂₇ (GRP), porcine GRP₁₈₋₂₇

[Neuromedin C (NMC)], porcine NMB₁₋₃₂ (NMB-32), and porcine NMB₂₃₋₃₂ (NMB-10) (Peninsula, CA) were individually run through the HPLC in order to obtain their particular elution profile using identical conditions as for the hypothalamic extracts. The elution time of the peptides peaks was monitored using a 484 absorbance detector (Waters) set at 214 nm.

Statistical analysis

Relevant data are expressed as mean $\pm$ S.E.M. A two-way analysis of variance (ANOVA) repeated over the factor time was performed, followed by Tukey post-hoc comparisons. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The perfusates from the PVN contained measurable levels of BN-like peptides. The majority of rats ingested two or three discrete meals during the 4 hr testing period. Thus some animals yielded more than one set of data per experiment. When the results were analyzed in terms of changes in BLI associated with all meals ingested ($n = 15$, obtained from 8 rats), significant differences were observed across feeding states, $F_{2,14} = 10.9658$, $P = 0.0003$. Tukey post-hoc comparisons revealed a significant postprandial increase ($n = 14$) in BN-like peptide release ($P < 0.01$) as well as similar elevation of BN-like peptide levels in the preprandial state ($n = 13$, $P < 0.01$). The enhanced release

associated with pre and postprandial conditions were significantly different from BN-like peptide release recorded during food intake *per se* ($n = 15$), a period during which the interstitial peptide levels were markedly diminished (Tukey, $p < 0.01$)(Fig. 1). When the data from the first meal were excluded from the analysis, a similar trend in peptide alterations was observed ($F_{2,5} = 10.68$, $p = 0.0033$; data not shown). No significant relationship was found between meal size and the levels of BN-like peptides observed either before, during or after spontaneous ingestion of a meal.

Analysis was also performed on BLI associated with the first meal ($n = 10$) of the testing session and revealed a significant effect of the feeding state, $F_{2,9} = 10.1496$, $P = 0.0011$. Tukey post-hoc comparisons indicated significant postprandial ($n = 10$) increase in BN-like peptide release in the PVN after initial food ingestion, when compared to values observed either during food intake ($n = 10$, $P < 0.01$) or prior to food intake ($n = 9$, $P < 0.05$)(Fig.2).

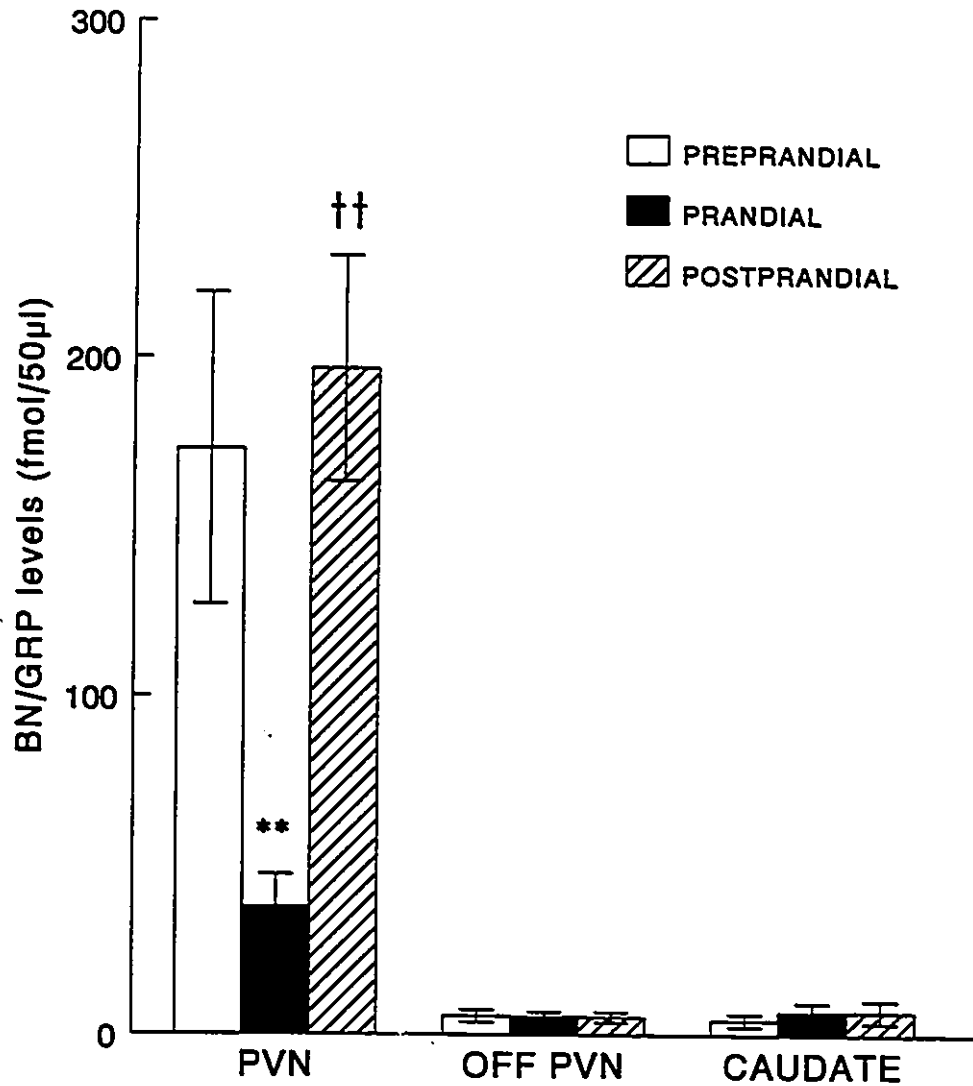


Fig.1 Prandial changes in BN/GRP release when all spontaneous meals of the testing session are considered. Extracellular BN-like immunoreactivity (means $\pm$ SE) is displayed for rats with implants at the anterior parvocellular portion of the PVN (referred as PVN; $n = 8$), from rats with more posterior or extra PVN implants (referred in the figure as OFF PVN; $n = 5$), as well as from rats with implantation sites within the caudate nucleus (referred to as Caudate; $n = 5$). **Significantly different from pre- and postprandial release ($p < 0.01$). †† Significantly different from prandial release ($p < 0.01$).

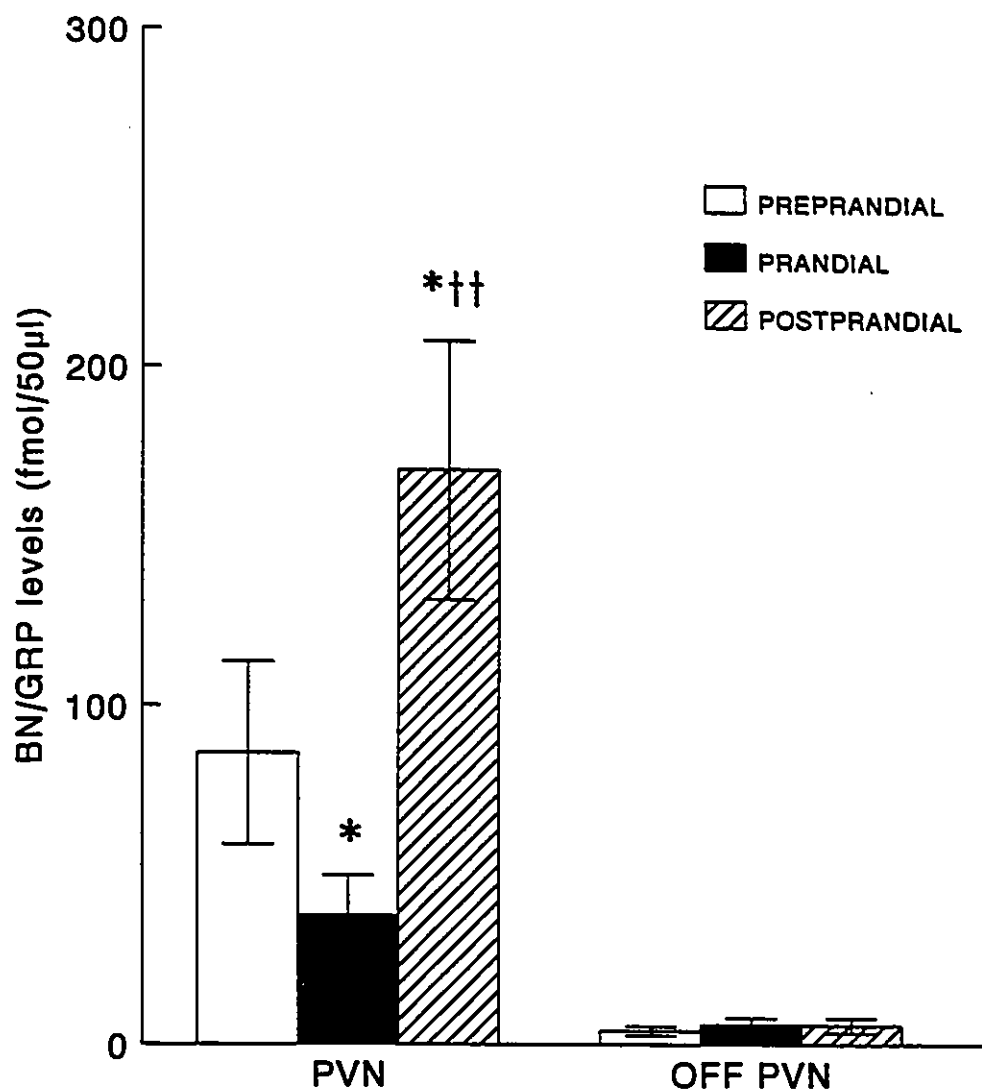


Fig 2 Prandial changes in BN/GRP release associated with the first spontaneous meal of the testing session. See Fig. 1 legend for details. *Significantly different from preprandial release ($p < 0.05$). †† Significantly different from prandial release ($p < 0.01$).

Representative profiles of BN/GRP release from an individual rat on days where meal patterns were quite different are presented in Fig. 3. Consistent with the main statistical analyses, the top panel reveals a marked decrease in interstitial BN-like peptide levels during food ingestion as compared to conditions where no food was ingested. Of interest is the profile of this same animal on the second testing day when the rat ate almost continuously over the entire 4 h sampling period (Fig.3, bottom panel). Typically, when rats exhibited similar eating patterns, low levels of interstitial BN-like peptide levels were recorded at all times even, as in the present example, even when the animal paused occasionally between two periods of ingestion. Hence, higher levels were associated with the cessation or suppression of food intake.

In terms of cannula localization, animals that demonstrated higher BLI and definite meal related peptidergic changes, were the rats with cannulae positioned at the anterior parvocellular portion of the PVN, between -1.3 mm and -1.7 mm posterior to bregma ($n = 8$). When the perfusion sites were further posterior (in the medial parvocellular portion of the PVN; A.P -1.8 and -2.0 mm) or distal to the parvocellular portion of the PVN ($n = 5$), statistical analysis of BLI did not reveal a meal-related release profile (Fig. 4). However, the values obtained from these sites were usually at the lower limit of detection and hard to quantify reliably. Data obtained from an extrahypothalamic site, namely the caudate putamen ($n = 5$), also failed to reveal meal related changes in BLI (see Fig.1).

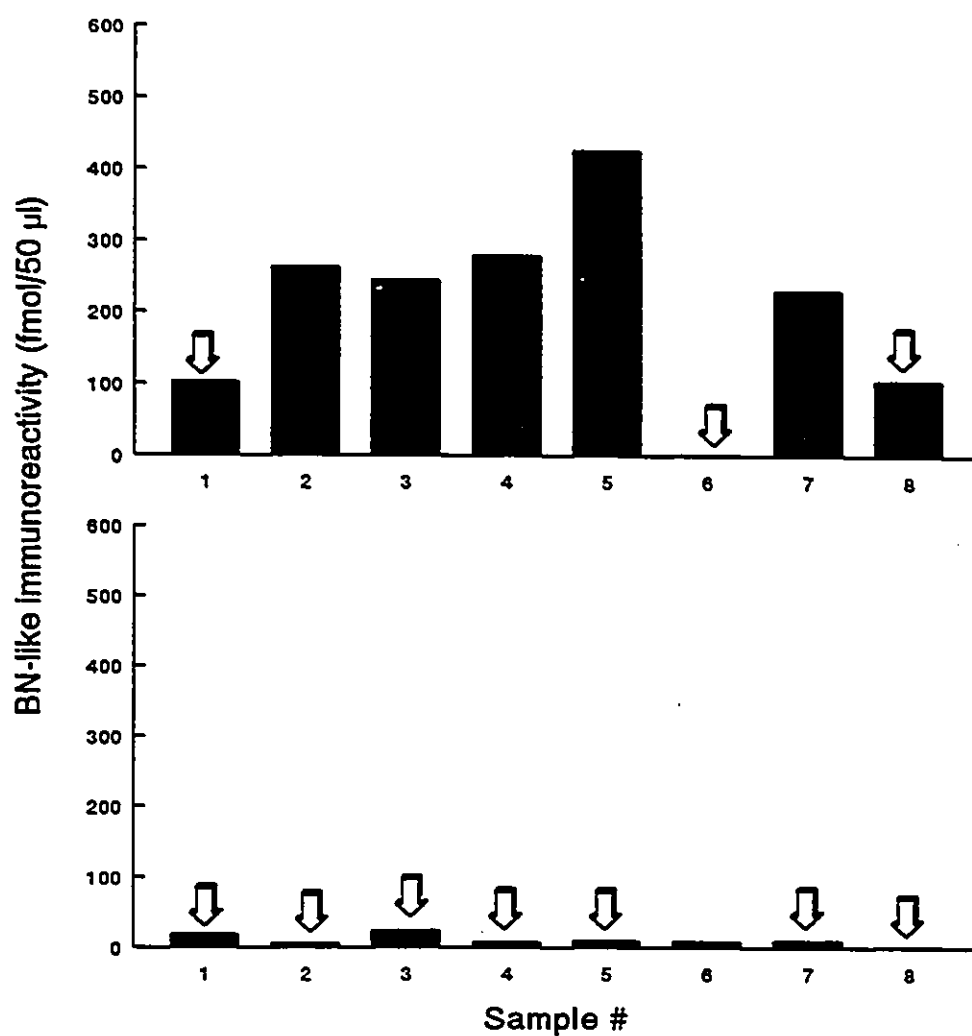


Fig. 3 Representative BN/GRP release profiles from an individual rat on days where meal patterns were quite different. The top figure panel displays BN-like peptide release profile over the whole testing period with arrows indicating samples which coincided with meal ingestion. The bottom panel depicts BN/GRP release profile when continuous food ingestion was recorded over the sampling period.

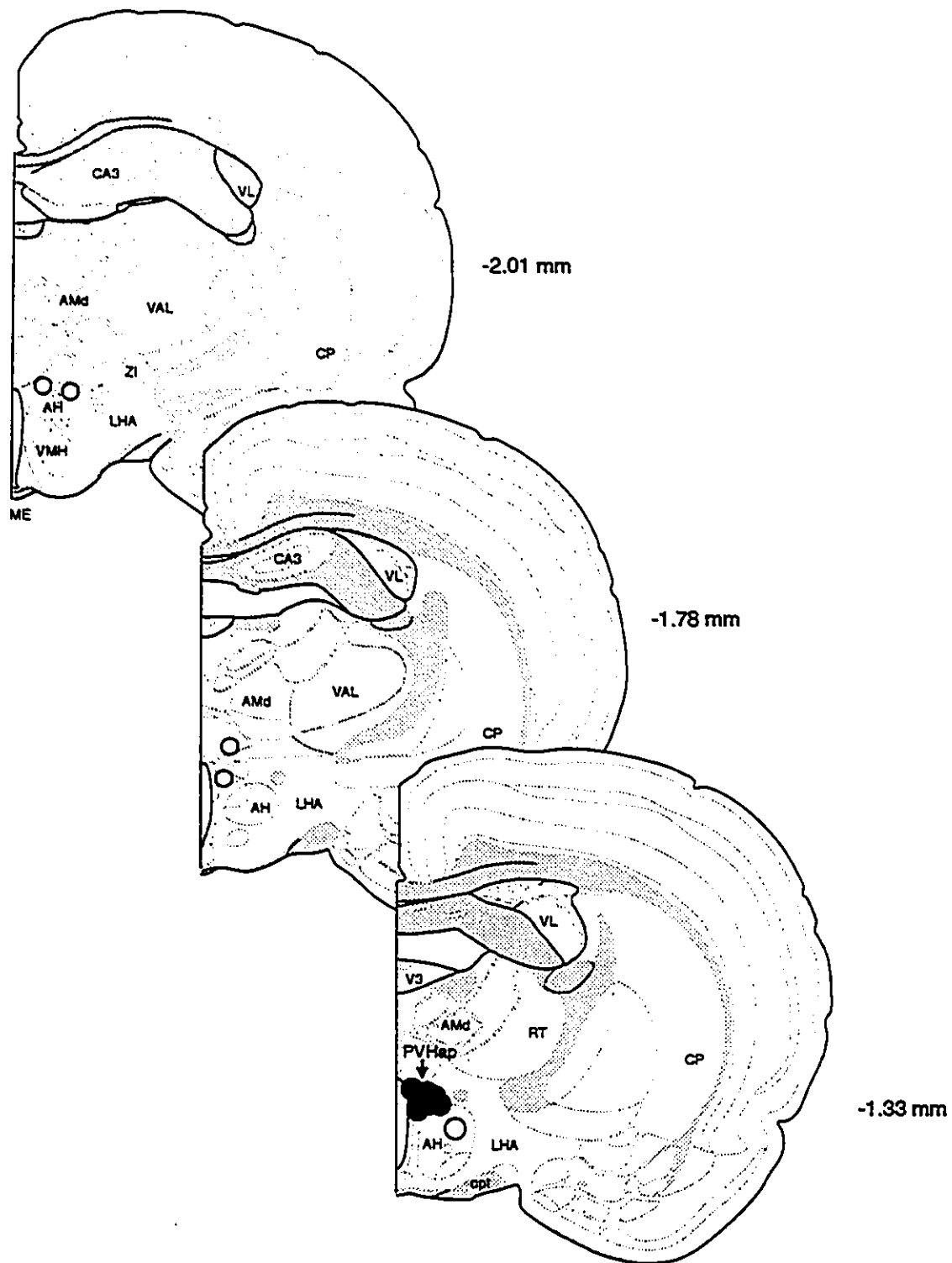


Fig. 4 Schematic diagrams of coronal sections of the rat brain based on Swanson's atlas³⁵⁵ showing hypothalamic PVN active (closed circles) versus inactive (open circles) sites at which push-pull perfusions were undertaken.

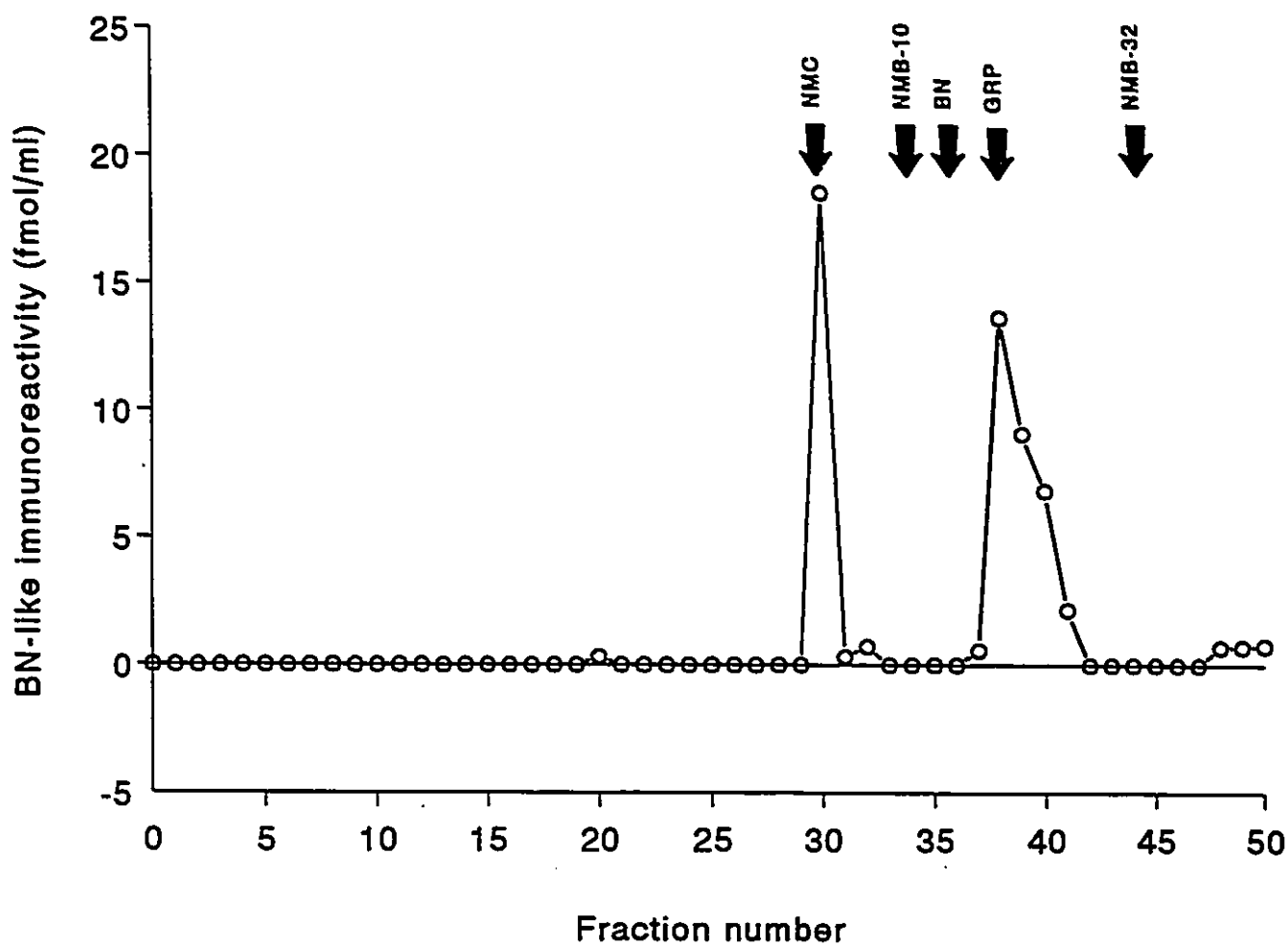


Fig.5 HPLC elution of hypothalamic BN-like immunoreactivity. The arrows indicate the retention times of authentic porcine GRP₁₋₂₇ (GRP), GRP₁₈₋₂₇ (NMC), NMB₁₋₃₂ (NMB-32), NMB₂₃₋₃₂ (NMB-10), and amphibian BN.

HPLC of NMC, NMB₂₃₋₃₂, BN, GRP, and NMB₁₋₃₂ standards revealed peak retention times corresponding to fractions 30, 35, 36, 39 and 45, respectively. The hypothalamic extracts when resolved using HPLC, yielded two peaks of BLI that eluted at fractions 30 and 38 respectively (Fig. 5) and revealed that the BLI detected using our RIA reflect changes in GRP-like peptides, specifically the GRP₁₈₋₂₇ fragment.

DISCUSSION

The working hypothesis behind this study was that if BN-like peptides are involved in the regulation of ingestive behavior then their release or availability should change in relation to the ingestive state at relevant central site(s). Thus we examined changes in the central *in vivo* release of BN-like peptides before, during and after spontaneous meals. The present data revealed that food intake was associated with a marked suppression of the extracellular levels of BN-like peptides at the PVN. Conversely, the release of BN-like peptides was significantly elevated immediately preceding and following food ingestion. These peptidergic fluctuations appeared to be site specific as they were observed at the anterior parvocellular portion of the PVN but not at more posterior PVN implants nor at extrahypothalamic perfusion sites within the caudate putamen. These results support an important physiological role for BN-like peptides in specific sites within the PVN and, suggest the possibility that more sparsely innervated PVN sites or extrahypothalamic sites, such as the caudate putamen, do not participate in this physiological response. Interestingly, Chronwall et al.⁶⁸ characterized a high density of BN-like fibers and

terminals in the anterior portion of the PVN, which were not present in more caudal hypothalamic sections. In addition, although BN-satiety effects have been reported to be independent of the central catecholaminergic system²⁵⁹, Leibowitz¹⁹⁷ indicated that the rostral aspect of the PVN was the most effective site for stimulating ingestive behavior with noradrenergic activation in the rat.

The currently known mammalian forms of BN-like peptides include gastrin-releasing peptide (GRP) and neuromedin B. In the present study, the antibody used in the RIAs recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with the mammalian peptide GRP (110%), BN (100%) and NMC (82%), but not with NMB-10 (<0.1%) or substance P (0.1%)^{245,247}. Due to the fact that relatively small amounts of the peptide(s) are released into the interstitial space, it was not possible to analyze the push-pull perfusates using HPLC methods. However, we did characterize the relative amounts of the various BN-like peptides in dissected hypothalamus. Peptides from hypothalamic extracts when resolved using HPLC revealed that the BLI detected by our RIA may mainly reflect changes in GRP-like peptides, specifically the COOH-terminal decapeptide of mammalian GRP, namely GRP₁₈₋₂₇ or NMC. Based on this observation, one can assume that the observed peptide changes in the perfusates also reflect changes in GRP-like peptides. This finding is concordant with mRNA studies indicating that GRP but not NMB-like peptides are primarily expressed in the PVN^{26,380}. Additionally, a clear signal for GRP receptor mRNA was observed in this brain structure while a very weak mRNA signal for NMB receptors was detected at the PVN, a finding which corroborated

the observed peptidergic distribution²⁶. Thus the meal related fluctuations in BLI observed at the PVN may be attributable to changes in GRP₁₈₋₂₇.

No clear relationship between extracellular peptide levels and the meal size was apparent in the present study. Interestingly, however, the preprandial peptide levels were found to be lower prior to the initial meal as compared to subsequent meals. The physiological relevance of this finding is not clear. One possible explanation may be that the release of BN-like peptides might have already been on its way down, in preparation for or anticipation of the rapidly impending first meal following dark onset. An alternative explanation for the lower preprandial BN-like peptide values associated with the initial meal of the testing session could be that these peptidergic fluctuations are influenced by circadian factors. Indeed, the 30 min. interval immediately preceding the first meal usually occurred in the light phase and was followed by dark onset and the meal ingestion. It could be proposed that circadian rhythms associated with nocturnal onset influenced the preprandial BN-like peptides release of this initial meal. Although the present study did not monitor circadian peptidergic fluctuations, a cursory examination of the daily fluctuations failed to reveal any consistent pattern. Microinjection of BN in the suprachiasmatic nucleus (SCN) has been demonstrated to modulate the firing pattern of SCN neurons *in vitro*^{291,359}. However, this SCN-dependent or light-associated circadian rhythm has been shown to be independent of the feeding-associated circadian rhythm which is detectable even after the destruction of the SCN and, therefore referred as SCN-independent rhythm¹⁴⁹. Diurnal variations in feeding response following

norepinephrine ^{36,363}, neuropeptide Y ³⁶³ and galanin ³⁶³ have been reported following injections into the PVN, suggesting that differential mechanisms might be involved in feeding activity observed at dark onset versus later during the dark cycle. However, in the case of BN-like peptides, since only the magnitude but not the pattern of release changed between the first and successive meals, the time after dark onset may not be as critical a factor for these BN/GRP effects.

The present data is consistent with the observed pharmacological effects of exogenously administered BN, principally the satiety-like effect observed following central or systemic administration of BN or related peptides. These observations are also concordant with those obtained from postmortem regional analysis demonstrating that tissue BLI changes in relation to food ingestion ¹⁶⁸. Thus, endogenously released BN-like peptide(s) may represent a physiological signal responsible for the onset and/or maintenance of satiety.

Notwithstanding the fact that increased BN release paralleled the beginning and termination of a spontaneous meal while an inhibition of release was observed during food consumption *per se*, it remains possible that other metabolic or behavioral events related to the ingestive process could participate in some of the observed peptidergic fluctuations. Indeed, food ingestion is associated with elevated blood glucose, as is central BN administration, via its activation of the autonomic nervous system ^{51,53,153}. Central administration of BN alters locomotor activity ²⁸⁶ and enhanced locomotor activity is

noted before and after spontaneous meal. Additionally, we cannot exclude the possibility that the fluctuations in the release of BN-like peptides observed in relation to spontaneous meal ingestion may be an epiphenomenon related to systems regulating corticosterone ¹³⁶, insulin and glucagon secretion ^{57,391} or gastrointestinal peptidergic secretion such as CCK ¹⁵⁸. Conversely, the activation or inhibition of PVN BN-like peptides release observed could as well play a role in the regulation of pancreatic or gastrointestinal expression of these meal related signals. Technical progress leading to faster sampling rate (time resolution < 30 min.) may provide some of the answers. At present, the precise mechanism(s) that bring about the changes in the BLI remain obscure.

Although each episode of food ingestion was consistently associated with a significant diminution in peptide release, the converse was not always true. That is on rare occasions, a drop in peptide level was not accompanied by food ingestion. Termination of a meal was also consistently associated with significant elevation in the PVN BLI, however, meal ingestion was never observed in the presence of elevated peptide levels. These observations suggest that increased availability of BN/GRP-like peptides at the PVN may mediate a powerful satiation signal associated with preabsorptive fullness. Typically, the intermeal interval was also associated with elevated peptide levels indicating the involvement of this family of peptides in the maintenance of more persistent postabsorptive satiety. However, in the later maintenance phase of satiety, the occasional fluctuations in peptide levels may not immediately affect feeding, and may be related to metabolic events not monitored in this study. Nevertheless, the tight association

consistently observed between the release of BN-like peptides at the PVN and the state of satiety, together with the observation that blockade of BN receptors partially reversed BN-induced satiety²³⁶ and facilitated feeding in satiated rats^{115,236} suggest a causal link between BN-like peptides and feeding status.

In conclusion, the observation of *in vivo* variations in the PVN BN-like peptide release associated with spontaneous feeding provide new insights on the physiological mechanism underlying BN-like peptide action and, suggest that hunger state, meal anticipation and/or meal initiation may be associated with an inhibition of BN-like peptide release whereas satiety is concomitant with an increase in the release of BN/GRP like peptides. These results further support the hypothesis that BN-like peptides may mediate a physiological signal in the PVN to induce and/or maintain satiety in the rat.

Chapter Four

Sustained bombesin (BN) exposure results in receptor down-regulation and tolerance to the chronic but not acute effects of BN on ingestion

Since the previous three sets of experiments strongly implicated the physiological participation of BN-like peptides in the regulation of food intake, it was of interest to examine the consequences of sustained exposure to relatively high levels of BN on the ingestive process. In other words, would chronic exposure to this satiety peptide induce long-term changes in food intake and weight gain like those noted in certain eating disorders, such as anorexia nervosa. As chronic agonist exposure often elicits receptor down-regulation, the next set of experiments also explored the effects of such prolonged exposure to BN on the receptor levels as well as their response to acute BN dose challenge.

Abstract

Acute intracerebroventricular (i.c.v.) administration of bombesin (BN) reduces meal intake in fasted rats. The overall objective of the present study was to determine the behavioral and ingestive effects of prolonged central administration of BN. In the first experiment, we characterized the effects of 8-day sustained central administration of BN (0, 0.01, 0.05, 0.1 and 5 $\mu\text{g}/0.5\mu\text{l/h}$) or its antagonist (BIM-26226; 0, 0.005, 0.05, 0.5 and 5.0 $\mu\text{g}/0.5\mu\text{l/h}$), in free feeding rats. Each dose was delivered over 48 h, in an ascending sequence. At the higher doses, the BN group consumed significantly less and the antagonist group significantly more food than the controls during the dark phase. Due to the limited 48 h exposure to these higher doses, we further investigated the role of BN-like peptides in a second study where a single dose of BN (0.25 $\mu\text{g}/0.5\mu\text{l/h}$) was infused over 7-day period. In this study we broadened our scope of investigation and determined the effects of increased availability of BN on a) daily spontaneous ingestive pattern, b) the rat's behavioral and ingestive response to an acute i.c.v. challenge with BN, c) anxiety-like behaviors and, d) BN receptor binding profile in various brain regions. Our findings revealed a significant satiating effect of BN infusion on spontaneous ingestion over the initial two days of chronic infusion. This effect was no longer present 72 h post-infusion. Upon acute challenge with bolus injection of BN (0.25 μg ; i.c.v.), both chronically BN-treated and control rats responded by decreased feeding and enhanced grooming behaviors. Rats chronically exposed to BN also appeared more anxious as they spent less time in the open zones of the elevated plus maze and through tunnel oval maze. Furthermore, there was a significant down-regulation of BN receptors in these rats at the

PVN and dentate gyrus. These findings represent the first demonstration of concomitant behavioral and receptor based changes consequent to sustained exposure to BN in relationship to feeding. These results suggest that tolerance to feeding suppressant effects of BN develop gradually and are partly mediated by down-regulation of BN/GRP receptors at specific brain loci. Furthermore, our results also suggest differential mechanism(s) in the tolerance development to the acute “emergency-like” versus the long term sustained effects of BN.

Introduction

Gibbs and co-workers were the first to demonstrate the ability of the tetradecapeptide bombesin (BN) to substantially inhibit food intake following peripheral administration in rats ¹²³. Since then, its action on feeding has been demonstrated in a variety of animal species, including man ^{21,264,349,393}. BN also suppresses feeding when injected directly at certain brain sites ^{113,162,186}. Furthermore, central administration of BN antagonists enhance feeding in sated rats ^{115,236} and central pretreatment with BN antiserum has been shown to prevent satiety effects of systemically administered BN ²³⁵. Meal-related fluctuations in endogenous levels and release of BN-like peptides have also been reported in food deprived ^{168,234} and *ad libitum* fed rats ²⁹³. Together, these findings suggest that BN-like peptides may play a physiological role in the control of food intake.

BN-like immunoreactivity and bindings sites have been shown widely distributed in the rat brain ^{68,245,247,281} and gut ^{50,249,378}. Recently, it has been reported that chronic agonist stimulation with BN can result in desensitization, internalization and down-regulation of BN/GRP receptors ^{32,218,239}. The functional relevance of such phenomenon on biological actions of BN is not clearly understood. The principal objective of this study was to determine whether tolerance develops to the ingestive effects of BN upon chronic infusion of this peptide. Tolerance to the poikilothermic effect of BN has been reported following i.c.v. infusion of the peptide (1 µg/µl/h) for 18 h ³⁸³. Conversely, BN-induced grooming has been reported to remain unaffected by chronic infusion of BN at doses of either 0.05 µg/h for 12 days or 0.18 µg/h for 7 days ¹²⁸. More recently, tolerance to the

ingestive and grooming effects of BN (assessed during a 30-min observation period following BN administration) was shown to gradually developed following daily acute injections of BN (0.025 ug; i.c.v.) for 8 days ⁷⁴.

At present, the effect of sustained infusion of BN on spontaneous ingestion are not known. In a preliminary study, we assessed the effects of 8-day sustained administration of ascending doses of BN or its antagonist BIM-26226 on spontaneous daily food intake. Although interesting results were obtained, two methodological confounds became apparent and needed to be addressed: 1) viability of the peptide for extended periods in implanted osmotic minipumps was not known and 2) feeding disturbances associated with postsurgical recovery confound with effects of peptides on food intake. In order to further investigate the role of BN-like peptides and control for these confounds, we performed a second study in which we deployed a drug delivery system that allowed for daily replenishment of fresh drug infusion solutions following a full week of postsurgical recovery. In this study, we determined the effects of increased availability of BN on a) daily spontaneous ingestive pattern, b) the rat's behavioral and ingestive response to an acute i.c.v. challenge with BN and, c) BN receptor binding profile in various brain regions. In light of recent findings from our lab suggesting a potential involvement of BN-like peptides in stress and anxiety ¹⁶⁹, we also examined the performance of rats chronically infused with BN on an elevated plus maze (EPM) and through tunnel oval maze (TTOM), to assess their anxiety level.

Material and Methods

Animals

Male Sprague-Dawley (350–400 g) were used in these experiments. The animals were housed individually in sound-attenuated chambers in a temperature (23–24° C), humidity (60%) and light (12 h light/dark cycle; lights on at 7:00 h) controlled environment. Rats were fed *ad libitum* and their food intake was recorded for 22 h using digital scales. The animals had free access to powdered Purina rat chow (through short tunnels - 6.5 x 6.5 x 10 cm - with grid floor) contained in a bin atop an electronic balance (accurate to 0.1 g, Omnitech Instruments, OH). Each balance was connected to a microcomputer that measured cumulative food intake throughout the entire period of recording. Rats were habituated to the testing apparatus and powdered food for at least 10–15 days before surgery. In addition to the above regimen, animals in the second experiment were presented with small quantities of chocolate chip cookies mashed in milk, on two or three occasions, since this palatable food was used to test the feeding suppressant effects of an acute i.c.v. BN challenge.

Apparatus

An elevated plus maze (EPM) and a through-tunnel oval maze (TTOM) were used to assess anxiety-like behaviors. The four arms of the EPM were of equal dimension (11 x 53 cm) and positioned at 90 degrees to one another. The arms were located 66 cm above the floor. Two opposite arms were surrounded by black walls, the closed zones. The two

other arms were flat planks, the open zones. All arms intercepted in the mid area (121 cm²).

The TTOM is a modification of the elevated “zero maze”³⁸⁵. This oval maze is made of closed tunnels in alternation with open corridors. Each tunnel or corridor was made of plastic tubing (I.D. = 10 cm). The pipe tubing was cut in half to make the open corridors. The closed entries were placed 50 cm away from each other and connected by open air corridors.

For both mazes, the rat was considered to be in the open or closed area of the maze if it had at least its head and two front paws were in that area. Risk assessment behavior was operationally defined as elongation of the neck with at least the snout protruding into the open zone while the paws and body remained in the closed area. The experiment was initiated once the rat had been placed at the extremity of one open arm facing the maze for the EPM and, at the beginning of an open corridor for the TTOM. The experimenter counted the number of entries into the three zones and time spent in each, during a 300 s observation session.

Surgery

Rats were anesthetized with pentobarbital (65 mg/kg; i.p.) and placed in a stereotaxic instrument with the skull leveled. Stainless steel cannulae (22 gauge) aimed at the third ventricle (4.3 mm posterior to bregma, 0.0 mm lateral to the midline and 4.3 mm

ventral to the skull surface) were implanted according to coordinates from Paxinos and Watson²⁸⁴. The cannulae were anchored by four stainless steel screws and acrylic dental cement. During the same surgery, osmotic minipumps (Alzet 2001, Palo Alto, CA) which had previously been filled with sterile saline and left to equilibrate in saline at 37° C for 19 h before implantation, were inserted subcutaneously in the midscapular area of the rat. The osmotic minipumps were attached using polyethylene tubing (PE 50) threaded subcutaneously, to an L-shaped stainless steel junction on the acrylic head cap. This L-shaped junction was connected at its other end to a short piece of PE 50 tubing (5.6 cm long; volume capacity = 15µl) connected to a removable injector maintained secure at the third ventricle through a guide cannula and a detachable plastic cap. The injector protruded 0.5 mm beyond the guide cannula tip. This assembly allowed continuous delivery (at a rate of 0.5 µl/h) of fresh drugs (i.e. the drug stored in the short piece of PE 50 was replaced daily by freshly dissolved compounds) and circumvented the potential problems of degradation associated with storage of the drug in the pumps at body temperature, over prolonged periods. This procedure also allowed us to provide the rats with a postsurgical recovery during which the rats only received a saline infusion.

The surgical procedure for the first study was identical to the one just described above with few exceptions related to the drug delivery system used. Thus, in this study, the 8-day drug regimen was stored in the abdominal cavity from the day of surgery and dispensed at a rate of 0.5 ul/h into the 3rd ventricle via a drug filled coiled polyethylene tubing attached at one end to the saline filled osmotic minipump and at the other end to an

L-shaped infusion cannula, using the Alzet brain infusion kit. Thus, one day prior to surgery, polyethylene tubing (I.D. = 0.7 mm) was coiled by wrapping 20 cm pieces around a cylindrical object (i.e. a syringe) placing them in boiling water for 20 sec and then in ice water. The osmotic minipumps were primed the night before implantation as described earlier. On the morning of surgery, the coiled tubes were either filled with a 3-day supply of saline followed by 2-day supplies of 4 ascending doses of either BN (0, 0.01, 0.05, 0.1, 0.5 ug/h) or BN antagonist, BIM-26226 (0, 0.005, 0.05, 0.5, 5.0 ug/h). Each dose of BN or BN antagonist was separated from the preceding and following drug solution by a small air bubble. The tubing was connected to the osmotic minipumps such that the three day quantity of saline would exit first. The control animals were not implanted in that initial study and received no chronic treatments.

Experimental protocol

Experiment 1: Sustained central infusion of saline, BN, and BN antagonist.

In this experiment, all rats received a 3-day infusion of saline postsurgically before their respective drug treatments begun. Spontaneous food intake was recorded for two weeks following implantation of the osmotic minipumps. Rats were monitored undisturbed in their cages during the first 11 postsurgical days except when their weight was taken every morning. At the end of the experiment, rats were sacrificed, their brain perfused with formalin and sectioned, to verify cannula placement.

Experiment 2: Sustained central infusion of BN and saline.

All rats received i.c.v. saline infusion during the six day postsurgical recovery period. Then, groups of rats either received continuous infusion of BN (0.25 $\mu\text{g}/0.5 \mu\text{l/h}$; i.c.v.) or saline for 7 consecutive days and were sacrificed on the morning of the 8th day. Spontaneous food intake was monitored every day for 22 h and rats weighed daily. Rats were also handled every morning, at which time the section of polyethylene tubing attached to the injector was replenished with fresh saline or BN solution.

Following 48 h of chronic saline or BN infusion, rats were tested on the EPM and TTOM. On that day, the animals' drug solutions were only replaced after testing, to minimize stress effects.

On the morning of the 6th day, and 30 min after temporary cessation of chronic infusion, saline- and BN-treated rats were challenged with an acute injection of BN (0.25 $\mu\text{g}/3 \mu\text{l}$; i.c.v.). A separate control group received i.c.v. saline injection. The amount of palatable food (mashed chocolate chip cookie in milk) eaten was determined for 1 h after acute i.c.v. treatments. Throughout the hour of the testing session, the following behaviors were visually monitored and rated using a time-sampling procedure (one 5-s observation/rat, every 15 s)²²⁹: eating (the rat is scooping, chewing and/or ingesting mashed cookie), grooming (rat is washing and/or scratching face/head or body/flank regions), exploring (rat is moving around the cage, sniffing or rearing) and resting (rat is

inactive with eyes open or lying in curled position with eyes closed). Chronic infusions were reestablished approximately 2 h after acute BN challenge.

Chronic infusion of BN was maintained until 12 h before sacrifice, at which time BN was replaced by saline infusion. On the morning of the 8th day, chronic treatment was discontinued in all animals prior to sacrifice. Autoradiography was used to determine density of BN binding sites in all rats.

Autoradiography procedure

Tissue preparation and sectioning

Rats in *Experiment 2* were sacrificed by decapitation, their brains quickly extracted and frozen in a rat stainless steel brain matrix resting on ice. 20 μ m brain slices were obtained using a Hacker 5030 cryostat set at -17° C, and thaw mounted onto gel coated 22 x 22 mm coverslips. A total of 20 brain slices were selected from each brain: five at the level of the accumbens (between bregma +1.70 and +1.2 mm), five at the level of the paraventricular nucleus (between bregma -1.4 and -1.8 mm) and five at the level of the dorsomedial and ventromedial hypothalamic nuclei (between bregma -2.8 and -3.3 mm). The remaining five sections aimed at the nucleus tractus solitarius were obtained from horizontal slices sampled around -8.1 mm behind bregma. Upon completion of brain sectioning, the slices were air dried at room temperature.

In vitro receptor autoradiography

Autoradiography was carried out using a modification of the procedure described by Wolf and colleagues³⁸⁹. Briefly, all incubations were carried out in autoradiography buffer containing 130 mM NaCl, 4.7 mM KCl, 5.0 mM MgCl₂*6H₂O, 1.0 mM EGTA and 10.0 mM HEPES, pH 7.4, with 0.1% bacitacin. The slices were preincubated for 20 min (to remove endogenous ligand) and then incubated for 1 h 45 min in the presence of ¹²⁵I-Tyr⁴-BN (at final concentration of 3 nM). Non specific binding was determined by incubating slices in presence of excess of unlabelled BN (1 μM). The brain sections were then rinsed, dried and affixed to a piece of cardboard together with [¹²⁵I] microscapes (RPA 523, Amersham, Canada). The sections were apposed to Hyperfilm βmax film (Amersham, Canada) for 36 h, which was then developed [Kodak D-19 developer solution for a period of 4 min.; Kodak rapid fixer for a period of 8 min]. Following development, the audiograms were analyzed using an image analysis system described below.

Staining

All brain sections were subsequently recovered and stained stained using thionin, to facilitate structural identification during image analysis.

Image Analysis

The autoradiograms were analysed using computer image analysis system (Imaging Research, St. Catherines, Ontario). Histological overlay of the stained section onto the autoradiogram facilitated identification of precise nuclei. The density of BN binding sites

was determined in 15 distinct brain regions and gray levels converted to nCi/mg using the [125 I] standard calibration scale (Amersham, Canada). The sampled hypothalamic nuclei included the anterior (AH), lateral (LH), paraventricular (PVN), arcuate (Arc), dorsomedial (DM) and ventromedial (VMH) nuclei. Extrahypothalamic regions included the frontal cortex (Fr), accumbens (Acb), caudate-putamen (CPu), hippocampal layers CA 1-3 of Ammon's horn (Hipp), dentate gyrus (DG), central nucleus of the amygdala (Ce), paraventricular thalamic nucleus (PVth), reuniens nucleus of the thalamus (Re) and the nucleus tractus solitarius (NTS).

Statistical Analysis

All results are expressed as means $\pm$ S.E.M. Two-way analysis of variance (ANOVA) were performed on the spontaneous feeding data with factor of treatment and time (infusion day). Palatable food intake upon i.c.v. BN was analyzed using one-way ANOVA. The 60-min visually monitored behavioral data was similarly analyzed by individual behavior. All behavioral data measures obtained in the EPM and TTOM were analyzed individually using a two-way ANOVA with factors of treatment and maze type. BN-like peptide receptor densities were analyzed by two-way ANOVA with factors of treatment and brain regions. Post hoc comparisons were conducted using Tukey's test, controlling for the α value in the multiple pair-wise comparisons.

RESULTS

Experiment 1: Effects of sustained central infusion of saline, BN, and BN antagonist in ascending doses on spontaneous feeding.

Statistical analysis revealed an overall interaction between drug treatment and days of infusion ($F_{24,216} = 2.4$; $P < 0.005$). This interaction appeared mainly attributable to increased food consumption in the antagonist group with ascending doses and decreased ingestion with ascending doses of BN, during the dark phase. Food consumption during the light phase was slightly but consistently higher for the rats receiving BN while it remained constant across test days for the control and antagonist groups. The small transient decrease in food consumption by the BN and antagonist groups at the saline infusion days was most likely due to acute effects of surgery as in the untreated control group, no significant difference in food consumption across days was noted (see Fig. 1).

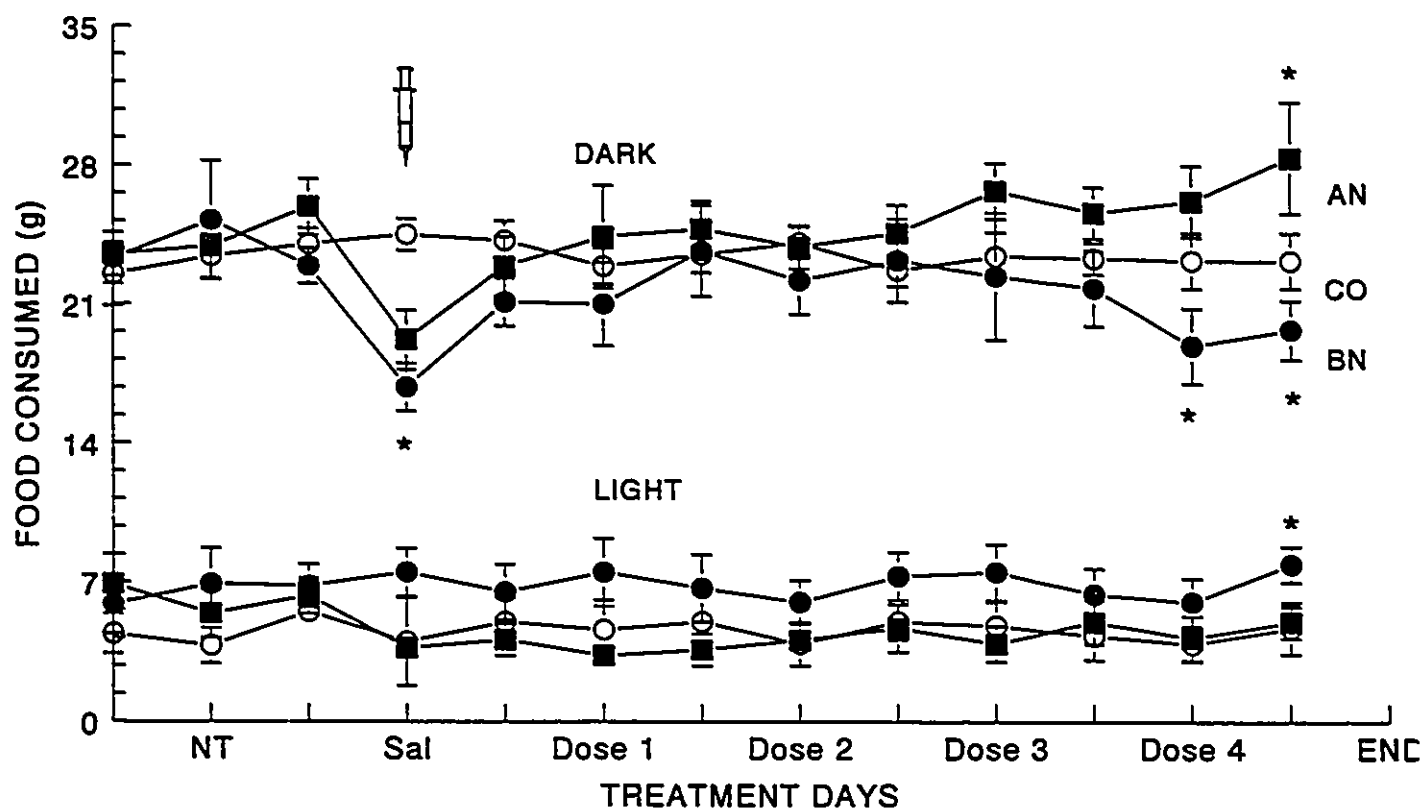


Fig. 1 Effects of sustained central BN and antagonist (BIM-26226) administration on food intake during the dark and light phase of the diurnal cycle, across treatment days. The syringe icon depicts start of infusion. The ascending doses of BN (0.01, 0.05, 0.1 and 0.5 $\mu\text{g/h}$) or BIM-26226 (0.005, 0.05, 0.5 and 5.0 $\mu\text{g/h}$) are indicated as dose 1-4, on the horizontal axis. Results are expressed as mean $\pm$ S.E.M. of the total grams of food consumed over the recorded period of time. * significantly different from control's spontaneous ingestion at $P < 0.05$.

Experiment 2: Sustained central administration of BN (0.25 $\mu\text{g}\cdot\text{h}$) or saline.

In the second experiment, we determined the effects of sustained BN or saline administration over 7 days on daily spontaneous ingestive patterns, and responses to an acute i.c.v. BN challenge. Anxiety-like behaviors and density of BN binding sites in various brain regions were also assessed. In contrast to *Experiment 1*, these rats were exposed to a single sustained dose of BN (0.25 $\mu\text{g}/0.5\text{ }\mu\text{l/h}$) and drug treatment begun only after a full week postsurgical recovery. Furthermore, a modified infusion procedure enabled daily replenishment with fresh BN or saline solutions, circumventing potential problems of drug degradation associated with prolonged storage in osmotic minipumps.

1. Daily spontaneous food consumption

Our findings revealed a significant overall interaction of drug treatment and infusion days on daily (22 h) food consumption ($F_{9,126} = 3.95$; $P < 0.0002$). Sustained BN infusion was found to significantly affect food consumption across days ($F_{9,63} = 9.14$; $P < 0.0001$) while the spontaneous food intake of saline-treated rats remained unaffected (see Fig. 2).

Statistical analysis revealed no significant difference in food consumption between saline- and BN-infused rats during the light phase (7:00 h to 19:00 h). However, ANOVA analysis revealed a significant overall interaction between drug treatment and infusion days on food consumption during the dark phase (19:00 h to 7:00 h) ($F_{9,126} = 4.66$; $P < 0.0001$). This effect was attributable to suppression of food intake by BN-treated rats on days 1 and 2 (see Fig. 3).

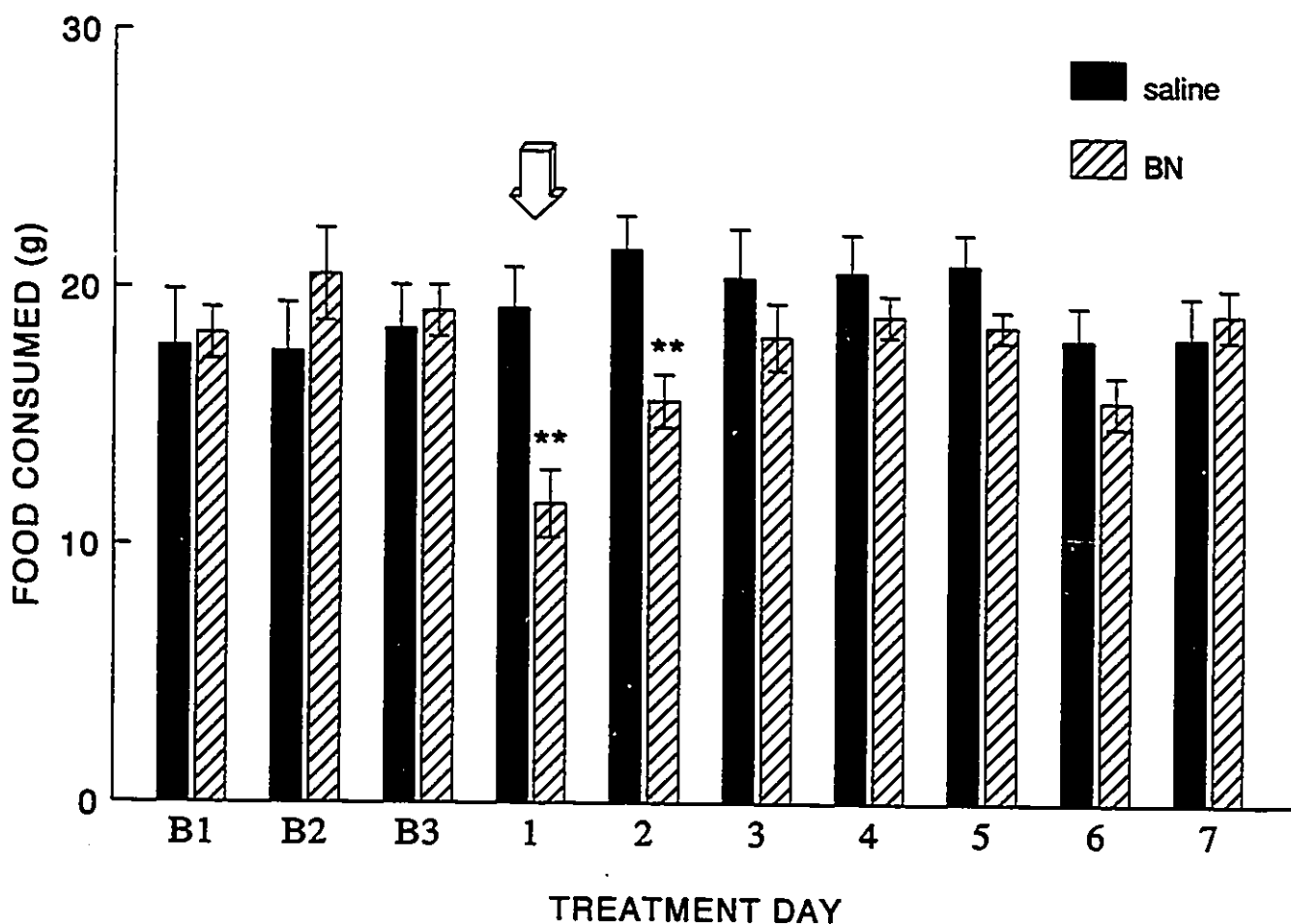


Fig. 2 Effects of sustained saline and BN infusion on total food consumption over treatment days. The arrow indicates the first day of drug infusion while B1, B2 and B3 represent spontaneous feeding ingestion during the 3 days preceding drug treatment. Results are expressed as means $\pm$ S.E.M. of the total grams consumed by rats over 22 h. **, significantly different from saline-treated rats on corresponding treatment day, $P < 0.01$.

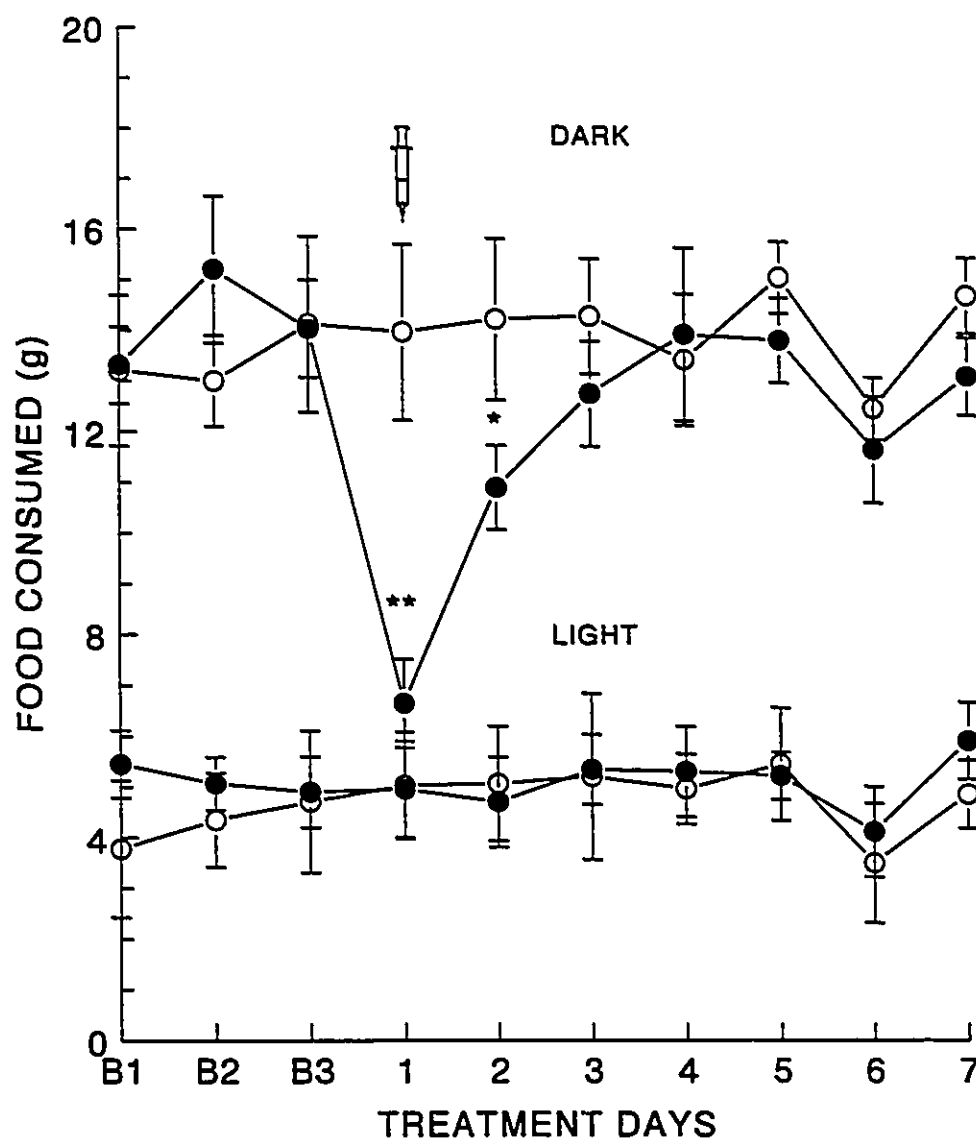


Fig. 3 Effects of sustained central BN administration on spontaneous feeding during the dark and light phase. The syringe icon depicts start of drug infusion while B1, B2 and B3 indicate the spontaneous ingestion during the 3 baseline days prior to drug treatment. Results are expressed as mean $\pm$ S.E.M. of the total food consumption over the corresponding diurnal phase. *, ** significantly different from saline rats ingestion on the corresponding treatment day as well as from baseline values for both rat groups at $P < 0.05$ and $P < 0.01$, respectively.

Analysis of variance also revealed a significant overall interaction between drug treatment and days of infusion on the latency to initiate the first meal after dark onset ($F_{9,126} = 2.29$; $P < 0.02$). Post hoc analysis revealed that BN infusion significantly delayed initial meal intake following dark onset at treatment day 1 as compared to saline-treated animals on that day. This effect was transient and only observed at drug treatment day 1 (data not shown).

ANOVA analysis revealed no significant difference in the daily number of meals eaten between the two groups. Furthermore, no significant difference in the number of meals consumed from day to day was observed. When the analysis was restricted to the number of meals during the dark phase, ANOVA revealed a significant overall interaction between treatment and days of infusion ($F_{9,126} = 2.16$; $P < 0.02$). On treatment day 1, rats infused with BN ate significantly fewer meals as compared to saline treated rats. On treatment day 2, BN treated animals also appeared to have consumed fewer meals as compared to saline animals (see Fig. 4).

2. Behavioral and ingestive responses to acute i.c.v. BN

ANOVA analysis revealed a significant effect of acute treatment (BN or saline; i.c.v.) on palatable food intake ($F_{2,22} = 7.94$; $P < 0.002$). Cookie intake was significantly suppressed in both chronically treated groups when challenged with an acute dose of BN (0.25 $\mu\text{g}/3 \mu\text{l}$; i.c.v.), as compared to the control (acute saline injection) condition, indicating that there was no tolerance to that acute dose of BN (see Fig. 5).

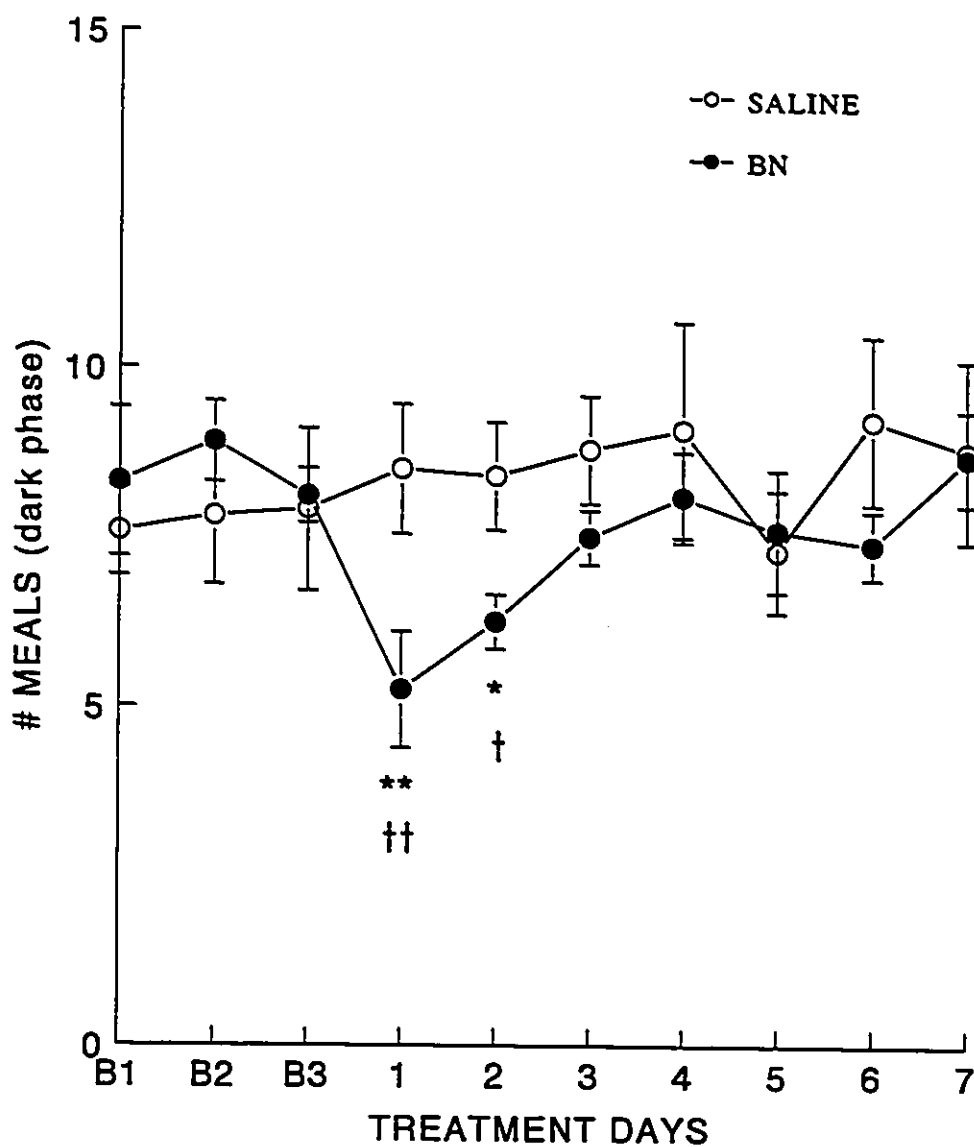


Fig. 4 Effect of sustained central infusion of BN on the number of meals ingested during the dark phase. *,** significantly different from saline treated rats on the corresponding day at $P < 0.05$ and $P < 0.01$, respectively. †, †† significantly different from BN animals on baseline days at $P < 0.05$ and $P < 0.01$, respectively.

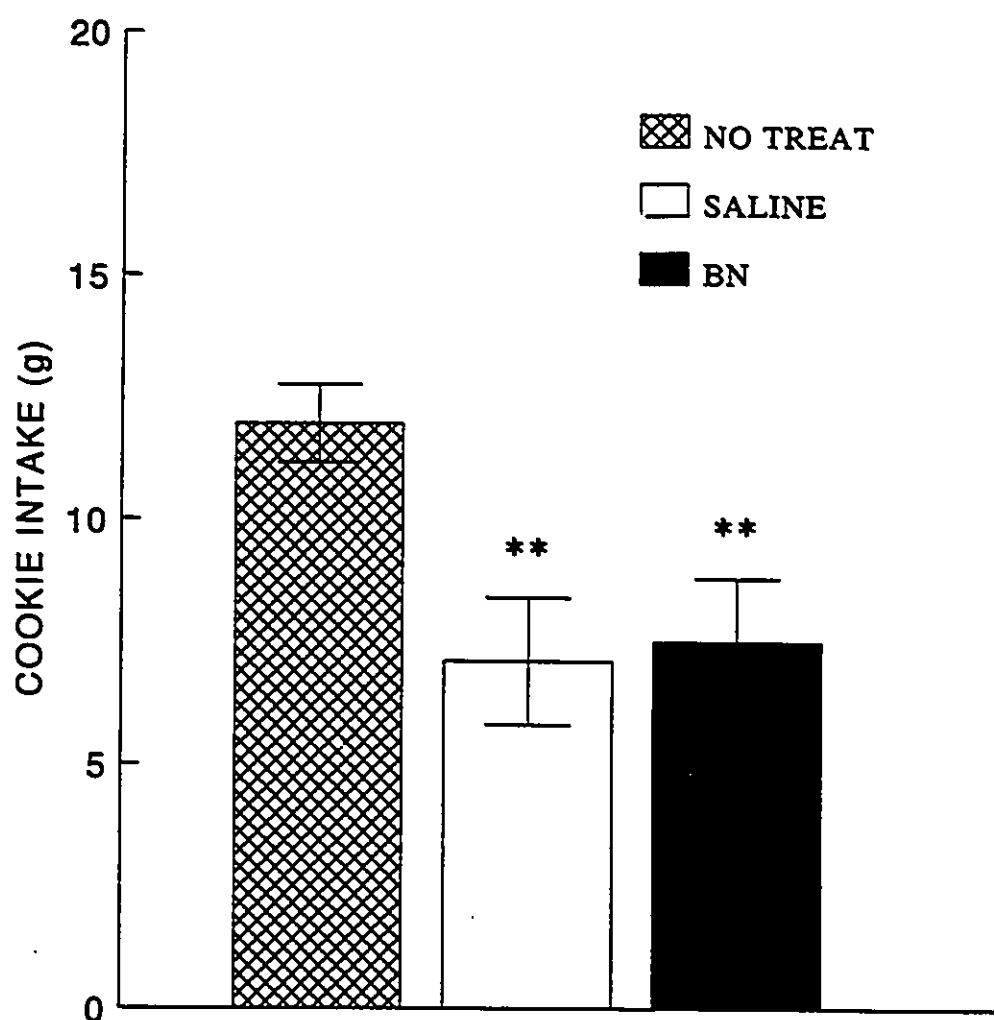


Fig. 5 Effect of bolus BN administration ($0.25 \mu\text{g}/3 \mu\text{l}$; i.c.v.) on palatable food intake during a 60-min test period. Both chronically infused rats groups (saline and BN) responded to acute BN administration by significant reduction of food intake at 1 h as compared to a non treated rat group receiving an i.c.v. saline infusion. **, significantly different from non treated rats receiving acute i.c.v. saline injection at $P < 0.01$.

During the hour of the test session, behavioral monitoring was also performed. ANOVA analysis of the frequencies of different behaviors revealed a significant effect of drug treatment for eating ($F_{2,21} = 119.74$; $P < 0.0001$), grooming ($F_{2,21} = 35.83$; $P < 0.0001$) and resting ($F_{2,21} = 6.35$; $P < 0.006$). The frequency of exploring was not significantly affected by acute BN administration (see table 1). Consistent with the amount of chow consumed (see Fig. 5), central BN administration significantly decreased the frequency of ingestive behavior in both saline- and BN-infused rats as compared to control rats injected with saline. A high incidence of grooming was also observed in rats acutely injected with BN. Concordant with such findings, these rats spent less time resting as compared to saline-injected rats. The frequency of exploring was slightly higher in rats that received acute BN as compared to control animals, although this difference was not significant (see table 1).

3. *Density of BN-like peptide receptor binding*

Statistical analysis revealed a significant overall interaction between drug treatment and brain regions ($F_{1,14} = 10.24$; $P < 0.001$). Post hoc comparisons revealed that BN/GRP receptor binding significantly decreased at the dentate gyrus and at the hypothalamic PVN following sustained 7-day infusion of BN. No significant alteration of BN-like peptide receptor binding was observed in the other hypothalamic and extrahypothalamic brain regions examined (see Fig. 6).

Table 1
Effect of acute administration of BN on behavior in rats chronically infused with BN or saline

Treatment	<i>Eating</i>	<i>Grooming</i>	<i>Exploring</i>	<i>Resting</i>
Control (saline; icv)	87.2 $\pm$ 4.8	24.2 $\pm$ 1.5	32.1 $\pm$ 2.6	25.2 $\pm$ 2.7
Saline (BN; icv)	17.6 $\pm$ 2.6**	109.2 $\pm$ 6.5**	41.8 $\pm$ 6.4	2.66 $\pm$ 1.3**
BN (BN; icv)	16.1 $\pm$ 3.5**	100.6 $\pm$ 11.7**	45.8 $\pm$ 4.3	6.55 $\pm$ 5.5**

Note. Each cell represents the mean $\pm$ S.E.M. of frequency of eating, grooming, exploring and resting during the 60-min test interval. Control rats received central saline administration as indicated in brackets. Both groups of rats chronically infused with saline or BN were injected with BN (0.25 μ g; icv). This acute test was performed on the morning of the 6th day of sustained drug administration.

** Significantly different from control rats at $P < 0.01$ (Tukey's test).

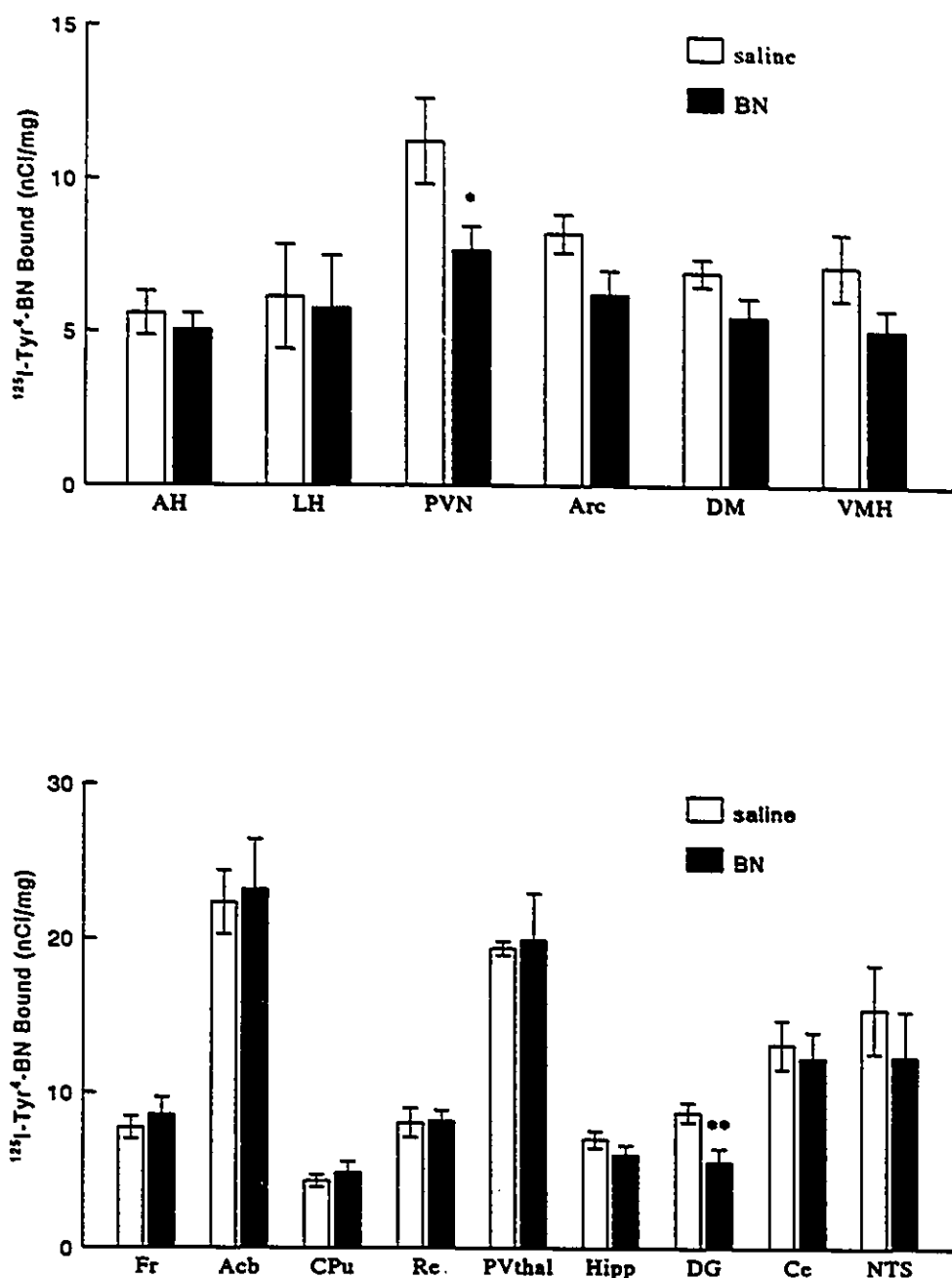


Fig. 6 BN-like peptide receptor binding in various brain regions following 7-day sustained central administration of BN or saline. The upper panel displays receptor binding in hypothalamic sites including the anterior (AH), lateral (LH), paraventricular (PVN), arcuate (Arc), dorsomedial (DM) and ventromedial (VMH) nuclei. The lower panel indicates receptor binding in extra-hypothalamic sites namely the frontal cortex (Fr), accumbens (Acb), caudate-putamen (CPu), reuniens thalamic nucleus (Re), paraventricular thalamic nucleus (PVthal), hippocampus (Hipp), dentate gyrus (DG), central amygdaloid nucleus (Ce) and nucleus tractus solitarius (NTS). Results are expressed as mean $\pm$ S.E.M. BN-like peptide receptor densities are expressed in nCi/mg. *,** significantly different from BN receptor binding values of saline-treated rats at $P < 0.05$ and $P < 0.01$, respectively.

Table 2
Performances in the EPM and TTOM 48 h following sustained central infusion of BN or saline.

	<i>Time spent (min)</i>		<i>Number of entry or occurrence (per 300 s)</i>	
	EPM	TTOM	EPM	TTOM
Open Area				
Saline	47.12 ± 7.0	55.03 ± 9.7	1.45 ± 0.4	2.91 ± 1.0
BN	13.44 ± 3.8*	28.15 ± 7.0**	1.10 ± 0.3	1.63 ± 0.5
Closed Area				
Saline	230.71 ± 18.3	206.85 ± 17.4	9.36 ± 1.0	7.73 ± 1.5
BN	247.21 ± 11.8	226.81 ± 9.06	9.45 ± 1.3	7.09 ± 1.1
Risk Assessment				
Saline	32.02 ± 9.9	33.40 ± 5.1	4.81 ± 0.8	6.64 ± 1.3
BN	31.31 ± 6.2	46.16 ± 9.4	5.36 ± 1.1	6.63 ± 1.0

Note. Each cell represents the mean ± S.E.M. of the time spent in the open or closed areas of the mazes or risk assessing or, of the frequency of entries in the open or closed areas or occurrences of risk assessment behaviors. *, ** significantly different from saline-infused rats at $P < 0.05$ and $P < 0.01$, respectively.

4. *Performances in the EPM and TTOM.*

ANOVA analysis revealed a significant main effect of drug treatment on the time spent in open areas of the mazes ($F_{1,22} = 22.28$; $P < 0.0001$). Post hoc analyses revealed that BN-treated rats spent significantly less time (min) in the open areas of both the EPM and TTOM. However, no significant differences were observed in the time spent in the closed areas of the mazes or performing risk assessment behaviors. Moreover, BN and saline treated animals appeared equally active as ANOVA analysis failed to reveal differences in the number of entries into the open or closed areas or in the number of times these groups engaged in risk assessment behaviors (see table 2).

DISCUSSION

Findings from our initial study suggested that infusion of BN at doses of 0.01, 0.05 and 0.1 μg for 2 days each, failed to significantly affect total daily ingestion. However, at the 0.5 μg dose, BN slightly diminished spontaneous ingestion in the dark phase in as compared to that of control rats. Conversely, at the higher dose, BIM-26226 slightly enhanced feeding following 24 h of sustained i.c.v. infusion. Although promising, these findings were tainted by a few methodological problems that prevented a clear analysis of the potential physiological mechanisms underlying the observed changes. Thus, lack of adequate postsurgical recovery period appeared to have interfered with the drug effect per se, perhaps limiting the degree to which BIM-26226 may have influenced spontaneous feeding. In addition, prolonged storage of the drug in a reservoir placed into the animal's

body raised the concern that the peptide and/or antagonist may have degraded in situ over time. Moreover, the observation that drug effects were restricted to higher doses of BN or its antagonist combined with the short 48 h delivery period for each dose did not allow for clear assessment of possible tolerance effects developing in response to longer exposure to these compounds. Nonetheless, these findings suggested that tolerance to the ingestive effects of BN appeared to take longer than 48 h to develop and thus, dissociated BN's feeding action from its hypothermic effects for which tolerance has been shown to rapidly develop within 18 h of sustained BN infusion ³⁸³. The increased spontaneous food ingestion observed on the second day of infusion of the highest dose of BIM-26226 also suggests that the organism does not adapt too rapidly to sustained blockade of BN receptors and that endogenous BN-like peptides may play a role in the regulation of food intake. Furthermore, these findings suggested that sustained central administration of BN does not have the same magnitude of effect on feeding behavior that has been found with acute BN administration ¹⁸⁶. However, most studies have focused on feeding behavior in food deprived animals and it remains possible that BN plays a different role during emergency situations of extreme hunger and non-emergency situations. Another possible explanation for relatively small effects at lower doses may be related to some loss of drug availability due to molecular breakdown of BN.

Thus, in order to gain better understanding of the role of BN-like peptides, we performed a second experiment in which we controlled drug infusion parameters by using daily freshly dissolved BN administered chronically at a fixed dose over a more prolonged

(7-day) infusion period. We also provided the animals with a full week postsurgical recovery before drug infusions began. This second study characterized the effects of sustained administration of BN (0.25 μ g/0.5 μ l/h) on spontaneous feeding, behavioral and ingestive response to acute BN, receptor binding profile and anxiety-like behaviors. Our findings revealed significant decrease in daily spontaneous ingestion in BN treated animals during the initial 48 h of drug infusion. These results were mainly attributable to effects of ingestion during the dark phase of diurnal cycle. Decreased efficacy of BN to inhibit feeding was apparent by the third day of daily i.c.v. administration of BN, time at which significant differences were no longer observed between saline- and BN-treated rats. Following acute daily injections of a lower BN dose (0.025 μ g; i.c.v.), the grooming and food intake effects of the peptide were also significantly attenuated by the third day of daily i.c.v. injections but 7 days were necessary to completely abolish BN effects ⁷⁴. In contrast, at higher doses (1 μ g; i.c.v.) repeated injections of BN for six consecutive days prior acute testing failed to alter BN-induced feeding suppression and grooming behavior ^{128,179}. In a similar way, receptor and behavioral changes observed following repeated versus sustained central administration of oxytocin appear quite different. Thus, while repeated daily oxytocin administration failed to alter receptor binding and was shown to induce overt signs of motor disturbances, sustained administration of the peptide at comparable daily doses resulted in considerable receptor down-regulation and failed to alter behavior ¹⁵⁵. However, although the daily dose of oxytocin received by both the acutely and chronically infused rats was of comparable magnitude, continual infusion

allowed drug exposure to be sustained over 24 h, a difference which may explain the observed findings.

The physiological mechanism(s) underlying the observed differences in feeding regulation consequent to acute and chronic treatment with BN are not understood. It is possible that differences in these acute and tonic action of BN may be related to mediation of these effects by distinct physiological mechanism(s). Thus, while the effects of BN on spontaneous feeding appeared attenuated by the third day of sustained BN infusion in our study, acute i.c.v. injection of BN at infusion day 6 induced comparable feeding suppression and enhanced grooming in chronically infused saline and BN rats. These findings suggest that BN neurons remain responsive to certain types of stimuli such as those present in acute or “emergency-like” situations. Pharmacological and lesioning studies suggest that other physiological systems may also function in a similar way ¹⁻³. In this context, tonic and phasic pain have been shown to be associated with distinct pharmacological responses to morphine administration. Thus, the apparent lack of tolerance to repeated morphine administration in animal models of tonic pain contrast with the rapidly decreased efficacy of this compound to alleviate acute pain episodes ¹. These findings suggested the existence of different neuronal pathways mediating tonic and phasic pain ³. In a similar way, it is possible that the intact behavioral response observed in our study following acute BN challenge in rats chronically infused with BN may be related to the existence of different neural systems mediating acute “emergency-like” situations and long term regulation of food intake. These findings together with the observation of

enhanced feeding following infusion with BIM-26226 at the higher dose suggest that BN-like peptides may physiologically contribute to the regulation of spontaneous feeding. The return to baseline spontaneous food intake within 48 to 72 h of sustained BN administration is biologically adaptive and, suggests the presence of other endogenous signals, peptidergic or not, compensating for the attenuated physiological input of BN-like peptides. However, such mechanisms of action remain hypothetical and do not appear standard to all physiological systems. Thus, in contrast with our findings with BN, chronic intraventricular administration of cholecystokinin (CCK) failed to significantly alter spontaneous feeding at doses twice as high as the one used for BN in the present study while at daily doses 20 times higher than what we used, sustained CCK infusion resulted in significant prolonged suppression of spontaneous feeding over 7-8 days as compared to saline infused rats³²⁵.

The presence of counterregulatory processes and/or a down-regulation of BN receptors may explain the progressive return to control food consumption. Recent *in vitro* studies have demonstrated that BN/GRP receptors are capable of remarkable plasticity and respond by desensitization, internalization and down-regulation following agonist exposure^{32,177}. In the present study, we demonstrated site-specific down-regulation of BN receptors at the PVN and DG following sustained administration of BN. These changes are unlikely to be attributable to residual BN in the tissue as brain sections were extensively washed and central infusion was discontinued 10-12 h before the rats were sacrificed. The observed down-regulation of BN receptors at the PVN is consistent with

pharmacological data supporting the participation of that structure in controlling feeding^{186,197,199,387}. Interestingly, increased BN concentrations have been demonstrated in both hypothalamic and hippocampal brain tissues following feeding, in food deprived animals²³⁴. Although the role of the hippocampus in feeding has not been well characterized, it is thought that this postprandial increase in BN content at that structure may be related to the affective qualities of food, such as memories of particular taste inputs or other relevant memories associated with the timing, duration or presentation of a meal²³⁴. Indeed, BN-like peptides have been shown to enhance memory retention following i.c.v. administration or microinjection into specific brain nuclei^{112,385}. In our study, the fact that tolerance to BN's feeding suppressant action was evident only after 3 days of sustained peptidergic infusion may suggest that learning and/or memory processes are involved in the development of tolerance to this peptide and perhaps explain why down-regulation of BN/GRP receptors was observed at that site.

Our findings also suggest that rats chronically infused with BN appeared more anxious and spent less time in the open zones of the EPM and TTOM when tested on the morning of the third day of sustained drug administration. In light of some preliminary data collected in our lab, showing alteration of BN-like peptides in specific brain nuclei following restraint stress, it is possible that these peptides may contribute to regulation of emotional and/or stress-related processes. However, the observed independence of BN's satiety action from its effect on secretion of ACTH may suggest that the dissociation of

the effects of BN on feeding from its potential involvement in the control of anxiety.

More research is required to confirm this hypothesis.

In conclusion, our findings suggest that tolerance to the feeding suppressant action of BN gradually develops in the first 72 h of sustained administration of the peptide. We also demonstrated that acute administration of BN remained equally potent at suppressing feeding and eliciting grooming in rats chronically infused with saline or BN. It is not clear how these effects are mediated. In our study, the chronic desensitization of BN receptors to agonist exposure appear partly mediated by receptor down-regulation. This result is concordant with findings from other studies which also concluded that chronic desensitization of BN-like peptide receptors is mediated by receptor down-regulation^{239,280}. Interestingly, BN receptor internalization is a much faster process which appears kinetically independent of chronic desensitization and down-regulation and is at least partly mediated by different intracellular processes than those mediating chronic down-regulation or desensitization³². Such phenomenon may partly explain why rats showing tolerance to BN's suppressant action on spontaneous feeding in our study displayed comparable behavioral responses as controls when acutely infused with the peptide on the 6th day of chronic infusion. More research is required to further characterize the effects of sustained administration of BN antagonist and determine more precisely the latency and duration of its stimulating action on spontaneous feeding.

Chapter Five

Participation of corticotropin-releasing factor but not oxytocin receptors, in the mediation of bombesin-induced suppression of food intake.

As the various neurotransmitters and/or peptidergic systems are interconnected, we wanted to clarify whether BN-like peptides act directly or indirectly via interactions with other peptidergic systems. Since CRF and BN share many biological and behavioral actions, the next set of experiments explored our working hypothesis that BN-like peptides may mediate some of their effects via the release of CRF. This possibility was assessed using specific CRF and OX receptor antagonists.

Abstract

In light of important functional similarities between pharmacological effects of bombesin (BN) and corticotropin-releasing factor (CRF), the present study aimed to test our working hypothesis that BN may elicit its biological effects through the release of CRF. Central pretreatment with CRF antagonists, α -helical CRF₉₋₄₁ (α -CRF₉₋₄₁) or [DPhe¹², C ^{α} MeLeu³⁷]CRF₁₂₋₄₁ (C α MeCRF), blocked the effects of centrally administered BN on food intake and related behaviors, and partially attenuated the satiety effects of systemically administered BN. We then attempted to characterize the specificity of this interaction through the combined utilization of BN with the oxytocin (OX) antagonist, [d(CH₂)₅, Tyr(OMe)², Orn⁸] vasotocin (vasotocin). Central pretreatment with vasotocin failed to alter BN-induced behaviors suggesting the absence of pharmacological interaction between these two peptidergic systems. Finally, we verified, using C α MeCRF, a potential role for CRF in the mediation and/or modulation of OX ingestive effects. The CRF antagonist failed to reverse OX-induced suppression of food intake. Thus, the present experiments support the contention that BN partly mediates its satiety effects through interactions with CRF. The specificity of this interaction is supported by the lack of interaction between BN and/or CRF with OX.

Introduction

Bombesin (BN), a tetradecapeptide of amphibian origin and its mammalian counterparts, gastrin-releasing peptides (GRP₁₋₂₇ and GRP₁₈₋₂₇) and neuromedin Bs (NMB₁₋₃₂ and NMB₂₃₋₃₂) are present in discrete brain sites^{68,246,247,281}. Central administration of BN elicits a number of biological and behavioral actions including elevation of blood catecholamines⁵³, glucose⁵², corticosterone¹³⁶, plasma adrenocorticotrophic hormone (ACTH)^{319,366} and, elicitation of grooming and locomotor activity^{162,180,286}. Central or systemic administration of BN-like peptides have also been shown to induce a satiety-like state in various species ranging from rats to humans^{21,186,264,349}. The evolutionary conservation of this pharmacological response suggests a physiological role for these peptides in the control of food intake. Thus, central blockade of BN/GRP receptors have been shown to enhance feeding in rats^{115,236}. Furthermore, increase in endogenous levels of BN-like peptides have been observed in postmortem rat hypothalamus, following feeding^{168,234}. More recently, meal-related *in vivo* fluctuations in the release of BN-like peptides at the rat paraventricular nucleus of the hypothalamus (PVN) have also been demonstrated²⁹³.

Despite these advances, the exact physiological mechanisms by which these peptides exert their action on feeding remains largely unknown. It has been demonstrated that satiety induced by systemically administered BN is abolished following total afferent disconnection of the gastrointestinal tract from the brain³⁵⁰. Furthermore, effects of systemically administered BN are also attenuated in rats centrally pretreated with BN

antiserum²³⁵. These observations suggest the participation of neuronal populations in BN's ingestive effects. Attempts to identify these neural substrates using brain microinjections and lesions have revealed that certain hypothalamic and hindbrain structures (such as the PVN¹⁸⁶ and the nucleus tractus solitarius (NTS)^{114,162}) are particularly sensitive to BN's satiating effects.

Like BN, central administration of the 41-amino acid polypeptide, corticotropin-releasing factor (CRF), has been reported to exert potent anorectic effects^{209,255}, enhance locomotion and grooming^{99,353}, and increase plasma glucose concentrations⁵⁵. CRF's autonomic effects also markedly resemble those of BN⁵⁴ in that centrally administered CRF dose-dependently elevates plasma epinephrine and norepinephrine concentrations⁵⁵. Moreover, central administration of CRF stimulates the release of pituitary adrenocorticotrophic hormone (ACTH) and β -endorphin in rats^{56,307,370}. Interestingly, central administration of subthreshold doses of GRP have been shown to potentiate the ACTH-releasing effects of CRF and vasopressin²⁷³. This effect was inhibited by about 60% in rats pretreated with antiserum to either CRF or vasopressin and was completely blocked by combined pretreatment with these antisera²⁷³. Together, these findings suggest that central GRP might trigger ACTH secretion by stimulating the central release of CRF and/or vasopressin. Indeed, we have recently found that many of the autonomic and endocrine effects of BN are blocked by CRF antagonists²³⁷.

In addition to the shared behavioral, endocrine and ingestive effects, BN and CRF also exhibit some anatomical overlap in their distribution pattern^{68,83,281}. For example, high concentrations of CRF-containing neurons originate in the parvocellular portion of the paraventricular hypothalamus^{292,384}, a site which also contains numerous immunoreactive BN/GRP neurons^{68,77,282}. The central nucleus of the amygdala, the arcuate nucleus and the NTS are also among brain regions where CRF and BN-positive neurons are densely distributed^{68,83,247,278}. Similarly, the presence of both BN and CRF binding sites has been demonstrated in the amygdala, PVN and the NTS^{85,245,396}.

In light of the important behavioral, anatomic, autonomic and endocrine similarities between CRF and BN, the aim of the present study was to test our working hypothesis that BN may mediate its effects on feeding and related behaviors via the release of CRF. Thus, the first series of experiments attempted to elucidate the potential role of CRF in the mediation and/or modulation of the behavioral and ingestive effects of BN, by utilizing two different CRF receptor antagonists [α -helical CRF₉₋₄₁ (α -CRF₉₋₄₁) and [DPhe¹², C ^{α} MeLeu³⁷]CRF₁₂₋₄₁ (C α MeCRF)] prior to central or systemic administration of BN.

To better determine the specificity of such interactions and potential mechanisms of action involved, we investigated the effect of blockade of oxytocin (OX) receptors on BN-induced satiety. Oxytocin was selected as this peptide shares similarities with BN in relationship to its feeding effects^{14,274} and immunohistochemical distribution^{354,357,367}, while eliciting some distinct, and at times, opposite biological actions^{100,212}. Furthermore,

central administration of CRF has been shown to stimulate pituitary secretion of OX in rats⁵⁹ and blockade of OX receptors has been reported to inhibit CRF-induced satiety²⁷². Thus, the second set of experiments aimed to determine potential interactions between BN with OX through the combined utilization of BN with the oxytocin antagonist [d(CH₂)₅, Tyr(OMe)², Orn⁸] vasotocin (vasotocin). Thirdly, attempts were made to further characterize the specificity of interactions with CRF through the assessment of a potential role of CRF in the mediation and/or modulation of OX effects.

Material and Methods

Animals

Male Sprague-Dawley rats (300-350 g) were used in all experiments. The animals were housed individually in sound-attenuated chambers in a temperature (23-24° C), humidity (60%) and light (12 h light/dark cycle; lights on at 7:00 h) controlled environment. All rats were trained to consume their daily food ration during a 4 h food access period between 10:00 and 14:00 during the light phase. During this time, rats had free access to powdered Purina rat chow (through short tunnels 6.5 x 6.5 x 10 cm, with grid floor) contained in a bin placed atop an electronic balance (accurate to 0.1 g, Omnitech Instruments, OH). Each balance was connected to a microcomputer that measured cumulative food ingestion throughout the 4 h session. Prior to testing, the animals were extensively habituated to the testing apparatus and the feeding paradigm until their food consumption stabilized (10-14 days). Rats had *ad libitum* access to water throughout. All experiments were carried out between 10:00 h and 14:00 h when food

was available. Rats were tested on alternate days and were left undisturbed on non-test days.

Surgery

Rats were anesthetized with pentobarbital (65 mg/kg; i.p.) and placed in a stereotaxic instrument with the skull leveled. Stainless steel cannulae (22 gauge) aimed at the third ventricle (4.3 mm posterior to bregma, 0.0 mm lateral to the midline and 4.3 mm ventral to the skull surface) were implanted according to coordinates from Paxinos and Watson²⁸⁴. The cannulae were anchored by four stainless steel screws and dental acrylic cement, and plugged by a stainless steel wire stylet. During post-surgical recovery and before the start of the experiment, all animals were acclimated to handling for a minimum of 7 days.

Behavioral Measurement

Throughout the first hour of the testing session, behavior of each rat was visually monitored using time-sampling procedure (one 5 sec observation every 20 sec)²²⁹. The behaviors monitored included: 1) *Eating*: Scooping, chewing and/or ingesting rat chow, 2) *Drinking*: Licking the water spout, 3) *Scratching*: Contact of the hind paw with the side of the face/head or body/flank followed by a scratching action, 4) *Washing*: wiping of the face and crown regions with circular movements of the forelimbs or active licking of the

abdomen and/or thorax, 5) *Resting/Sleeping*: the animal is inactive with eyes open or lying in curled position with eyes closed, and 6) *Exploring*: the animal is moving around the cage, sniffing or rearing.

Peptide and Drug Treatments

Saline, BN, two CRF antagonists, α -helical CRF₉₋₄₁ (α -CRF₉₋₄₁) and [DPhe¹², C^αMeLeu³⁷]CRF₁₂₋₄₁ (C α MeCRF), OX and the OX antagonist d(CH₂)₅, Tyr(OMe)², Orn⁸] vasotocin (vasotocin) were used in these experiments. All drugs were freshly dissolved in 0.9% saline prior to use and administered in the third cerebral ventricle 10-15 min prior to food presentation. Drugs were obtained from Bachem (California) except for the CRF antagonist [DPhe¹², C^αMeLeu³⁷]CRF₁₂₋₄₁ which was generously provided by J. Rivier, Salk Institute's Clayton Foundation Laboratories for Peptide Biology. The i.c.v. injections were made in awake, unrestrained animals through a stainless steel cannula which extended 0.5 mm below the tip of the guide cannula and into the third cerebral ventricle. The injection cannula was connected by polyethylene tubing to a Harvard infusion pump and the 3 μ l of drug solutions were infused at a constant rate over a 30 sec period. The cannula was left in place for an additional 1 min to minimize back flow along the guide cannula. The injection cannula was then withdrawn, the stylet replaced and the animal put back in its home cage. For systemic injections, drug solutions were delivered in a volume of 1 ml/kg body weight.

Histology

Upon completion of each experiment, the animals were sacrificed with an overdose of pentobarbital and intracardially perfused with saline followed by 10% formaldehyde solution. India ink (0.5 μ l) was then microinjected through the cannula. Brain slices (30 μ m) were then obtained using a cryostat and successful cannula placement confirmed by the presence of ink in the 3rd ventricle.

Experimental Procedures

Experiment 1: Effects of central blockade of the CRF receptors on BN effects.

Using a group of animals ($n = 8$ to 9) entrained to a 4 h food access test sessions as described previously, this experiment determined the effects of the CRF antagonist α -CRF₉₋₄₁ on BN-induced ingestive and behavioral effects. Animals were injected with α -CRF₉₋₄₁ [0 (saline), 5 or 10 μ g; i.c.v.], which was followed 15-20 min later by administration of BN (0.5 μ g; i.c.v.) or saline. All drug treatments were repeated across subjects and were interspersed by at least 48 h.

In the next experiment, the effects of central blockade of CRF receptors on the satiety induced by systemically administered BN were determined. Rats ($n = 9$) were entrained, treated and monitored as in the first study with the exception that only the most

effective dose of α -CRF₉₋₄₁ (10 μ g; i.c.v.) was used, followed by peripheral administration of saline or BN (6 μ g/kg; i.p.).

In order to ascertain that these findings were not unique to the particular CRF antagonist deployed, a third group of rats ($n = 8$ to 9) was entrained, treated and monitored as in the first experiment except that a different CRF antagonist, C α MeCRF, was used in the place of α -CRF₉₋₄₁. Preliminary experiments had revealed that optimal doses of C α MeCRF were 2 to 5 μ g, lower than those of α -CRF₉₋₄₁. This is concordant with recent report indicating that C α MeCRF was more potent than α -CRF₉₋₄₁ in antagonizing CRF-induced locomotor and anxiogenic effects in the rat²²⁸. Thus, as in earlier experiments, rats were injected with C α MeCRF (0 (saline), 2 or 5 μ g; i.c.v.), followed 15-20 min later by administration of BN (0.5 μ g; i.c.v.) or saline (control).

All drug treatments were performed 10-15 min before food presentation. The cumulative amount of food consumed was recorded at 0.5, 1, 2, and 4 h time intervals. The incidence of expression of other behaviors was monitored during the first hour of the test session.

Experiment 2: Effects of central blockade of oxytocin receptors on BN effects.

A separate group of rats ($n = 8$) was entrained to the feeding and testing regimen of *experiment 1*. In the present study, rats were alternately treated with i.c.v. vasotocin [0

(saline) or 8.9 μg ; i.c.v.] 15 min before central administration of BN (0.5 μg ; i.c.v.) or saline (3 μl ; i.c.v.; control condition).

Experiment 3: Effects of central blockade of CRF receptors on OX effects.

A separate group of rats ($n = 7$ to 8) submitted to identical feeding regimen and behavioral monitoring as the earlier experiments, was used to determine the potential participation of CRF in OX-induced satiety and related behaviors. Rats were injected with C α MeCRF (0 (saline), 5 μg ; i.c.v.), followed 15-20 min later by administration of OX (10 μg ; i.c.v.) or saline (control).

Statistical Analysis

Repeated measures analyses of variance (ANOVA) were performed on the feeding and locomotion data with factors of time and treatment. The visually monitored behavioral data were similarly analyzed by individual behavior with factor of treatment and time. Post hoc comparisons were conducted using Tukey's test, controlling the α value in the multiple pairwise comparisons.

RESULTS

Experiment 1: Effects of central blockade of the CRF receptors with α -CRF₉₋₄₁ on effects of centrally applied BN.

Statistical analysis revealed a significant effect of the drug treatment on food intake ($F_{4,38} = 11.56$, $P < 0.0001$) as well as a significant overall interaction between the effects of treatment and time ($F_{12,114} = 8.69$, $P < 0.05$). As illustrated in Fig. 1, central administration of BN (0.5 μ g) suppressed food intake for up to 4 hr. Central administration of α -CRF₉₋₄₁ prior to BN attenuated BN-induced satiety, at both the 5 and 10 μ g doses. This effect was specific as central administration of α -CRF₉₋₄₁ (10 μ g) alone failed to alter food intake.

ANOVA analyses of the frequencies of different behaviors revealed significant overall interaction for eating ($F_{12,96} = 2.47$, $P < 0.007$) and grooming ($F_{12,96} = 2.49$, $P < 0.007$) and an effect of drug treatment for exploring ($F_{4,32} = 9.14$, $P < 0.0001$) and drinking ($F_{4,32} = 9.05$, $P < 0.0001$). The frequency of resting behavior was relatively low and was not significantly altered by any of the drug treatments (data not presented). Consistent with the amount of chow consumed (data presented in Fig. 1), central BN treatment (0.5 μ g; i.c.v.) was associated with decreased frequency of ingestive behavior. Under saline and α -CRF₉₋₄₁ conditions or α -CRF₉₋₄₁ plus BN, data also paralleled the rat's food intake. Rats under these treatment conditions spent most of the hour ingesting food (see Fig. 2). A high incidence of grooming was observed in the BN condition, which was also blocked by α -CRF₉₋₄₁ at the 10 μ g dose and partially blocked with the lower dose (5 μ g). Frequencies of exploring were relatively low in all groups. However, BN

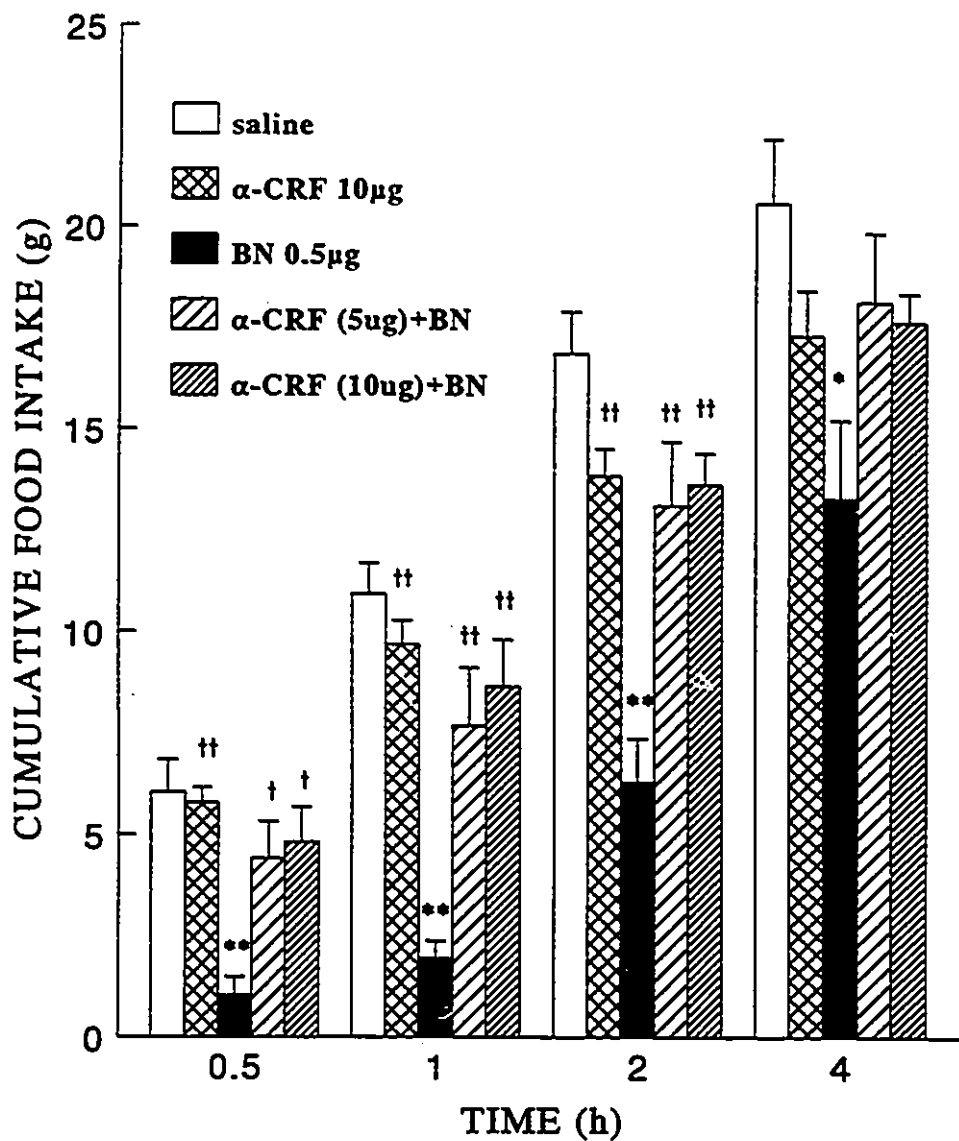


Fig. 1 BN-induced satiety is blocked by central administration of α -CRF. Each column represents the mean $\pm$ S.E.M. of the cumulative food consumed (g) at designated time points during the rat's daily 4 h food access period.

*, ** significantly differences from saline at $P < 0.05$ and $P < 0.01$, respectively.

†, †† significantly difference from BN at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

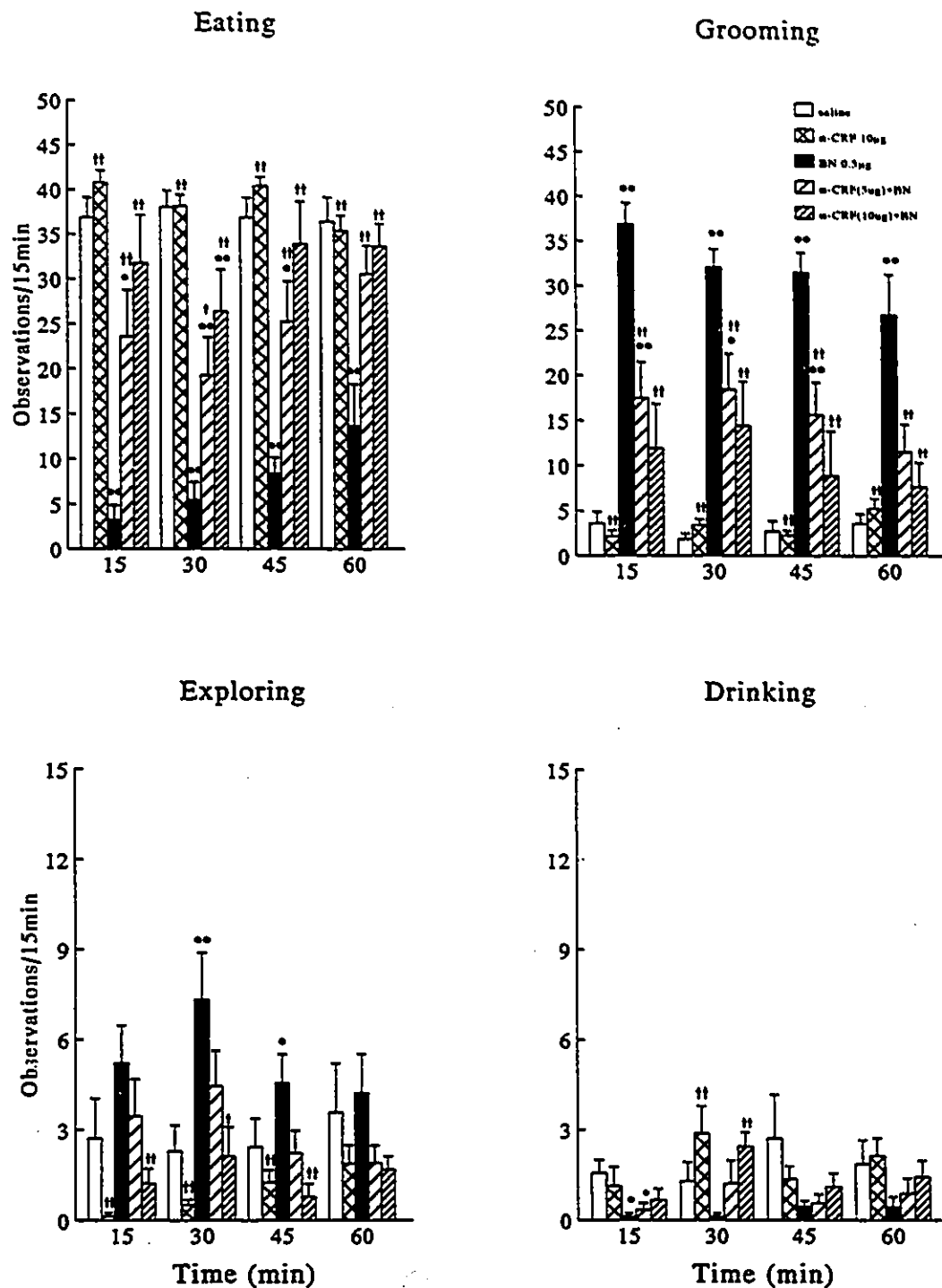


Fig. 2 Effect of blockade of CRF receptors by α -CRF on BN-elicited behavioral profile. Each column represents the mean $\pm$ S.E.M. of the frequency (incidence/15 min time bins) of eating, grooming, exploring and drinking during the 60 min test session.

* ** Significant differences from saline at $P < 0.05$ and $P < 0.01$, respectively.

†, †† Significant differences from BN at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

significantly enhanced exploration at the 30 and 45 min interval, a behavior blocked by the high dose of α -CRF₉₋₄₁. Finally, the drinking incidence was low for all treatment conditions (see Fig. 2).

2. Effects of the CRF antagonist, α -CRF₉₋₄₁, on effects of systemically administered BN.

To determine whether central CRF receptors also participate in satiety induced by systemically administered BN, we assessed the efficacy of α -CRF₉₋₄₁ (10 μ g; i.c.v.) in attenuating the effects of systemic BN (6 μ g/kg; i.p.). Statistical analysis of variance revealed a significant effect of the drug treatment on food ingestion ($F_{4,40} = 14.29$, $P < 0.0001$) as well as a significant overall interaction between the effects of treatment and time ($F_{12,120} = 2.58$, $P < 0.004$). Systemic administration of BN (6 μ g/kg; i.p.) suppressed food intake (by about 50%) in the first hour of testing. This suppression was significantly attenuated by central administration of α -CRF₉₋₄₁ (10 μ g; i.c.v.), as illustrated by Fig. 3.

Based on results from the first experiment, the behavioral data was compiled for two 30 min bins and submitted to ANOVA analysis. Analyses of the frequency of the monitored behaviors during the hour of behavioral monitoring indicated a significant overall interaction between drug treatment and time for eating ($F_{3,31} = 3.25$, $P < 0.03$), grooming ($F_{3,31} = 3.08$, $P < 0.04$) and exploring ($F_{3,31} = 4.21$, $P < 0.01$). No significant effects on the drinking and resting behavioral frequencies were observed. As mentioned above, central administration of α -CRF₉₋₄₁ partially blocked the satiety induced by peripheral BN administration. In agreement with this finding, central pretreatment with α -CRF₉₋₄₁ (10 μ g; i.c.v.) significantly increased eating frequency in BN treated rats.

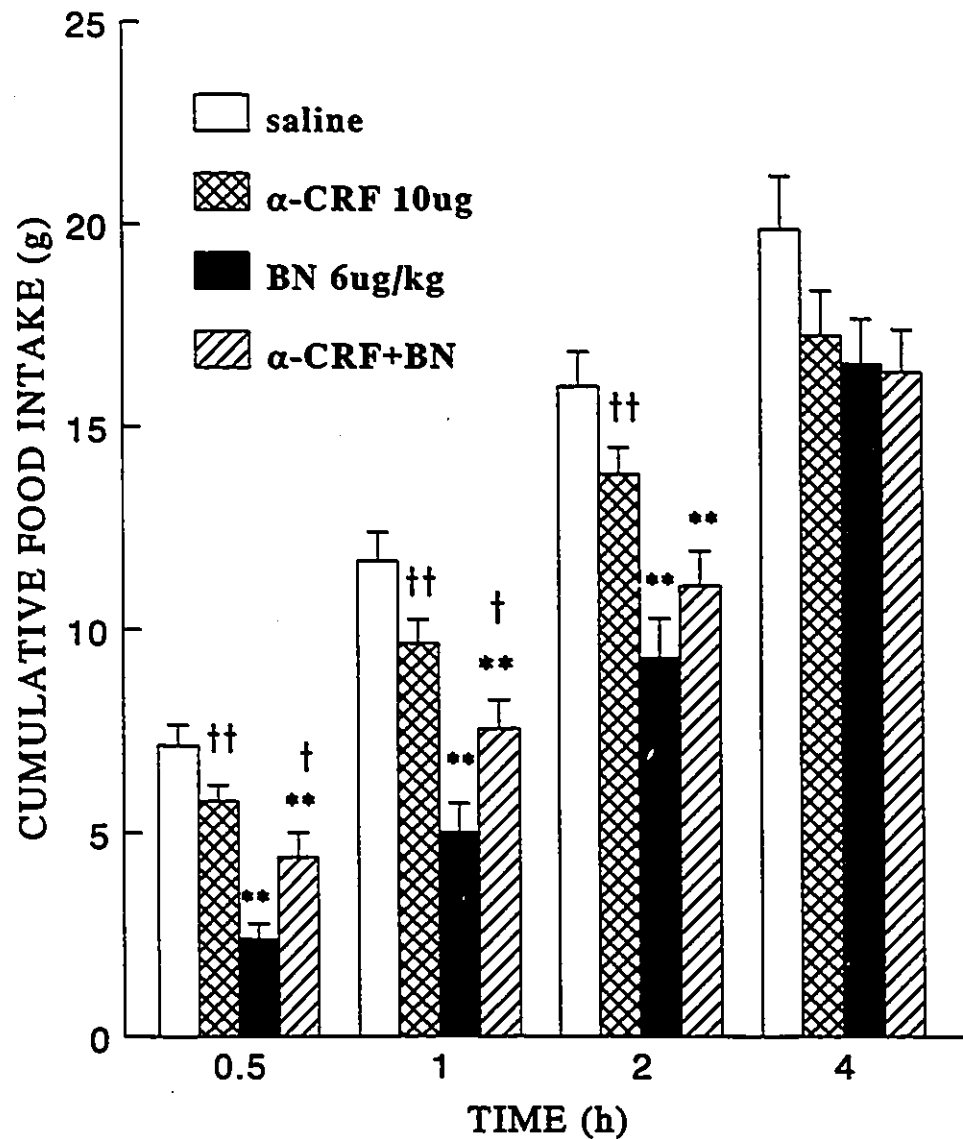


Fig. 3 Central administration of α -CRF attenuates the satiating effects of systemically administered BN. Each column represents the mean $\pm$ S.E.M. of the cumulative food consumed (g) at designated time points during the 4 h access period to food.

** significant differences from saline at $P < 0.01$.

†, †† significant difference from BN at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

The frequency of grooming was relatively low throughout the test hour but slight grooming enhancement was observed in BN-treated animals in the first 30 min testing interval, an activity which appeared to be reversed by α -CRF₉₋₄₁ pretreatment (see table 1). The frequency of exploration was also elevated by BN administration and this effect was only blocked by α -CRF₉₋₄₁ at the 60 min interval. Finally, the drinking and resting incidence were not significantly altered by any of the drug treatments (data not shown).

3. Effects of the newer CRF antagonist, C α MeCRF on effects of central BN.

This experiment examined whether the above findings were unique to α -CRF₉₋₄₁ or could be reproduced using another CRF receptor antagonist. Thus a more recently synthesized CRF antagonist, C α MeCRF, was deployed. Statistical analysis of food intake revealed a significant overall interaction between the effects of treatment and time ($F_{12,117} = 2.03$, $P < 0.02$). Administration of C α MeCRF (5 μ g; i.c.v.) by itself failed to affect food intake. However, like α -CRF₉₋₄₁, central administration of C α MeCRF, 15 min prior BN administration (0.5 μ g; i.c.v.) dose-dependently blocked BN-induced satiety. Complete blockade of BN-elicited satiety was attained with the higher dose of C α MeCRF (5 μ g), while only a partial attenuation was obtained with the lower 2 μ g dose (see Fig. 4).

Analyses of the behavioral data revealed a significant main effect of drug treatment for eating ($F_{4,39} = 21.97$, $P < 0.0001$) and grooming ($F_{4,39} = 23.45$, $P < 0.0001$). ANOVA analyses failed to reveal significant differences between groups for drinking, resting or exploring behaviors.

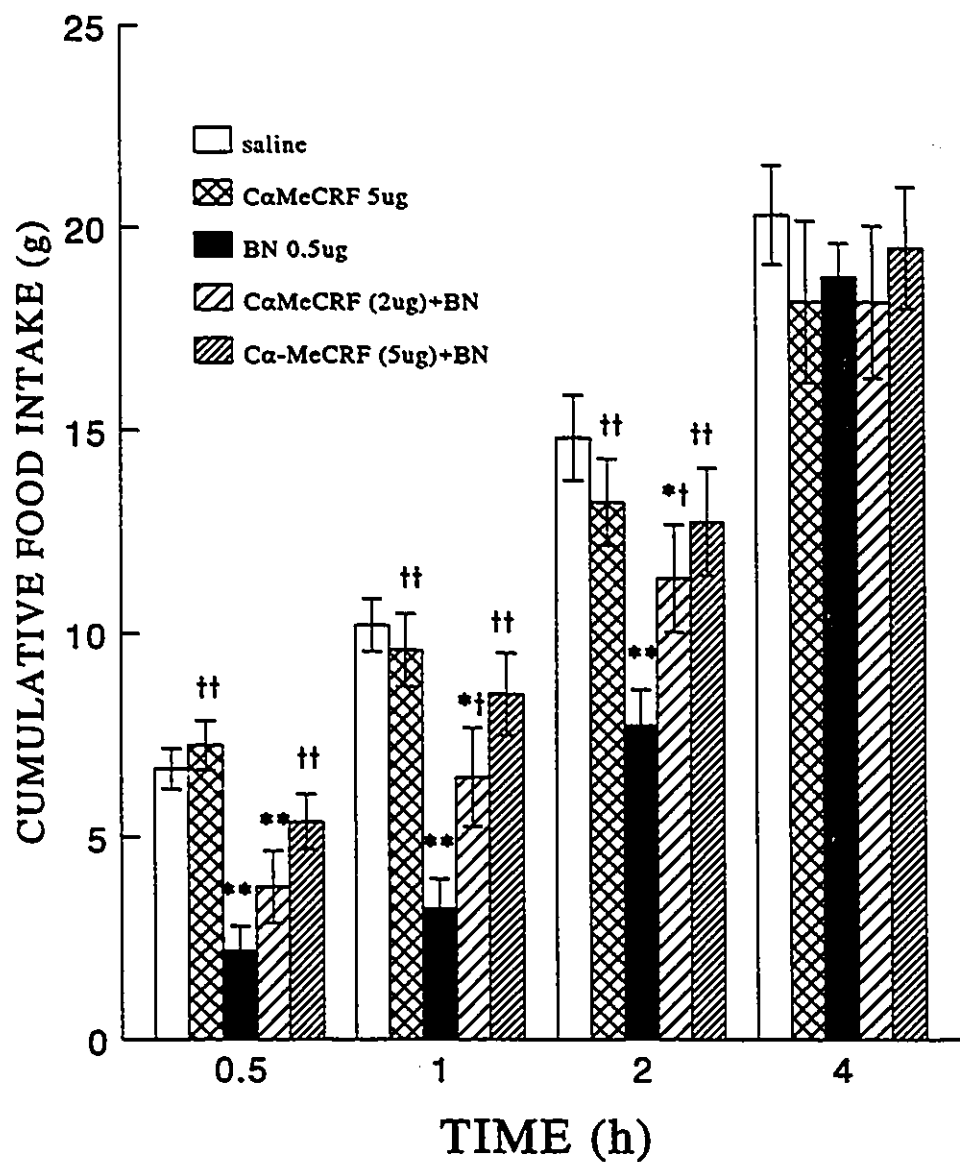


Fig.4 Central blockade of CRF receptor using CoMeCRF dose-dependently blocked BN-induced satiety. Each column represents the mean $\pm$ S.E.M. of the cumulative food consumed (g) at designated time points during the rat's daily 4 h food access period. *, ** significantly differences from saline at $P < 0.05$ and $P < 0.01$, respectively. †, †† significantly difference from BN at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

Under the saline and C α MeCRF conditions, rats spent most of the hour ingesting food. Consistent with the food intake data presented in Fig. 4, BN alone was associated with decreased frequency of ingestive behavior during the entire observation period. The frequency of this behavior was also diminished in rats pretreated with the 2 μ g dose of C α MeCRF prior to BN (see table 1).

Under the saline and C α MeCRF (5 μ g) conditions, the frequency of grooming was low and remained unchanged during the entire hour. In contrast, BN administration alone or in combination with the 2 μ g dose of C α MeCRF enhanced grooming during the entire hour of behavioral monitoring (see table 1).

Experiment 2: Effects of central blockade of OX receptors on BN-elicited behaviors.

Statistical analysis of food intake revealed a significant overall interaction between the effects of treatment and time ($F_{9,63} = 5.75$, $P < 0.0001$). As expected, BN treatment (0.5 μ g; i.c.v.) significantly suppressed food ingestion, this effect being mainly attributable to reduction of feeding during the initial hour of testing. Pretreatment with the selective OX antagonist, vasotocin (8.9 μ g; i.c.v.), failed to affect BN-induced satiety. Administration of vasotocin by itself had no effect on feeding (see Fig. 5).

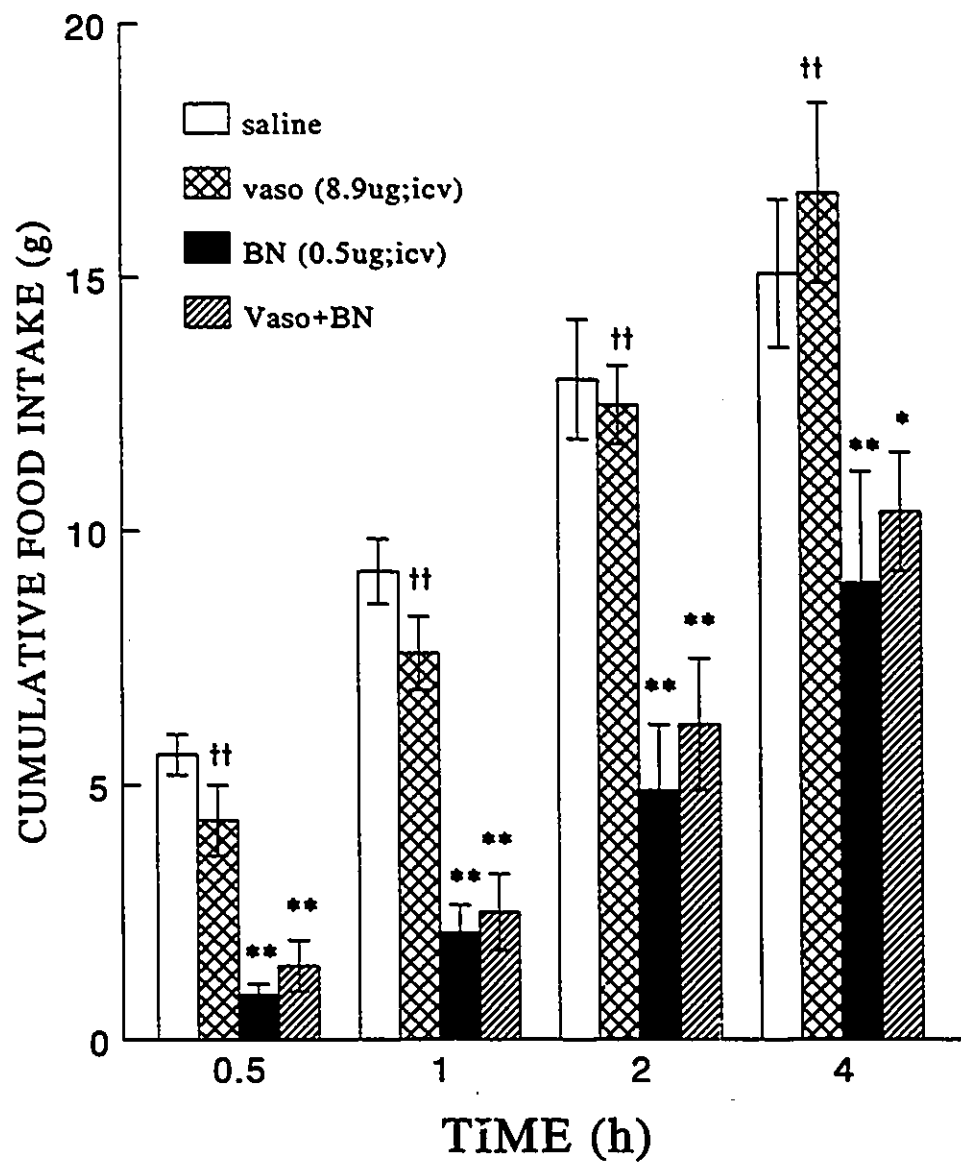


Fig. 5 Central blockade of oxytocin receptors by vasotocin failed to block BN-induced satiety. Each column represents the mean $\pm$ S.E.M. of the cumulative food consumed (g) at designated time points during the rat's daily access period to food.

** significant differences from saline at $P < 0.01$.

†† significant difference from BN at $P < 0.01$ (Tukey's test).

Analyses of the frequency of various behaviors during the first hour of testing revealed significant overall interactions for eating ($F_{3,28} = 4.89$, $P < 0.007$) and grooming ($F_{3,28} = 5.01$, $P < 0.006$). Consistent with the food intake data presented in Fig.5, BN treatment was associated with decreased frequency of ingestive behavior during the entire hour of monitoring. There were no significant differences between conditions where rats received BN alone or vasotocin plus BN. Similarly, both these drug treatments enhanced grooming frequency throughout behavioral testing. In contrast, saline and vasotocin treated animals spent most of the time ingesting food and their grooming frequency remained relatively low throughout the test hour (see table 1). The frequencies of exploring, drinking and resting behaviors were low for all treatment conditions and no overall statistical differences were found between treatments (data not presented).

Experiment 3: Effects of central blockade of CRF receptors on OX effects.

This experiment aimed to determine whether CRF receptors participated in the expression of OX effects. Statistical analysis of food intake revealed a significant interaction between the effects of drug and time ($F_{15,144} = 1.73$, $P < 0.05$). As illustrated in Fig. 6, central administration of OX (10 μ g; i.c.v.) attenuated feeding by about 40% in the initial hour following drug administration. Central pretreatment with the CRF antagonist C α MeCRF (5 μ g; i.c.v.) prior to OX administration failed to alter OX-induced feeding suppression. No significant alteration in feeding was obtained following central administration of the lower 5 μ g dose of OX. Similarly, C α MeCRF (5 μ g) failed to alter feeding when administered on its own (see Fig.6).

Analyses of the behavioral data revealed significant effect of drug treatment for eating ($F_{3,42} = 17.87$, $P < 0.0001$) and grooming ($F_{3,42} = 6.17$, $P < 0.0002$) and, an overall interaction between drug treatment and time for drinking ($F_{3,42} = 4.62$, $P < 0.001$). The reduction of food intake observed following central administration of OX was accompanied by a concomitant reduction in eating frequency in OX-treated animals as well as in rats pretreated with C α MeCRF (5 μ g) prior to OX administration. The reduced eating frequency was proportional to the treatment effects on food consumption, and lasted for the entire observation hour (see table 1). The frequency of grooming also appeared enhanced during the entire hour of testing in both the OX-treated rats and the rats pretreated with C α MeCRF (5 μ g) prior to OX administration (see table 1). Finally, the drinking frequency was low for all treatment groups but rats administered with C α MeCRF (5 μ g) alone drank more frequently than saline-treated rats during the initial 30 min of behavioral observation. At the 60 min interval, the drinking incidence of both saline and C α MeCRF treated rats appeared more elevated than the other test groups (data not shown).

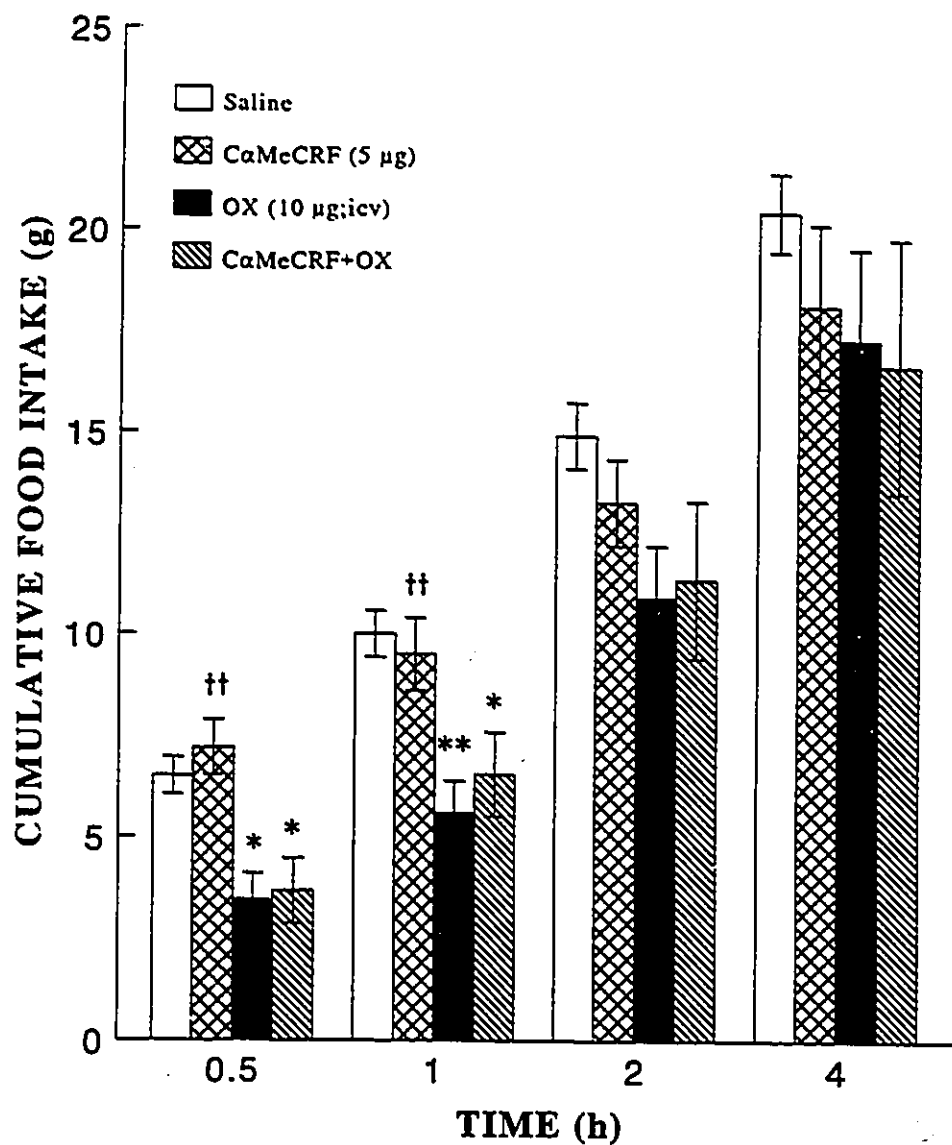


Fig. 6 Central blockade of CRF receptors failed to reverse OX-induced satiety. Each column represents the mean $\pm$ S.E.M. of the cumulative food consumed (g) at designated time points during the rat's daily access period to food.

*, ** significant differences from saline at $P < 0.05$ and $P < 0.01$, respectively.

†† significant difference from BN at $P < 0.01$ (Tukey's test).

Table 1 Effect of the various drug treatments on eating and grooming behaviors

Treatment	Eating		Grooming	
	30	60	30	60
Experiment 1				
Saline (i.p.)	79.9 ± 1.0	76.0 ± 1.7	5.4 ± 0.9	7.6 ± 1.3
BN (6µg; i.p.)	29.1 ± 6.3**	45.6 ± 7.7**	15.6 ± 2.7**	11.0 ± 1.6
α-CRF+BN	61.7 ± 8.8*	68.4 ± 5.0	9.0 ± 1.7	7.7 ± 1.6
α-CRF(10 µg; i.c.v.)	78.9 ± 1.7	75.8 ± 1.7	5.5 ± 1.2	7.5 ± 1.1
Experiment 2				
Saline (i.c.v.)	77.8 ± 3.2	75.4 ± 5.7	3.3 ± 1.4	5.2 ± 1.4
BN (0.5µg; i.c.v.)	6.4 ± 2.0**	18.0 ± 4.9**	71.6 ± 3.8**	61.9 ± 5.7**
CαMeCRF (2µg)+BN	48.7 ± 12.0**	49.6 ± 7.4**	33.0 ± 10.3**	30.7 ± 8.1**
CαMeCRF (5µg)+BN	63.6 ± 8.3	62.6 ± 5.5	19.3 ± 8.1	18.0 ± 4.2
CαMeCRF (5µg)	72.8 ± 3.7	71.6 ± 4.2	9.8 ± 2.4	7.1 ± 1.7
Experiment 3				
Saline (i.c.v.)	80.0 ± 3.6	65.4 ± 5.2	4.0 ± 1.4	10.4 ± 3.3
BN (0.5µg; i.c.v.)	9.0 ± 3.6**	16.8 ± 4.9**	75.9 ± 3.8**	65.3 ± 4.9**
Vaso (8.9µg) + BN	22.6 ± 9.9**	19.1 ± 6.4**	59.9 ± 9.6**	62.9 ± 7.9**
Vaso (8.9µg; i.c.v.)	79.3 ± 2.7	77.9 ± 2.9	5.1 ± 1.4	5.1 ± 1.3
Experiment 3				
Saline (i.c.v.)	80.0 ± 3.6	65.4 ± 5.2	4.0 ± 1.4	10.4 ± 3.3
OX (10µg; i.c.v.)	33.0 ± 6.8**	41.1 ± 4.1**	24.3 ± 4.5**	20.4 ± 3.8*
CαMeCRF (5µg)+OX	28.6 ± 6.5**	37.6 ± 6.5**	31.4 ± 5.8**	24.6 ± 4.2**
CαMeCRF (5µg)	73.4 ± 3.5	71.6 ± 4.2	9.8 ± 2.4	7.1 ± 1.7

Note. Each cell represent the mean ± S.E.M. of frequency of eating and grooming at the 30 and 60 min test intervals. *, ** significantly different from saline condition at $P < 0.05$ and $P < 0.01$, respectively (Tukey's test).

DISCUSSION

The exact role of CRF in the regulation of food intake is not understood. However, various findings support the contention that CRF plays a central role in the control of various feeding-related events. Thus, evidence has accumulated suggesting that CRF may mediate the anorectic effects of certain stressful stimuli associated with increased CRF release^{174,306,331}. Dagnault et al. have also shown the ability of α -helical CRF₉₋₄₁ to block the feeding suppressant effects of centrally infused estradiol⁸⁴. Furthermore, administration of α -helical CRF₉₋₄₁ has been shown to potentiate NPY-induced feeding at the PVN¹³⁹.

Based on functional and anatomical overlap between BN and CRF, the present study aimed to elucidate the potential participation of CRF in BN-induced satiety. Our findings provide evidence for the mediating role of CRF in BN-induced satiety. Central administration of both the CRF antagonists tested (α -CRF₉₋₄₁ and C α MeCRF) blocked the behavioral and ingestive effects of centrally administered BN. When administered alone, α -CRF₉₋₄₁ and C α MeCRF, had no significant effect on food intake, suggesting that the blockade of BN-induced satiety was not attributable to intrinsic properties of these drugs on feeding. Moreover, these pharmacological interactions appeared quite specific as BN did not interact with OX and, C α MeCRF failed to alter OX-induced satiety. These results led us to believe that BN partly mediates its behavioral effects through the release of CRF. The fact that α -CRF₉₋₄₁ attenuated the satiety induced by systemically administered BN,

suggests that at least some of the interactions between BN and CRF might be occurring within the CNS. The participation of distinct anatomical sites where the peptides might interact has not been confirmed yet. However, the PVN might potentially be an important site as endogenous increase in the release of BN-like peptides have been observed before and after food intake at that brain structure²⁹³. Moreover, Krahn et al.'s findings revealed that among other brain sites CRF suppressed feeding in rats when microinjected into the PVN, but not following injection into the lateral or ventromedial hypothalamic nuclei, globus pallidus or striatum¹⁷⁶. Furthermore, exogenous administration of CRF into the PVN has been shown to site-specifically increase grooming and locomotion^{176,260}. Thus, it is possible that elevated BN release at the PVN before and after spontaneous meal intake stimulates the release of CRF from neurons emanating from the PVN or at anatomically related sites, which would induce and/or maintain a satiety-like state in the animal. Such interactions between BN-like peptides and CRF have been proposed in relationship to pituitary ACTH secretion. Indeed, Olsen et al. have demonstrated that GRP-induced ACTH release was reduced by approximately 60% in rats pretreated with an antiserum to CRF and totally blocked by combined administration of CRF and vasopressin antisera²⁷³. Furthermore, we have found that α -CRF pretreatment blocks endocrine and autonomic effects of central BN²³⁷. These findings support the contention that some of the effects of BN-like peptides may be directly mediated through the release of CRF.

The precise mechanisms by which BN stimulates CRF activity remains unknown. Blockade of CRF receptors using α -CRF₉₋₄₁ failed to alter BN membrane binding

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(unpublished observation) suggesting the inability of BN to directly bind CRF receptors. The presence of BN receptors on CRF neurons remains to be determined. In terms of specific physiological mechanisms by which these peptides may regulate feeding related behaviors, their shared endocrine, autonomic and behavioral actions offer different possibilities for their common actions. Although central administration of BN and CRF have been shown to stimulate ACTH release from the pituitary^{307,319,366}, it is unlikely that the feeding suppression and grooming induced by these peptides is related to their pituitary activation, as hypophysectomy failed to alter the behavioral effects of these peptides^{128,255}.

Central administration of CRF or BN induce hyperglycemia associated with elevation of plasma glucagon levels and relative lowering of insulin concentrations^{52,55}. These glucostatic effects are thought to be secondary to adrenomedullary epinephrine secretion^{55,57}. In light of the transient decline in glucose levels observed immediately preceding the meal initiation⁶⁵, the satiety effects of these peptides may partly be secondary to their hyperglycemic action. Indeed, glucose infusions have also been shown to inhibit or postpone food intake²⁷⁰. Interestingly however, although adrenalectomy failed to alter CRF-induced hyperglycemia⁵⁵, this procedure significantly attenuated the satiety effects of centrally-infused CRF¹³¹. This alteration of CRF suppression of feeding has been shown to be mainly attributable to adrenomedullary epinephrine secretion and not to corticosterone levels¹³¹. Furthermore, CRF's suppressant action on feeding appears to be dependent on direct CRF activity within the brain as intravenous administration of CRF

antiserum failed attenuate i.c.v. CRF-elicited epinephrine release ⁵⁴. In contrast to CRF, the central effects of BN on circulating levels of glucose, glucagon, and insulin were prevented by adrenalectomy ⁵⁷. It is of interest to note that adrenalectomy failed to alter the satiety induced by systemically administered BN ¹²⁴, whereas its effects on centrally administered BN have yet to be tested. While inhibition of stress-induced eating by central administration of BN remained present in adrenalectomized rats, the satiating power of BN was significantly reduced in these animals as compared to normal rats, suggesting the possibility that BN satiety is partially mediated by its action on the adrenal glands ²³⁴. Interestingly, the effect of BN on adrenomedullary epinephrine secretion has been observed following central, but not peripheral, administration of BN ⁵³. This could also explain why no alterations in feeding have been reported following peripheral administration of BN in adrenalectomized rats. Further studies assessing the participation of central BN administration in rats with lesions affecting the autonomic nervous system will help to elucidate the role of adrenomedullary epinephrine in BN-induced satiety.

It is also of interest to note that central administration of BN or CRF have been shown to inhibit norepinephrine (NE)-induced feeding ^{209,258}. Moreover, CRF administration reduces NE turnover in the PVN ¹¹. These findings suggest that the peptides in question may exert an inhibitory control over central NE release. Thus, the prevention of such inhibitory control over NE release through blockade of CRF receptors may contribute to the blockade of BN-induced satiety observed in the present study.

Centrally administered BN potently elicits grooming/scratching activity. This effect was also attenuated in rats pretreated with CRF antagonists. While the BN doses used in the present study did not allow for differentiation between its various behavioral effects, previous studies have revealed that the ingestive effects of BN can be dissociated from its locomotor and grooming effects, suggesting that these effects are mediated by different neural substrates^{113,191}. Qualitatively, BN and CRF-induced grooming appear distinct. Thus, while grooming induced by central BN is principally characterized by intense scratching of the face and neck regions by the hindlimbs with fewer bouts of face and body washing¹²⁸, CRF-induced grooming is mainly characterized by increased body grooming, face washing and forepaw grooming with little or no effect on the scratching component⁹⁹. In the present study, both the washing and scratching components of BN-elicited grooming were attenuated in rats pretreated with either one of the CRF antagonists. Although the mechanism(s) underlying grooming behavior has not been clearly established for either of these peptides, both BN and CRF-induced grooming have been shown independent of pituitary ACTH secretion^{99,128,255}. Diazepam has been shown to attenuate grooming behavior induced by BN (1 µg; i.c.v.) at a presumably non-sedative dose⁸². Moreover, administration of α -helical CRF₉₋₄₁ into the amygdala reversed the anxiety-like effects elicited by the elevated plus-maze or by ethanol withdrawal, at doses which failed to reverse stress-induced pituitary ACTH and corticosterone secretion^{49,300}. In contrast to localized microinjections, i.c.v. administration of such low doses of α -CRF₉₋₄₁ were without effect in the elevated plus-maze²²⁸ and, it is not known whether at higher effective doses this action of the CRF antagonist would remain independent of

activation of the pituitary-adrenal axis. Furthermore, it is noteworthy that the degree to which exogenous administration of CRF potentiates grooming appear strongly dependent on the familiarity of the animal's environment. Thus, i.c.v. doses of CRF that produce marked behavioral activation in familiar environment failed to alter or even inhibited grooming in a novel, potentially stressful environment^{33,98,100,353}. It is thus unlikely that the decreased grooming observed in the present study in rats pretreated with CRF antagonists, is exclusively attributable to anxiolytic properties of these antagonists. Thus, although CRF appears to play a role in BN-induced grooming it is not the only substance involved in the mediation of this complex behavior^{232,288} and, at present, the mechanisms underlying this ubiquitous behavior remains largely unknown.

The specificity of the functional interactions between BN and CRF were further assessed by elucidating potential interactions with OX, a different satiety peptide. We selected OX because this peptide not only suppresses food intake^{14,274} but also induces grooming in rats^{64,274,285}. Moreover, since CRF has been shown to elevate plasma OX secretion⁵⁹ and, OX receptor blockade to antagonize CRF-induced anorexia²⁷², we wished to determine whether OX might be a further link in the chain of biological events contributing to BN-induced satiety. Furthermore, brain OX receptor blockade has been shown to significantly attenuate the satiety effects of systemically administered CCK²⁷¹. In the present study, vasotocin failed to alter BN-induced satiety. Furthermore, OX-induced satiety was not affected by pretreatment with a CRF antagonist. These findings further support the existence of different mechanisms of action for BN- and CCK-induced

satiety; BN-elicited satiety requiring the participation of CRF receptors but being independent of OX receptor participation. These findings are also consistent with the observation that while increased pituitary secretion of OX is observed following systemic administration of CCK, which is thought to contribute to CCK's satiety effects³⁷⁶, BN failed to alter pituitary OX secretion in rats³⁷⁷. It thus seems likely that the effects obtained in the present study are not secondary to blockade of CRF-activated oxytocinergic pathways that induce satiety. Furthermore, the fact that pituitary secretion of OX is observed after many stressors^{121,195} may suggest that CRF-induced OX secretion might contribute more specifically to stress-induced anorexia and not spontaneous satiety. Finally, the inability of CRF receptor blockade to alter OX-induced anorexia suggests that the interaction between these two peptides is not bi-directional and further support the specificity of the functional interaction of BN with CRF.

In conclusion, the present findings suggest the participation of CRF in BN-induced satiety. Our results demonstrated that central blockade of CRF receptors blocked BN's suppressant action on feeding and attenuated the stimulating effects of this peptide on grooming behavior. This pharmacological interaction appears to be specific as BN did not interact with OX and the blockade of CRF receptors failed to alter the anorectic effects of OX. The observation that BN-induced satiety remains unaltered by blockade of OX receptors suggests that BN's anorectic action is not mediated through CRF activation of oxytocinergic neurons. Furthermore, this finding provides additional evidence of the existence of distinct physiological mechanisms underlying BN and CCK satiety effects.

Taken together, our findings suggest that BN may activate central CRF pathways to produce its effects on feeding and related behaviors. It is possible that BN and/or CRF may play a role in the pathophysiology of eating disorders such as anorexia nervosa, cancer anorexia, bulimia and obesity.

General Discussion

The principal objective of this thesis was to try to elucidate the physiological participation and potential mechanism(s) of action of BN-like peptides in the control of food intake. As an initial step towards that objective, we investigated the action of these peptides on feeding during ontogenic development. Thus, we characterized the response profile of neonatal rats to BN and/or its antagonist DesMet. Our findings revealed that BN binding sites are pharmacologically functional and participate in the regulation of feeding from few hours following birth. While the observation that BN binding sites are functional prior to the expression of endogenous BN-like peptides is intriguing its significance is not clearly understood. It is possible, however, that BN-like peptides in maternal milk^{34,157} may provide the ligand for those receptors during development, regulating the amount of food a neonate ingests. Furthermore, it is of interest to note that administration of the BN antagonist (DesMet) alone, failed to potentiate feeding in infant rats until PD 15, when it augmented milk intake. This observation is concordant with the findings of Gillati and Moody, which demonstrated that BN-like peptides become immunohistochemically detectable only in the second postnatal week and reach mature concentrations in the third week postpartum¹²⁷. Since the enhancement of feeding following DesMet administration was of relatively small magnitude, conclusions about the physiological role of these peptides at that stage of development should be made with caution. Interestingly, the efficacy of other BN antagonists to enhance food intake in adult rats has been shown to largely depend upon the animal's state of deprivation and was

clearly evident only in sated or partially sated rats^{115,236}. Thus, it remains possible that in our study, food deprivation may have led to large test meal consumption, causing a ceiling effect (related to physical limit of the stomach), which limited further food intake and might have masked the desatiating effect of DesMet. More research is thus required to ascertain these tantalizing results supporting the physiological participation of these peptides in feeding regulation very early during development. However, these results demonstrate that in addition to the well established evolutionary conservation of the satiety-inducing effect of BN, there is also a developmental continuity of this response, implicating the role of this family of peptides in rudimentary processes regulating ingestive processes. In this context, the distinct ingestive profile of neonatal rats, characterized by repeated ingestion periods at short intervals, may be associated with differences in the availability of BN-like peptides. This possibility is suggested by our observation that *in vivo* levels of BN-like peptides fluctuated differently in situations where a rat ingested food almost continuously over the testing period versus situations where its meals were interspaced by longer intermeal intervals suggesting that different feeding profiles may be associated with distinct patterns of release of satiety peptides. Thus, it is possible that the endogenous availability of satiety peptides may be limited (due to little or no expression) early in life as the neonates are breast fed many times a day and at short time intervals. As the pups get older, endogenously synthesized BN-like peptides are more fully expressed and may provide stronger satiety signals leading to longer intermeal intervals. Such phenomenon could also explain why OX and BN antagonists had relatively small effects on enhanced feeding in our neonatal studies. However, the physical characteristics of

neonates greatly limit experimental manipulations on that population and such hypothesis appears difficult to verify.

Thus, in an attempt to better elucidate the physiological participation of BN-like peptides in the control of ingestion, we examined endogenous fluctuations in BN-like peptides associated with spontaneous feeding in adult rats. Using brain micropunch and radioimmunoassay, we aimed to identify specific loci where peptidergic alterations may be occurring. Fifteen different brain nuclei were micropunched in animals sacrificed in the preprandial, prandial and postprandial ingestive states, in free-feeding (non food deprived) rats. Our results demonstrated site-specific alterations in BN concentrations at the Acb, PVN, Arc-ME and at the DM in relationship to the feeding status. These results strongly imply physiological participation of BN-like peptides in the mediation of feeding. However, alterations in the tissue levels of BN-like immunoreactivity are difficult to interpret since increased tissue peptide levels such as those noted at the PVN during food ingestion may be related to different factors including: 1) increased synthesis of the peptide, and/or 2) decreased release of the peptide, or 3) increased release of the peptide accompanied by even greater rate of replenishment (synthesis). Similarly, decreased tissue levels of the peptide in question may be the result of 1) decreased synthesis and/or 2) increased release (or utilization), or 3) increased synthesis with even greater rate of release. Some of these possibilities are depicted in Figure 1.

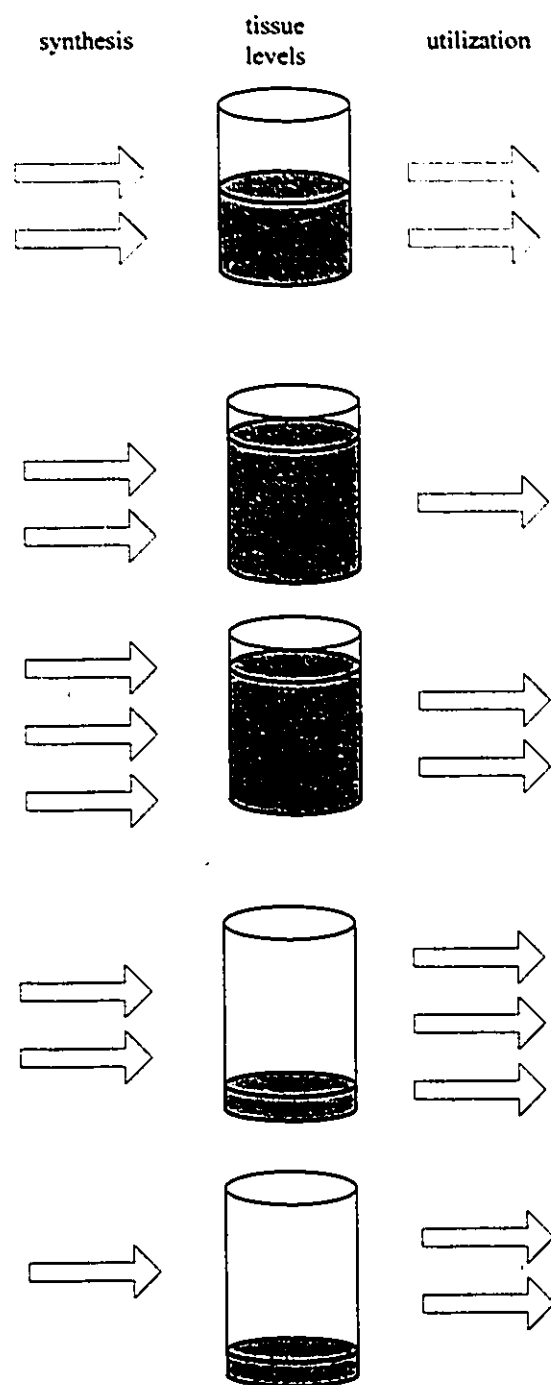


Fig. 1 Relationship between tissue levels, and the rates of synthesis and utilization.

In the next set of experiments, we set out to determine what these meal-related increases or decreases in postmortem tissue levels meant in terms of *in vivo* availability of these peptides at the synaptic level. Thus push-pull perfusion followed by RIAs was used to investigate the *in vivo* release of BN-like peptides during spontaneous feeding. We selected the PVN as the target site for these studies because a) its participation in the regulation of feeding has clearly been demonstrated^{129,186,197,199,387}, and b) our postmortem studies had revealed meal-related fluctuations in endogenous levels of BN-like peptides at this nucleus. Results from this experiment revealed that feeding was associated with a marked suppression of the interstitial availability of BN-like peptides at the PVN. Conversely, the release of BN-like peptides was significantly elevated before and after the meal. These peptidergic fluctuations appeared specific as they were observed at the anterior parvocellular portion of the PVN but not at more posterior PVN implants nor at extrahypothalamic perfusion sites within the caudate putamen. These results further support the role of the PVN in the regulation of food intake and suggest that BN-like peptides released at this brain nucleus may mediate a physiological signal to induce and/or maintain satiety in the rat. From our results, it appears that BN-like peptides do play a role in satiety as increased release of the peptide was observed in the sample following ingestion as well as in consecutive samples and a drop in the peptide levels was accompanied by ingestion in most cases. However, whether BN/GRP peptides participate in satiation processes that bring about the termination of feeding remains to be clearly elucidated. The concentration of BN-like peptides present in our sample and the sensitivity of the RIA used did not allow for sampling intervals shorter than 30 min.

Consequently, we do not know the precise temporal sequence or rapidity of peptide release in context of the meal. However, the amplitude and the robustness of the observed postprandial increases in the release of BN/GRP-like peptides suggest that these peptides might also provide a powerful satiation signal associated with preabsorptive fullness.

Furthermore, findings from this *in vivo* study together with feeding-related alterations in BN concentrations observed at the PVN in micropunched tissue, helped us gain more insight on the physiological mechanisms underlying these effects. Thus, the observation of opposite changes in the content versus release of BN-like peptides at the PVN during each of the feeding states (i.e. increases in the release of BN/GRP peptides versus diminished tissue content of BN both pre- and postprandially, and inhibition in the release accompanied by elevation of tissue content during feeding) indicates that increased release is not immediately accompanied by compensatory upregulation in the peptide synthesis and suggests the existence of a delay between the utilization and replenishment (increased synthesis) of the tissue stores of BN-like peptides. At present, it is difficult to compare our findings with those obtained with other satiety peptides, in terms of whether similar relationship between postmortem tissue levels and *in vivo* release profile holds true, since few studies have assessed the endogenous release of peptides. Moreover, experimental protocols often vary widely between studies, rendering cross-comparisons difficult. For such reasons, it is difficult to interpret the exact meaning of the endogenous alterations in CRF concentrations that we observed in relationship to spontaneous feeding in the micropunched LH, VMH and Ce. Further research assessing the release of CRF

during spontaneous feeding will hopefully shed some light on the exact meaning of the observed endogenous fluctuations in CRF-like immunoreactivity at these nuclei.

The demonstrated feeding-related alterations in BN content and release at specific CNS sites strongly support the physiological involvement of BN-like peptides in appetite control. In our next experiment, we thus aimed to determine the consequences of chronic disturbance of this peptidergic system. Thus effects of sustained administration of BN agonist on spontaneous feeding, behavioral profile, ingestive response to acute BN, as well as changes in BN receptor density within the CNS were assessed. Our results revealed that chronic BN administration significantly reduced feeding during the first 48 to 72 h of infusion in rats, period following which tolerance effects to the peptides became apparent. Concordant with the decreased behavioral efficacy of the chronically infused peptide, autoradiograms revealed a global trend towards down-regulation of the related receptors in several brain regions. At some hypothalamic nuclei, the reduction of receptor density was slight whereas at the PVN and the hippocampal dentate gyrus, a significant drop in the BN-binding site density was elicited by the 7-day sustained administration of BN. Surprisingly, however, upon an acute i.c.v. challenge with BN, no behavioral differences were noticeable between saline- or BN-infused rats; both groups responded to acute BN (0.25 µg; i.c.v.) by decreased ingestion of palatable food and enhanced grooming. These findings suggest that BN neurons remain responsive to marked changes in peptide level fluctuations such as those present in acute or "emergency-like" situations. Whereas the animal may show tolerance to sustained "steady" levels of the same peptide. Other physiological systems may also function in a similar way. For example, while many

individuals suffering from chronic pain become able to abstract from this pain and participate in some daily activities, these people would still feel the pain if for instance, they suddenly hit their thumb with a hammer. Interestingly, these types of pain have also been associated with distinct pharmacological responses to morphine administration ^{1,3}. Thus, the apparent lack of tolerance to acute morphine administration in animals models of tonic pain contrasts with the rapidly developing tolerance to morphine of rats exposed to phasic pain episodes ¹. These findings suggested the existence of distinct neuronal pathways mediating tonic and phasic pain ^{2,3}. In a similar fashion, it is possible that the intact behavioral response observed in our study following acute BN challenge in rats chronically infused with BN, may be related to the existence of different neural systems mediating acute “emergency-like” situations versus long term regulation of food intake. These findings combined with enhanced feeding observed following infusion of BN antagonists suggest that BN-like peptides may physiologically contribute to spontaneous feeding regulation. The return to baseline daily ration of spontaneous food intake within 48 to 72 h of sustained BN infusion is biologically adaptive and, suggests that other endogenous signals, peptidergic or not, may compensate for the attenuated physiological input of this peptidergic system. The recent development of oligonucleotide antisenses to mRNAs for BN-like peptides receptors appear as very promising tools to further determine the physiological role of BN-like peptides in long-term regulation of food intake. In light of the anxiogenic-like responses of the rats chronically infused with BN as compared to saline-treated rats, such technique could also be used to better characterize

the physiological contribution of these peptides in anxiety and/or stress-related processes, as well.

Numerous neurotransmitter, metabolic and peptidergic signals have been proposed as instrumental in the control of ingestion. In the introduction, examples of pharmacological interactions between some of these endogenous signals have been discussed. The final set of experiments presented in this thesis were prodded by existing similarities between the behavioral, endocrine, autonomic and ingestive actions of BN and CRF which led us to investigate potential interactions between BN and CRF in the regulation of food intake. Our results support the existence of pharmacological interactions between the two peptidergic systems as central blockade of CRF receptors reversed BN-induced satiety and related behaviors. Such an interaction appeared specific as central blockade of CRF receptors failed to alter the OX's suppressant effects on feeding. Moreover, BN did not interact with the satiety peptide OX, as central blockade of OX receptors failed to alter BN-induced satiety. At present, the exact physiological mechanism(s) by which these peptides regulate feeding is not known. Interestingly, adrenalectomy significantly attenuated the satiating effects of CRF, an effect shown attributable to adrenomedullary epinephrine secretion ¹³¹. Although adrenalectomy failed to alter the effects of peripherally administered BN, the role of the adrenal glands on feeding suppressant effects of centrally administered BN are not known. Interestingly, elevated epinephrine levels have been reported following central but not peripheral administration of BN. Furthermore, the observation that central blockade of CRF attenuated BN-induced satiety suggests that this interaction may be mediated, at least in

part, within the CNS. The mechanisms subserving the action of these peptides on feeding and/or their pharmacological interaction remains to be determined and warrant further research. In this context, studies assessing the *in vivo* release of CRF following administration of BN could be performed to verify the contention that BN may induce satiety partly through endogenous release of CRF. CRF-like immunoreactivity has now been successfully measured in microdialysis perfusates^{238,287} and this technique could be used to measure the release of CRF following central and/or peripheral administration of BN. In light of anatomical connections between the NTS and PVN³⁰⁴, the demonstrated role of these brain nuclei in feeding^{162,186,197,387} as well as the presence of BN and CRF immunoreactivity and binding sites at these loci^{68,281,334,357}, one could possibly microinject BN into the NTS and verify its impact on endogenous release of CRF from the PVN. Interestingly, oxytocinergic neurons in the parvocellular portion of the PVN have been shown to send projections to the amygdala⁶¹. It is not known whether similar pathways may exist for BN-like peptides. However, findings from our micropunch study revealed significant feeding-related alterations in BN content at the PVN while similar patterns of CRF-like immunoreactivity were observed at the Ce in relationship to feeding status in the same study. These reductions in the tissue content of BN have been shown to correspond to increased release of BN-like peptides pre- and postprandially while reduced release has been characterized during ingestion. Thus, it may be possible that the observed fluctuations in CRF-like immunoreactivity at the Ce result from a priori changes in BN-like peptides at the parvocellular portion of the PVN. This possibility could be verified

through push-pull perfusion or microdialysis experiments combined with RIA for the detection of CRF release from the Ce following BN infusion at the PVN.

It may also be possible that like the pharmacological interactions observed between CCK and 5-HT, interactions between BN and CRF are bi-directional, an assumption that also needs to be further investigated. In this context, Dourish and Cooper have proposed a model based on assumptions of cooperativity and interdependence between CCK and 5-HT⁷². Thus, the cooperativity assumption states that CCK and 5-HT exert reciprocal influences on each other such that elevated CCK release following ingestion will enhance 5-HT release and action, and vice-versa. The interdependence assumption of the model suggest that CCK and 5-HT act as interdependent parallel systems which individual actions at distinct receptor sites are both required for satiety be fully expressed. Consequently, blocking either component will affect the ability of the other component to inhibit food intake. Further studies characterizing the effects of BN receptor blockade on CRF-induced anorexia are required to determine whether interactions between BN and CRF are bi-directional. However, our studies clearly show that blockade of CRF receptors block the satiety effects of exogenously administered BN. Thus CRF receptor based system seems to play a permissive role in the full expression of satiety effects of BN (see Figure 2). Moreover, in order to ascertain whether the actions of these peptides on feeding are mediated in part through mutual cooperativity, one needs to determine the influence of one peptide on the endogenous release of the other.

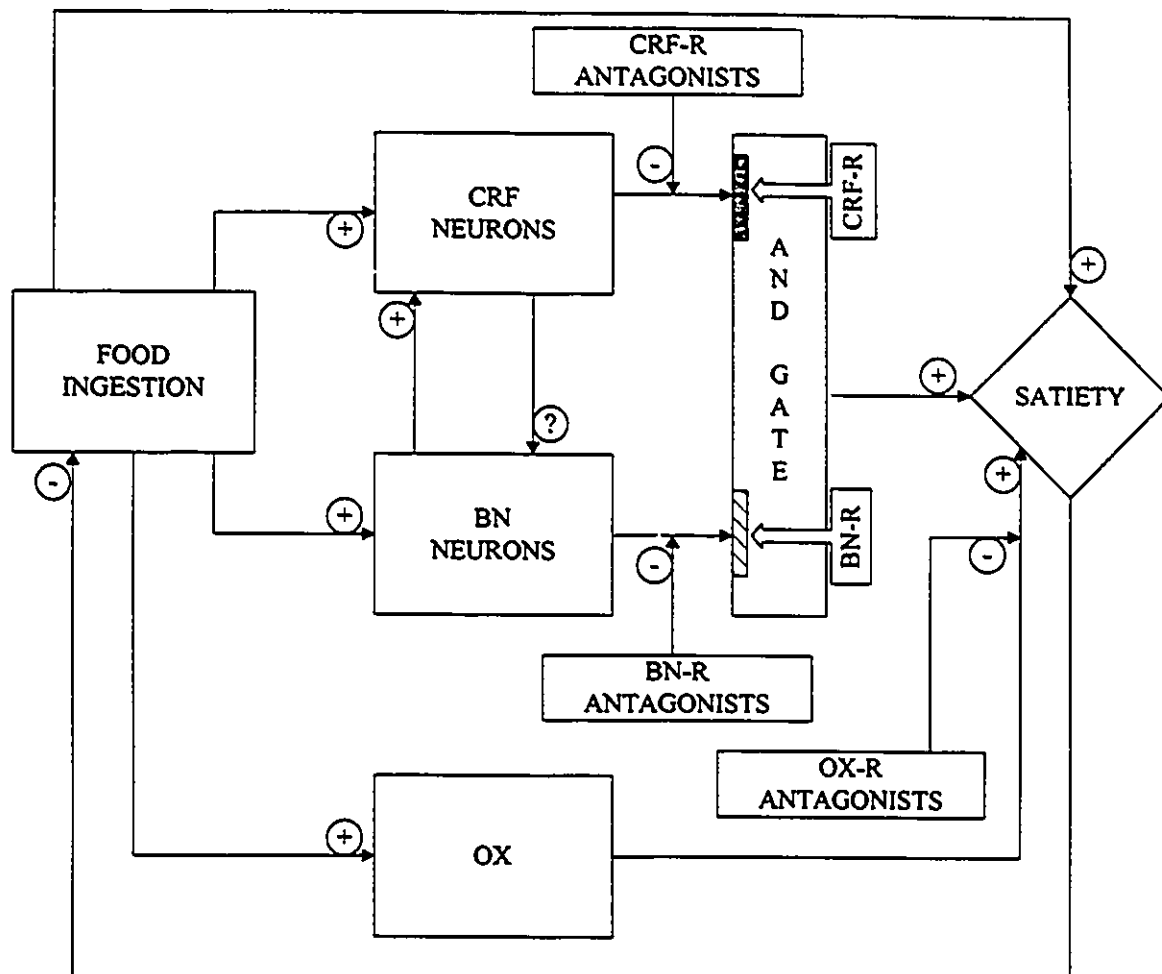


Fig. 2 Schematic model illustrating proposed mechanism(s) by which BN and CRF may exert their action on feeding regulation.

Conclusions

In conclusion, our findings from this multiple faceted study provide novel and strong support for the contention that BN-like peptides may act as physiological satiety signals, and shed some light on the potential mechanism(s) by which BN-like peptides elicit their effects. The data from the above experiments when considered as a whole yield the following specific conclusions:

- 1) Receptors for BN and OX are pharmacologically functional and participate in the regulation of feeding related behaviors as early as 1 day after birth. BN is thus able to induce satiety prior to measurable expression of BN-like peptides, in neonatal rats. One could speculate from these observations that BN-like peptides in maternal milk may provide exogenous ligand(s) for those receptors during ontogenic development, regulating the amount of food the neonate consumes.
- 2) In this study, we determined endogenous fluctuations in BN and CRF concentrations in relation to feeding status. Changes were characterized in fifteen distinct hypothalamic and extrahypothalamic nuclei of *ad libitum* fed rats and represent peptidergic alterations associated with a spontaneous meal. To our knowledge, this is the first study to report meal-induced fluctuations of CRF content and, the first demonstration of changes in BN concentrations in discrete brain nuclei in relationship to spontaneous feeding. BN-like peptides at

the hypothalamic PVN, DM and Arc-ME were reduced during the pre- and postprandial ingestive states as compared to levels during ingestion. In contrast, meal-related changes in CRF content were restricted to the LH, VMH and Ce, and varied differently, based on the nucleus. These data clearly indicate distinct locus- and peptide-specific endogenous fluctuations in BN and CRF concentrations over the course of the first spontaneous meal of the dark phase, implying their involvement in regulation of food intake.

- 3) The observation of *in vivo* variations in the PVN BN-like peptide release associated with spontaneous feeding provide new insights on the physiological mechanism underlying BN-like peptide action and, suggest that hunger state, meal anticipation and/or meal initiation may be associated with an inhibition of BN-like peptide release whereas satiety is concomitant with an increase in the release of BN/GRP like peptides. These results further support the hypothesis that BN-like peptides may mediate a physiological signal in the PVN to induce and/or maintain satiety in the rat.
- 4) In this study, we demonstrated the effects of sustained infusion of BN on a) daily spontaneous ingestive pattern, b) the rat's behavioral and ingestive response to an acute i.c.v. challenge with BN, c) anxiety-like behaviors and, d) BN receptor binding profile in various brain regions. Our findings revealed a significant satiating effect of BN infusion on spontaneous ingestion over the

initial two days of chronic infusion. This effect was no longer present 72 h post-infusion. Upon acute administration of BN (0.25 μ g; i.c.v.), both chronically BN exposed and control rats responded by decreased feeding and enhanced grooming behaviors. BN-treated rats also appeared more anxious as they spent less time in the open or “anxiogenic” portions of the mazes. Following chronic BN, significant down-regulation of BN receptors was observed at the PVN and dentate gyrus. These findings represent the first demonstration of changes in receptor based and behavioral responses consequent to sustained BN exposure. Furthermore, our results suggest different mechanisms mediating the acute “emergency-like” versus the long-term effects of BN.

- 5) Our findings suggest the participation of CRF in BN-induced satiety, as central blockade of CRF receptors blocked BN’s suppressant action on feeding and attenuated the BN-elicited grooming response. This pharmacological interaction was specific as BN did not interact with OX and the blockade of CRF receptors failed to alter the anorectic effects of OX. The observation that BN-induced satiety was unaffected by blockade of OX receptors suggest that BN’s action is not mediated through CRF activation of oxytocinergic neurons. Taken together, our findings suggest CRF receptor based systems may play a permissive role in full expression of BN-elicited behaviors.

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