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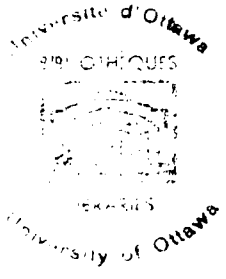
Rapid Alterations in the Levels of Bombesin-Like Peptides and Their Receptors in Response to a Single Meal

Claude Christian Kateb



A thesis submitted to the School of Graduate Studies of the University of Ottawa as partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Acknowledgments

"The fool fails by behaving without regard to his condition, position, origin, or friendships"
Gracian, "The Art of Worldly Wisdom", 1647.

I would especially like to thank my mentor, supervisor, and friend Dr. Zul Merali. You've put up with my fits, my impatience, my dishes, my dog - the list goes on. I've been extremely fortunate to have worked with you. You were always there to guide and support me, to "roll up your sleeves" when the situation called for it, and to listen when I needed you to. You have always been, both personally and academically, forthright, honest, and dedicated. I can only hope that I have learnt well from you. Thank you for showing me that the right way is seldom the easy way, and that persistence will lead to success. But mostly, thank you for always treating me as a friend first, and student second.

I would like to thank my family for never faltering in my eyes. To my parents, Cyril and Josephine Arnold, I extend my deepest gratitude for your support and encouragement, and especially for going through the paces of my frustrations with me as if they were your own. Whether I was right or wrong, you have been in my corner throughout. Thank you for being there when I needed you most, and for never losing faith in me.

I would also like to thank my sister, Claudette Somogyvari. You have never allowed me to achieve less than I was capable and have stood silently and steadfastly beside me for longer than I can remember. There have been many times I have succeeded, only because I knew you believed I could. You are now, more than ever, a better sister and friend than I could ever hope for. Thank you for never letting me fall short of the mark.

Special thanks go to my brother (in-law), Frank Somogyvari, my big brother for the last 23 years. You taught me to play chess at eight, to assist you in rat surgery at ten, and to drive at sixteen. You also taught me the difference between accuracy and precision, a distinction which is particularly salient to my chosen career. I could not have chosen a smarter, more capable, or better man to emulate than yourself. Thank you for always giving my energies a focus and a purpose.

To my nephew, Philip, and my niece, Diana, thanks for being so perfect! And thanks for thinking that I am (... almost). Maybe now I'll finally have time to let you beat me at Nintendo!

To all my friends, including Hugh, Qadeer, Hélène, Sue, Pam, Bobbie, Judith, Tamara, Monika, Lisa, Janet, and Thomas - thanks for the coffees, the hallway heart-to-hearts, and the friendship. You guys are the best! A special thanks to Pierre, Kate, and George for being such good friends and always having my best interests at heart.

Paul, Pino, Tony, and Dino - Lest we forget our roots! Thanks guys!

I would also like to thank my thesis proposal committee members, Dr. Catherine Bielajew, Dr. George Fouriezos, and Dr. Simon Lemaire, for their much needed advice and guidance during the preparation of the research project.

I saved the best for last. For her limitless patience, tolerance, and support I wish to thank my best friend, my wife, my "sweetie" Catherine Gow. Thanks for encouraging my every effort, for never allowing me to feel defeated, and for being there, beside me, every step of the way. You are my greatest pride and joy, my inspiration, and the brightest light in my life. Thank you for all that you've done to make this happen, and thank you for having shared it with me.

Yes Trista, *now* we can go to the park.

Abstract

It has been suggested that bombesin (BN)-like peptides may play a physiological role in the control of food intake. The working hypothesis behind the experiments presented in this dissertation was that if this family of peptides is involved in the mediation of satiety, then the availability of the peptide(s) and/or its receptors should change with feeding status. Thus, the specific objectives of this research were to elucidate the changes in the availability of central and peripheral BN-like peptides and their receptors during a meal. In our preliminary experiments, all rats were food deprived for a 12 hr period. Half the animals were then permitted to eat solid food until satiated (35 min). Techniques of Radioimmunoassay (RIA) and quantitative autoradiography (QAR) were employed to measure peptide and receptor levels respectively in several brain and gut sites. Our results indicated significant increases in BN-like peptide levels, specifically gastrin-releasing peptide (GRP) GRP_{1-27} and/or GRP_{18-27}, in the hypothalamus, hippocampus, and antral region of the stomach after food ingestion. The autoradiography study indicated an effect of food intake on central, but not gastrointestinal, BN-like peptide receptors, but failed to identify specific loci where these differences were significant between feeding states. The objective of the next series of experiments was to elucidate the pattern and degree of change in the levels of BN-like peptides and their receptors at different timepoints during different feeding states. These experiments included four groups of animals: 1) Rats that were food deprived for a 12 hr period 2) and then given access to food for either 10 min or 3) 35 min. The fourth group constituted of non-deprived controls (*ad libitum* fed). RIA of hypothalamic, hippocampal, and medullary (control) tissues replicated and extended our earlier findings and showed that, in relation to the *ad libitum*

condition, hypothalamic BN-like peptide concentrations were reduced by fasting, while hippocampal levels were elevated by feeding. The autoradiographic studies of several central areas demonstrated decreased binding in the hypothalamus, fundus striatum, and nucleus accumbens of the postprandial animals, as compared to the food deprived group. In the next experiment, an attempt was made to identify whether the changes noted at the receptor level were related to changes in the receptor number or affinity. The saturation experiments did not reveal significant alterations in the K_D and B_{max} of BN-like peptide receptors in response to food intake. Thus, the mechanisms underlying these receptor changes were not successfully established. The rapid alterations in peptide and receptor levels may support the contention that BN-like peptides play a physiological role in the regulation of ingestive behavior.

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Rapid Alterations in the Levels of Bombesin-Like Peptides and Their Receptors in Response to a Single Meal

There has been a large body of research aimed at understanding the mechanisms by which food ingestion is regulated in mammals. During the course of this research, and of research in other domains, scientists have intentionally or serendipidously uncovered a number of substances that affect the ingestive process. Throughout the course of this doctoral research, our intent had been to elucidate whether BN-like peptides are involved in the control of ingestion, and to explore the underlying mechanisms by which these effects may be elicited. Although BN-like peptides are amongst several neuropeptides known to affect food intake, they have a unique behavioral and physiological profile which sets them apart from the others. In the review that follows, I will first briefly describe some of the so-called "classical neurotransmitters", namely the dopaminergic, noradrenergic, and serotonergic systems, which are thought to play a role in ingestive regulation. A brief description of the effects of a few representative peptides on food intake will follow, namely the opiate peptides, cholecystokinin (CCK) and glucagon, with a focus towards possible sites of action. The introduction will continue with an overview of past and recent studies involving BN-like peptides, with a separate section outlining and discussing their potential role in the control of food ingestion. Finally, the objectives and hypotheses of the present research will be outlined.

The study of the control of appetite has proven to be an exceedingly complex pursuit, implicating the involvement of several neurotransmitter systems, various regulatory substances, including gastrointestinal peptides, and the

concurrent modulation of related physiological and psychological mechanisms such as sensory taste stimuli, reward, gastric function, and nutrient content. The now extensive pool of literature in this domain has repeatedly illustrated that the comprehensive intercorrelation of these systems and mechanisms is as elusive a goal as is their differentiation. This is not to say that research has not been fruitful in elucidating their potential roles in the control of ingestion, but only to stress that each progressive study, in providing often critical insight into the possible underlying mechanism(s) of appetite regulation, plays an active role in the increasing complexity of the system under investigation. This preamble is meant to introduce the following sections, which attempt to offer a largely simplified overview of the mechanisms thought to be involved in the regulation of food intake, primarily in the laboratory rat. We do not pretend to have addressed all relevant aspects of the research domain, but only to offer a broad written and schematic representation of what is generally accepted or understood. We begin with an outline of the proposed roles of neurotransmitters in the central control of ingestion.

Central Neurotransmitters and the Control of Food Intake

Dopamine

Dopamine (DA) is one neurotransmitter that has been associated with many aspects of feeding behavior. Figure 1 shows the origins and projections of the mesolimbic, mesocortical, and nigrostriatal dopaminergic systems. The mesocorticolimbic system originates in the ventral tegmental area (VTA) and projects through the medial forebrain bundle (mfb) to the nucleus accumbens (Acb), amygdala, bed nucleus of stria terminalis (Bst), olfactory tubercle (TU), and various regions of the cortex. Cells from the ventral striatum, hypothalamus, and preoptic area project into the VTA, and seem to function at least in part through the release of γ-aminobutyric acid (GABA).

Neurotensin, an excitatory neuropeptide mainly associated with the DA system, and noradrenergic terminals in the VTA region have been suggested to be active in the regulation of DA cell activity in the VTA. Other projections from the VTA are those in the mesocortical DA system, innervating the septum, prefrontal and amygdaloid cortices, as well as the hippocampus. Elevations in DA levels in the prefrontal cortex have been shown to occur in response to feeding, suggesting a role for cortical DA in the modulation of food ingestion (Hernandez & Hoebel, 1990). The authors of that study suggest that such a role may be related to the cognitive aspects of searching for food reward, or to the cortical control of lower brain stem feeding nuclei, such as the nucleus tractus solitarius (NTS), which receive projections from the prefrontal cortex (van der Kooy et al., 1982).

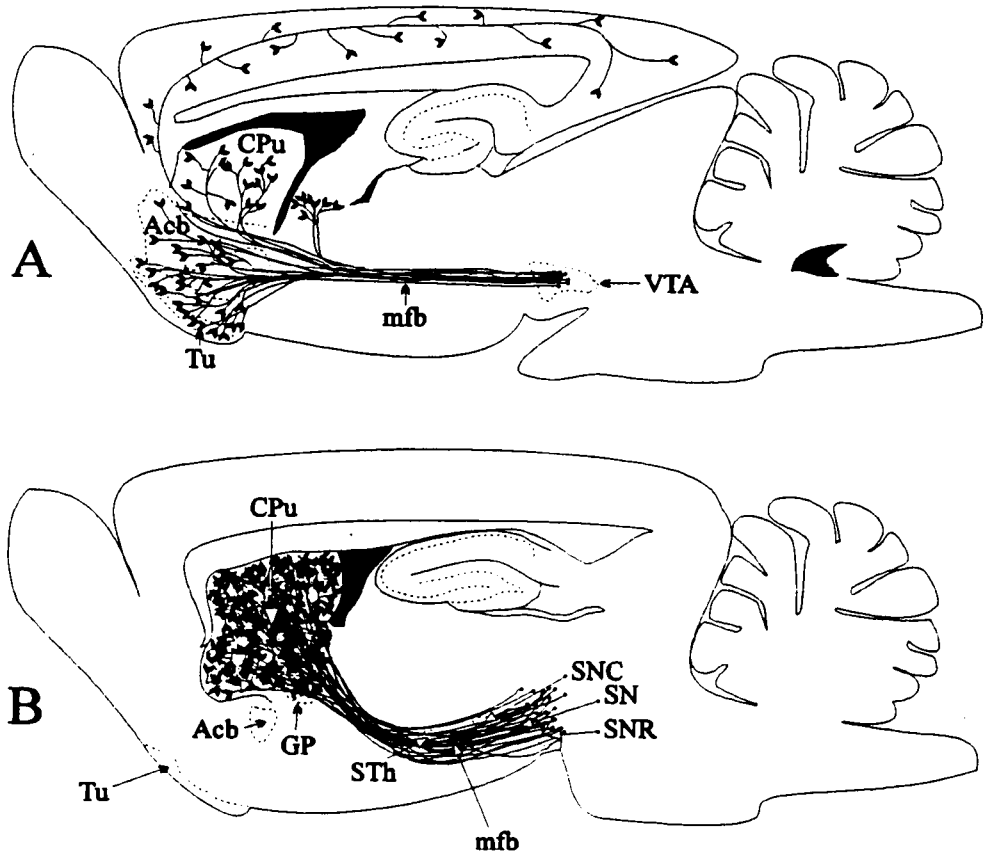

Figure 1. (A) Drawing of a sagittal section of a rat brain showing the mesolimbic and mesocortical DA pathways at 2.4 mm lateral to the midline. (B) The nigrostriatal DA pathway at a level 1.4 mm lateral to the midline. VTA, ventral tegmental area; Tu, olfactory tubercle; CPu, caudate putamen; Acb, nucleus accumbens; SN, substantia nigra; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; STh, subthalamic nucleus; GP, globus pallidus. (Adapted from "Dopaminergic systems in the brain and pituitary" by K. Fuxe et al., 1985. In "The Dopamine System", ***Basic and Clinical Aspects of Neuroscience***, 1, p. 15.)

Mesocorticolimbic DA neuron activity has a considerable role in the activation of motor function (such as locomotion and exploratory behavior), especially in the Acb. Understimulation of this system results in decreased locomotion and exploration, and inhibits operant behavior, implicating DA function in the Acb in the regulation of motivated behaviors (Mogenson, 1982; Wise, 1982; Hoebel et al., 1989). DA antagonists directly applied to the Acb have been shown to prevent operant response to i.v. self-injection, food, and self-stimulation, strongly associating this brain site with reinforcement mechanisms and suggesting that the mesolimbic system is a necessary component of reinforcement behavior (Hoebel et al., 1989; Corrigall et al., 1992; Fibiger et al., 1992). Conversely, food reward, cocaine or amphetamine administration, and electrical stimulation of the lateral hypothalamus (LH) have each been shown to increase extracellular DA in the Acb (Hernandez & Hoebel, 1988). Mogenson et al (1980) have suggested that the Acb is a central component of limbic-motor communication, through which emotional and motivational factors influence motoric behavior. An example of such an influence is indicated by the attenuation of amphetamine-induced operant behavior in the Acb in response to basolateral amygdala lesions (Everitt et al., 1989; Cador et al., 1991). These results would suggest that the amygdala may provide direct input to the Acb concerning the status of reinforcement, which is then translated into action by DA-dependent processes (White, 1989; Blackburn et al., 1992).

Nigrostriatal pathways originate primarily in the substantia nigra (SN) and travel through the mfb, densely innervating the caudate putamen (CPu), and partially innervating the globus pallidus (GP). The SN receives inputs from GABAergic pathways and from neuronal systems containing substance P or

dynorphin, an endogenous opiate. Further, the cell bodies within the SN possess highly immunoreactive neurotensin nerve terminals and contain receptors for neurotensin. Neuronal fibers leaving the SN pass through the LH *en route* to the CPu. Lesions of the SN or destruction of nigrostriatal fibers with 6-hydroxydopamine (6-OHDA) have been shown to produce aphagia, adipsia, and a decrease in motor behavior (Ungerstedt, cited in Carlson, 1986; Parker et al., 1991; Nitsch et al., 1993). Electrical stimulation of the SN has been shown to be reinforcing in operant paradigms, potentially implicating the nigrostriatal system in reinforcement mechanisms (Schwarting et al., 1991). It is also possible, however, that such stimulation produces its reinforcing effects through the comparatively few SN-projecting fibers that are part of the mesolimbic dopaminergic system. Both the nigrostriatal and mesolimbic systems send projections through the mfb, yet 2-[^{14}C]deoxyglucose studies showing changes in local energy metabolism during electrical brain stimulation reward of the mfb indicate metabolic activation in primarily mesolimbic areas such as the Acb, olfactory tubercle, lateral septum, and VTA (Porrino et al., 1990).

Norepinephrine

There are two major noradrenergic pathways in the brain. The central tegmental tract originates in the medulla and in the pons, ventral to the locus coeruleus (LC), and terminates almost exclusively in the hypothalamus. The dorsal tegmental bundle, the second major pathway, originates in the LC and projects to neocortical regions, the hippocampus, thalamic nuclei, cerebellar cortex, and the medulla. Figure 2 illustrates both noradrenergic pathways. Both pathways, similarly to dopaminergic systems, travel rostrally in the brain through the mfb. Exogenous norepinephrine (NE) stimulates food intake when injected into the dorsomedial hippocampus, mammilary body, anterior thalamus, cingulate gyrus, lateral septal nucleus, and midline thalamic nuclei (Coury, cited in Milner, 1970), as well as in the ventromedial and paraventricular hypothalamic areas (VMH and PVN) (Leibowitz, 1978; Lichtenstein et al., 1985; Shimazu et al., 1986; de Rooy & Coscina, 1990; Ravazio & Paschoalini, 1992). Conversely, central NE receptor antagonism attenuates the stimulatory effect of NE on feeding (Capuano et al., 1992; Kurose et al., 1992). Disturbances in plasma and cerebrospinal fluid NE levels have been reported in normal-weight bulimics, reflected by elevated levels during binging and vomiting periods and reduced levels during abstinence from binging and vomiting, when compared to normal, non-bulimic subjects (Kaye et al., 1990; Jimerson et al., 1992). The PVN is highly immunoreactive to NE, and NE neurons from the NTS and dorsal vagal motor nucleus are the major source of noradrenergic innervation of the hypothalamus via the central tegmental tract (Steffens et al., 1988). Injection of NE into the PVN is 10-fold more potent at stimulating food intake than in the third ventricle (Capuano et al., 1992).

Interestingly, however, noradrenergic stimulation of the LH inhibits feeding (Leibowitz & Brown, 1980).

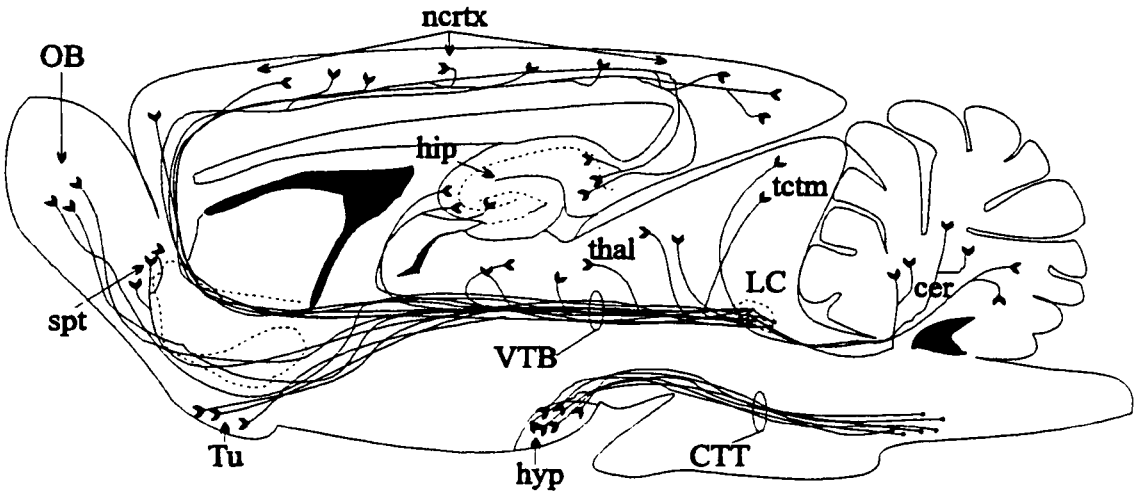

Figure 2. Drawing of a sagittal section of a rat brain showing the central tegmental tract (CTT) and ventral tegmental bundle (VTB) of the NE pathways. cer, cerebellum; tctm, tectum; thal, thalamus; hyp, hypothalamus; hip, hippocampus; ncrtx, neocortex; spt, septum; OB, olfactory bulb. (Adapted from "Classical transmitters and neuromodulators" by J. K. McQueen, 1985. In "Transmitter Molecules in the Brain", ***Basic and Clinical Aspects of Neuroscience***, 2, p. 11.)

Serotonin

Serotonin or 5-Hydroxytryptamine (5-HT) neurons are located in the raphé of the midbrain, and in the medulla oblongata (projecting to the spinal cord and lower brain stem). Cells in the dorsal raphé nuclei give rise to extensive projections to the forebrain, cerebral cortex, and hippocampus, as well as caudal projections to the cerebellum. There also appear to be 5-HT-containing cell bodies in the hypothalamus, substantia nigra, and spinal cord. Figure 3 illustrates these projections.

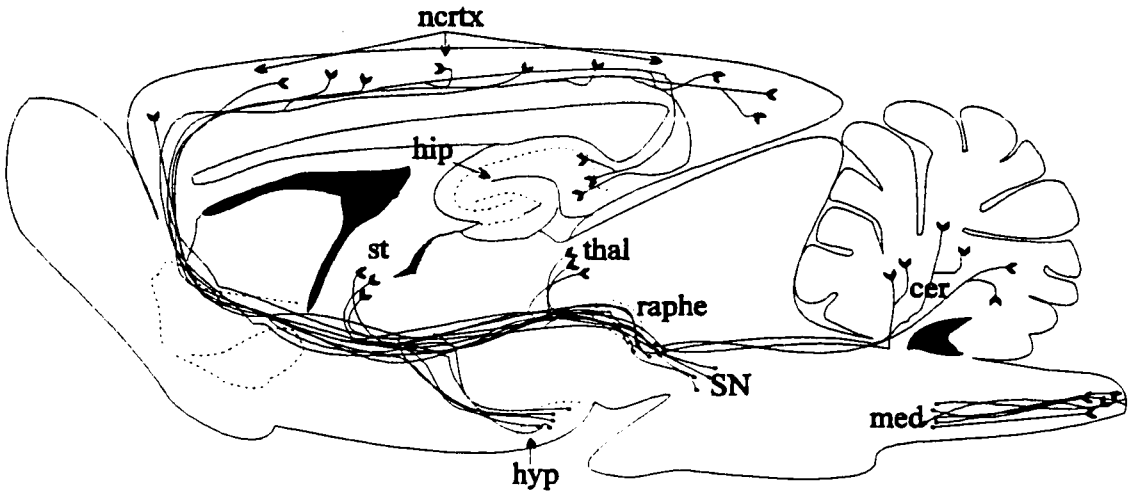

Figure 3. Drawing of a sagittal section of a rat brain showing the main 5-HT-containing pathways. med, medulla; st, stria terminalis. (Adapted from **Principles of Neural Science** (2nd ed.), by E. R. Kandel & J. H. Schwartz., 1985, p. 559)

Central administration of serotonergic agonists have been shown to decrease food intake in rats (Nielsen et al., 1992; McGuirk et al., 1992).

Conversely, intracerebroventricular (i.c.v.) injection of serotonergic antagonists stimulate ingestion (Stallone & Nicolaidis, 1989). Hoebel and colleagues (1989) have shown through microdialysis that, during a meal, an increase in the 5-HT metabolite, 5-HIAA, can be detected in the PVN. Orosco & Nicolaidis (1992) have similarly shown elevations in both 5-HT and 5-HIAA tissue levels during food consumption in the rat. Conversely, decreased levels of central 5-HT and 5-HIAA have been observed during periods of food deprivation (Bubenik et al., 1992). Exogenous CCK (administered centrally or systemically) significantly increases striatal and hypothalamic 5-HIAA levels (Gourch et al., 1990). Antagonism of CCK-A receptors has been shown to block the anorectic effect of 5-HT, suggesting a peptidergic involvement in serotonergic anorectic mechanisms (Cooper et al., 1992; Cooper & Dourish, 1990). Conversely, systemic pretreatment with a 5-HT agonist has been shown to significantly attenuate the satiating action of systemically administered CCK (Poeschla et al., 1992). Further, BN, as well as its structurally related mammalian homologues gastrin-releasing peptide (GRP_{1-27}) and neuromedin B (NMB_{1-32}), depolarize 5-HT neurons of the dorsal raphé, in an *in vitro* preparation (Pinnock & Woodruff, 1991). Saporito and Warwick (1989), in an *in vitro* hypothalamic preparation, have shown that BN and GRP_{18-27} decrease the evoked release of 5-HT, and that none of these BN-like peptides affected reuptake of 5-HT. The authors of that study suggest that BN-like peptides may alter neurotransmission in the hypothalamus by acting presynaptically on the 5-HT release mechanism.

Neuropeptides and the Stimulation/Inhibition of Ingestion

Of the neuropeptides known to affect food intake in the rat, those receiving the most attention in the literature have been CCK, glucagon, somatostatin, neurotensin, BN, neuropeptide Y, and the opiates. In this section, a brief outline of the stimulating effects of the opiates on feeding, and the inhibition of feeding by CCK and glucagon will be presented. These descriptions will be intentionally simple and selective. The rationale for this approach is, as it has been for the preceding sections, to indicate only those aspects of the literature that may shed light on our results with BN-like peptides. Almost certainly, other substances and mechanisms play an integral role, not only in the regulation of feeding, but in the elucidation of our experimental results. These alternate systems, as well as those discussed in this introduction, will be addressed in the body of the document as our experimental data necessitates.

The Opiates

The endogenous opiates, or endorphins (*endo*genous + mo*rphine*) are a group of peptides that bind to opiate receptors in various brain regions and thereby, like morphine, raise the pain threshold (induce analgesia). These peptides originally included the enkephalins Met^5-enkephalin and Leu^5-enkephalin (Hughes, 1975). Within a short period, a total of three endorphin ligand families had been identified, and are identified by their precursors: β-endorphin/ACTH precursor, enkephalin precursor, and the dynorphine precursor (Levine et al., 1985; Cooper et al., 1982). The brain, pituitary, and gastrointestinal (GI) tract each contain enkephalin and β-endorphin, in varying loci and concentrations.

Extremely high β-endorphin concentrations have been located in the intermediate lobe of the pituitary gland. Neurons containing β-endorphin seem to be present exclusively in the hypothalamus, with processes extending within the hypothalamus and to the septum, periaqueductal grey, and pons (Cooper et al., 1982).

Injection of opiates has been generally shown to stimulate feeding (McKay et al., 1981; Gosnell et al., 1984; McLean & Hoebel, 1983). β-endorphin infusion into the PVN and VMH results in a marked increase in food intake (McLean & Hoebel, 1983; Tepperman & Hirst, cited in Levine 1985). I.C.V. infusion of dynorphin peptides have also been shown to stimulate feeding responses in animals (Baldwin et al., 1990; Della-Fera et al., 1990; Inui et al., 1991). Conversely, central infusion of antibodies to dynorphins has been shown to suppress feeding (Carr & Bak, 1990). Several other studies have demonstrated the enhancement of feeding response in the presence of opiates at central sites (mainly hypothalamus, perifornical area, and amygdala), as well as inhibition of feeding in response to opiate-receptor antagonism. The literature is extensive and complex, involving the differential potencies of, not only opiate subtypes, but also of several receptor types (κ, δ, ε, σ, μ). These peptides have been linked to the NE system through studies that indicate attenuation of opiate-induced feeding by noradrenergic receptor blockade (Leibowitz & Hor, cited in Ritter, 1986), and by increases in NE turnover in the PVN in response to morphine infusion (Jhanwar-Uniyal et al., cited in Leibowitz, 1986). The effects of opiates on feeding have also been shown to be associated with DA function in the brain. Opiate antagonists will inhibit DA-mediated feeding (Fletcher, 1991). As well, chronic amphetamine administration has been shown to potentiate the stimulatory effect of

morphine on feeding (Nencini & Stewart, 1990). Figure 4 shows central loci in which feeding is stimulated with opiate agonists, and attenuated with the opiate receptor antagonist, naloxone.

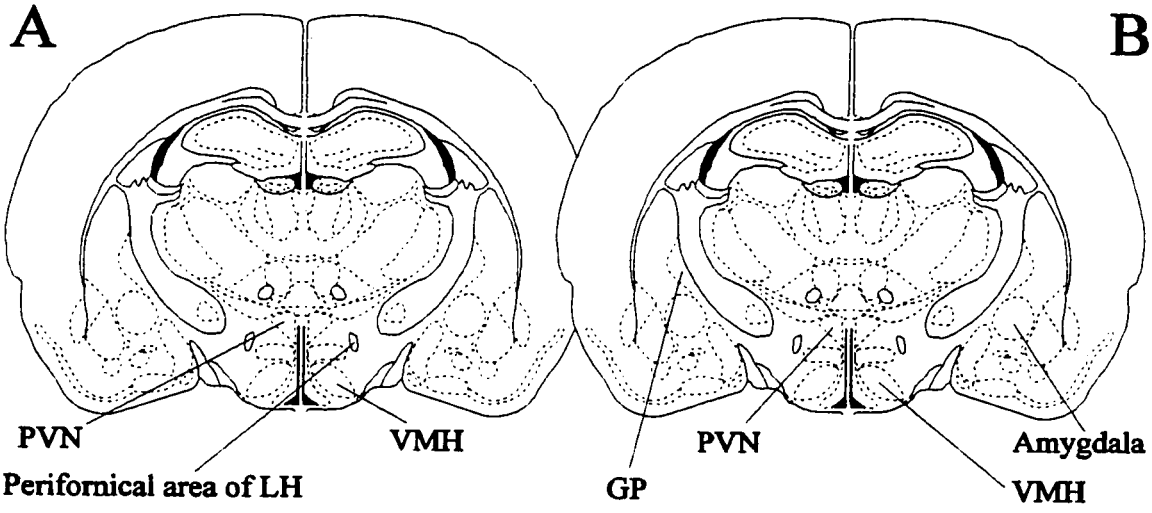

Figure 4. A) Areas in which opiate agonists increase feeding. B) Areas in which the opiate receptor antagonist naloxone decreases ingestive behavior. (Adapted from "Opioids and consummatory behavior" by A. S. Levine et al., 1985, ***Brain Research Bulletin***, 14, p.666.)

Cholecystokinin

In 1981, Kisseleff and colleagues showed that CCK could inhibit feeding in human subjects. These were not the first studies involving CCK and feeding suppression, but the result of several years of research that had begun with the initial report of feeding suppression by CCK in rats almost a decade earlier (Gibbs et al., 1973). Initially, CCK was isolated as a 33 amino acid peptide by Mutt and Jorpes (cited in Rehfeld et al., 1985), and showed homology to the C-terminal decapeptide sequence of gastrin. The C-terminal octapeptide fragment (CCK-8) is the predominant form of CCK found in the brain and periphery, and is most commonly found (99%) in its sulphated form (Dockray, 1976; Rehfeld, 1977). Therefore the concensus has gradually developed that CCK is essentially synonymous with CCK-8 (Rehfeld, 1985). Researchers have suggested that a large, 58 amino acid peptide (CCK-58) is the major circulating form of CCK in humans (Eberlein et al., 1987; Eysselein et al., 1990). CCK has been shown to inhibit feeding, through either peripheral or central routes of administration by several researchers over the last two decades (Gibbs et al., 1973; Bellinger & Bernardis, 1984; Conover et al., 1989, to name a few). Interestingly, although either central or systemic infusion of CCK reduces meal size in non-satiated animals, abdominal or selective gastric vagotomy abolishes peripheral CCK effects in this regard (Smith et al., 1981a). The selective lesioning of vagal afferent or efferent rootlets revealed that the critical lesions were afferent in nature (Smith et al., cited in Smith & Gibbs, 1985). CCK is thought to control feeding peripherally through inhibition of gastric emptying (Moran & McHugh, 1982), an effect attributed to CCK receptors in the pylorus (Smith et al., 1984; Moran et al., 1989).

In the brain, CCK co-exists with DA mesolimbic neurons stemming from the VTA to the Acb and TU, primarily (80-90% of the system) in the nerve terminal networks (Agnati & Fuxe, cited in Fuxe et al., 1985). It has been demonstrated that injection of CCK into the PVN or VMH decreases food intake (Farris, cited in Leibowitz & Stanley, 1986; Willis, 1984). Endogenous changes in CCK peptide levels and receptor binding in response to ingestion and fasting has been reported in the literature. Fasting has been shown to decrease hypothalamic levels of CCK, with a concurrent increase in receptors (Scallet et al., cited in Leibowitz & Stanley, 1986; Saito et al., 1981). Ingestion increases CCK levels exclusively in the hypothalamus (McLaughlin et al., 1985).

It is suggested that peripheral CCK, glucagon, and somatostatin inhibit feeding by activating the vagal afferents that (possibly via projections from the NTS to the PVN) lead to the inhibition of the adrenergic (α-agonist) feeding system (Morley et al., 1984). This idea is supported by the fact that CCK is present in some of the vagal afferent terminals in the NTS, and that local iontophoretic application of CCK inhibits the extracellular unit activity of neurons in this region (Moran et al., 1982), though it does not inhibit feeding (Crawley, 1985b). In addition, the functional integrity of both the NTS (van der Kooy, 1984) and PVN (Crawley, 1985a) is required for the inhibitory action of CCK on eating. Thus it is possible that CCK (and possibly other vagally dependent peptides) contributes to postprandial satiety in two ways: 1) There is the endocrine effect in the periphery that activates vagal afferent fibers, and 2) there may be a neurocrine effect in the projection site of vagal afferent terminals in the NTS.

Moran and colleagues (1986) proposed that pancreatic- and GI-type CCK receptors were to be described as CCK-A receptors, whereas brain-type CCK

receptors were to be referred to as CCK-B receptors. CCK-A receptors are found in many peripheral locations, including the pancreas, vagus, and pyloric sphincter (Moran et al., 1986; Zarbin et al., 1981; Smith et al., 1984). Although CCK-B receptors represent the predominant CCK receptor in the brain, there is evidence that CCK-A receptors are also present centrally, although their regional distributions may be different (Hill et al., cited in Cooper et al., 1992). At the present time, there seems to be some controversy as to which of these CCK receptor types is more closely involved with the anorectic action of CCK. Several researchers have shown that selective CCK-A receptor antagonism will attenuate the effect of CCK on feeding (Cooper & Dourish, 1990; Hewson et al., 1988; Reidelberger & O'Rourke, 1989; Smith et al., 1991). Similarly, CCK-B receptor antagonists have been demonstrated to block the anorectic effect of CCK (Cooper & Dourish, 1990; Dourish et al., 1989), however many studies have failed to show such an effect (Moran et al., 1992; Reidelberger et al., 1991; Corwin et al., 1991).

Glucagon

Schulman et al., in 1957, were the first group to report a decrease in food intake by human subjects following intramuscular injection of glucagon (cited in Geary, 1990). Since then, many studies have reported similar findings in both human and animal subjects (Penick & Hinkle, cited in Geary, 1990; Weick & Ritter, 1986; McLaughlin et al., 1986; Langhans et al., 1987; Geary & Smith, 1982). Glucagon's primary metabolic target organ is the liver. It has been suggested that the liver may represent the site from which glucagon exerts its satiety effect (MacIssac & Geary, 1985; Novin et al., 1985). During a meal, hepatic portal vein glucagon levels have been demonstrated to increase significantly (Langhans et al., 1987). Previously, Martin and colleagues had shown that hepatic-portal infusion of glucagon decreased feeding in rats, and that this effect could be abolished by vagotomy (Martin & Novin, 1977; Martin et al., 1978). A more selective lesioning of the vagus produced clearer results; transection of the gastric and celiac branches of the vagus had no effect on the satiety response to hepatic-portally infused glucagon and, in contrast, lesioning only the hepatic branch abolished this response (Geary & Smith, 1983). It has, thus, been suggested that the satiety signal attributable to glucagon is vagally mediated, through the hepatic branch of the vagus nerve.

Unlike other putative satiety agents, intraperitoneal (i.p.) glucagon will only inhibit food intake in mildly deprived (0-6 hr) animals, but fails to do so when animals are food deprived for 8 hr or more. Interestingly, at dark onset, glucagon will inhibit feeding in 12 hr, but not 0-4 hr, deprived animals. Only 2-3 hr after dark onset will glucagon inhibit food intake in 0-2.5 hr deprived animals. Geary (1990) suggests that this dynamic pattern of potency reflects glucagon's ability to

inhibit ingestion when rats feed the most and is consistent with the hypothesis that glucagon is an important endogenous satiety signal. This hypothesis is further supported by the finding that antibodies directed at endogenous glucagon stimulate feeding in rats (Langhans et al., 1982; Le Sauter et al., 1991).

Bombesin: An Overview of the Literature

BN represents one class of peptides biologically active in the central nervous system (CNS) and the periphery. Initially isolated from the skin of the European frog Bombina bombina (Anastasi et al., 1971), BN displays a structural homology with the decapeptide C-terminal regions of the mammalian gastrin-releasing peptide (GRP_{1-27}), neuromedin C (GRP_{18-27}), and neuromedin B (NMB_{1-32} and NMB_{23-32}). Endogenous BN-like immunoreactivity (BLI) has been detected in discrete regions of the brain of a number of mammals including the rat (Brown et al., 1978; Polak et al., 1978), pig (Yanaihara et al., 1981), cat (Namba et al., 1985), and man (Yamada et al., 1981). In the rat brain, BN-like peptides are widely distributed, but have been predominantly localized in limbic areas (see Figure 5). The highest concentrations of BN-like peptides amongst major brain structures are found in the hypothalamus, specifically in the arcuate nucleus and PVN (Walsh et al., 1979; Moody et al., 1981; Panula et al., 1982). BLI has been demonstrated in cell bodies of the PVN and NTS, as well as in fibers within both the VMH and LH (Panula et al., 1982). Centrally, BN-like peptides can also be found in the hippocampal areas, globus pallidus, medial geniculate nucleus, Acb, olfactory tubercle, and pituitary gland, as well as cortical areas such as the cingulate and retrospenial cortex. BLI has also been detected in the GI tract of the guinea pig (Costa et al., 1984), rat (Soveny et al., 1982), dog (Reeve et al., 1983) and man (Polak et al., 1976). In the rat GI tract, the highest levels of BN-like peptides are found in the stomach, particularly in the fundus, as well as in the duodenum and colon (Dockray et al., 1979). BN-like peptides are found in nerve fibers of the myenteric plexus, submucosal plexus, and in the mucosa of the stomach, and primarily in the myenteric plexus of the rat intestine (Hutchison et

al., 1981; Dockray et al., 1979; Kuwahara et al., 1983). Distinct distribution of cells expressing GRP_{1-27} or NMB_{1-32} mRNA centrally (Wada et al., 1990), and GRP_{1-27} mRNA systemically (Dimaline et al., 1992) have been identified. BN has been detected in the blood of animals (Rayford et al., 1991; Holmgren et al., 1989), as well as humans (Hernanz et al., 1989), although the concentrations detected may not be sufficiently elevated to suggest a blood-borne role for these peptides.

Similarly, receptor localization has been described within the CNS (Pert et al., 1980; Wolf et al., 1983; Zarbin et al., 1985; Moody et al., 1988) and GI tract (Jensen et al., 1978; Vigna et al., 1987; Moran et al., 1988) of several mammalian species, including humans (see Figure 5). High density receptor distribution can be found in the olfactory bulb and tubercle, Acb, Fstr, hypothalamic areas such as the suprachiasmatic nucleus and PVN, central-medial and paraventricular thalamic nuclei, dentate gyrus, NTS, as well as central and medial amygdaloid nuclei. Moderate receptor densities are seen in the hippocampus, Bst, VMH, LH, as well as most other areas where BN-like peptide binding is observed. Other than the NTS, hindbrain areas are virtually absent of BN-like peptide receptor binding and, along with the frontal, cingulate, and entorhinal cortical areas, represent the lowest receptor binding areas in the rat brain. Recent studies involving the solubilization and characterization of the BN receptor in Swiss 3T3 cells (Feldman et al., 1990; Naldini et al., 1990; Sinnett-Smith et al., 1990) have led to the cloning of the BN receptor from these cells (Battey et al., 1991).

The precise physiological role(s) of this peptide and/or its analogues has not yet been determined. However, BN-like peptides are not only endogenously ubiquitous but behaviorally and physiologically potent when administered through

both central and systemic routes. Although BN exhibits partial structural homology with a number of peptides, the term "BN-like peptides", within the context of the present research, refers to GRP_{1-27}, GRP_{18-27}, NMB_{1-32}, and NMB_{23-32}, which are currently the only mammalian peptides demonstrating structural homology with BN. A number of amphibian peptides also show such homology but have yet to be detected in mammalian tissues. It is conceivable that, in the near future these peptides, or peptides closely related, will be discovered in the mammalian system.

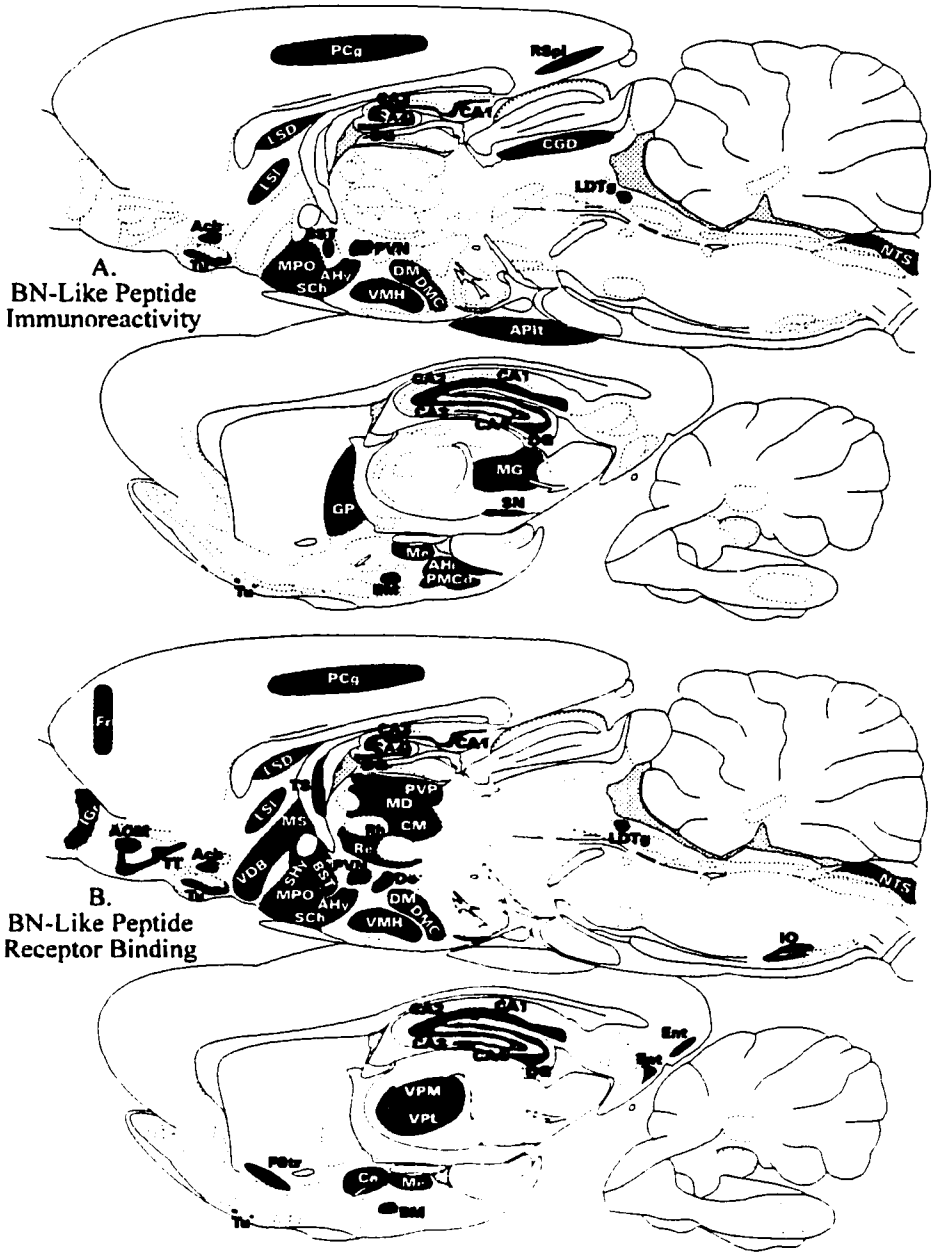

Figure 5. Schematic representation of central BN-like peptide distribution (A) and receptor localization (B), shown in black. Abbreviations are defined on the following page.

Acb	nucleus accumbens	LSD	lateral septal nucleus, dorsal
AHi	amygdalohippocampal area	LSI	lateral septal nucleus, intermediate
AHy	anterior hypothalamic area	MD	mediodorsal thalamic nucleus
AOM	anterior olfactory nucleus, medial	Me	medial amygdaloid nucleus
APit	anterior lobe of the pituitary	MG	medial geniculate nucleus
BM	basomedial amygdaloid nuclei	MPO	medial preoptic area
BST	bed nucleus of stria terminalis	MS	medial septal nucleus
CA1	field CA1 of Ammon's horn	NTS	nucleus of the solitary tract
CA2	field CA2 of Ammon's horn	PCg	posterior cingulate cortex
CA3	field CA3 of Ammon's horn	PMCo	posterolateral cortical amygdala
CA4	field CA4 of Ammon's horn	PVN	paraventricular hypothalamic area
Ce	central amygdaloid nucleus	PVP	paraventricular thalamus, posterior
CGD	central grey, dorsal part	Re	reuniens thalamic nucleus
CM	central medial thalamic nucleus	Rh	rhomboid thalamic nucleus
DG	dentate gyrus	RSpl	retrosplenial cortex
DM	dorsomedial hypothalamic nucleus	SCh	suprachiasmatic nucleus
DMC	DM, compact part	SHy	septohypothalamic nucleus
Do	dorsal hypothalamic nucleus	SN	substantia nigra
Ent	entorhinal cortex	TS	triangular septal nucleus
Fr	frontal cortex	TT	taenia tecta
FStr	fundus striatum	Tu	olfactory tubercle
GP	globus pallidus	VDB	vertical limb of the diagonal band
IGr	intergranular layer, olfactory bulb	VMH	ventromedial hypothalamus
Io	inferior olive	VPL	ventroposterior thalamus, lateral
LDTg	laterodorsal tegmental nucleus	VPM	ventroposterior thalamus, medial

Adapted from **The Rat Brain in Stereotaxic Coordinates**, by G. Paxinos & C. Watson, 1982.

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**

NMB_{1-32}.....X-Val-His-Pro-Arg-**Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**

NMB_{23-32}.....**Gly-Asn**-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**

Ranatensin.....pGlu-Val-Pro-**Gln-Trp-Ala-Val-Gly-His**-Phe-**Met-NH_2**

Ranatensin R.....X-Ala-Leu-Arg-Arg-Tyr-**Asn-Gln-Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**

Ranatensin C.....X-Glx-Thr-Pro-**Gln-Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**

Neurokinin A.....His-Lys-Thr-Asp-Ser-Phe-**Val-Gly-Leu-Met-NH_2**

Neurokinin B.....Asp-Met-His-Asp-Phe-Phe-**Val-Gly-Leu-Met-NH_2**

Litorin.....pGlu-**Gln-Trp-Ala-Val-Gly-His**-Phe-**Met-NH_2**

Rohdei-Litorin.....pGlu-Leu-**Trp-Ala**-Thr-**Gly-His**-Phe-**Met-NH_2**

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**

Alytesin.....pGlu-Gly-Arg-Leu-Gly-Thr-**Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2**

Figure 6. Structural homology of BN-like peptides and other amphibian peptides at the decapeptide C-terminal region. "X", represents the amino acid chain preceding the sequence indicated.

BN administration has been shown to elicit a wide array of behavioral and metabolic alterations in experimental animals, including marked effects on grooming behavior, locomotor activity, appetite, thermoregulation, cardiovascular

function, gastrointestinal function, and various endocrine, exocrine, and trophic mechanisms.

The profound alterations in homeostasis produced by both centrally and systemically administered BN are reflected not only in significant physiological and pharmacological changes but are most readily obvious in the behavioral transformations which occur post-injection. Behaviorally, central administration of BN induces a grooming response (Kulkosky et al., 1982; Merali et al., 1983) and locomotory activity (Pert et al., 1980; Schultz et al., 1984). The grooming behavior consists almost entirely of a facial scratching action performed with the hind paws and has been, as has locomotory stimulation, associated with the DA system (Merali et al., 1983; Merali et al., 1985; van Wimersma Greidanus et al., 1985). Attenuation of the grooming response without suppression of locomotion has been achieved through blockade of central muscarinic receptors (Merali et al., 1988), suggesting cholinergic involvement, whilst more recent experimentation has revealed a possible separation of BN effects through partial mediation by dopamine D-1 and D-2 receptor subtypes (Piggins & Merali, 1989). The grooming and exploratory effects are not seen following systemic administration of BN.

Apart from enhancing grooming and locomotory activity, central infusion of BN suppresses the consumption of food (Gibbs et al., 1981) and water intake (de Caro et al., 1984). The mechanisms though which BN's antidipsogenic effects are mediated are presently unknown. They appear, however to be centrally mediated, as systemic administration of BN does not attenuate water intake (de Caro et al., 1985). This observation contrasts the suppressive effects of BN on food intake, which can be seen through either systemic or central administration (Gibbs et al., 1979; Gibbs et al., 1981; Negri et al., 1985). It would appear that the central

mechanisms subserving BN's effects on food and water intake are, at least in part, distinct as local injection on BN into the LH will significantly attenuate food, but not water, intake (Stuckey & Gibbs, 1982). However, it is difficult to determine the specificity of this suppression since BN also elicits other behaviors (i.e., grooming and locomotion) which could interfere with food and water consumption (Kulkosky et al., 1982). A certain degree of dissociation between these effects has been attained. Central administration of relatively low doses of BN into the fourth ventricle (Ladenheim & Ritter, 1988; Flynn, 1989) and NTS (Johnson & Merali, 1988) have been shown to suppress food intake without increasing grooming or locomotor activity. Furthermore, peripherally administered BN does not stimulate grooming nor locomotion but has a profound attenuating effect upon feeding.

Systemic BN affects meal size (whether solid or liquid feed) by reducing the duration of the meal without altering the initial rate of consumption and without affecting the normal postprandial sequence of behaviors typical of the species under investigation (Gibbs et al., 1979). This effect has been observed in the rat (Gibbs et al., 1979), mouse (McLaughlin & Baile, 1981), domestic fowl (Savory & Hodgkiss, 1984), pig (Parrott & Baldwin, 1982), wolf pup (Morley et al., 1986), baboon (Woods et al., 1983), and man (Muurahainen et al., 1983). The nature and resilience of this feeding suppression has been of great interest within the research arena and has, subsequently, been the root of several encouraging findings.

Central, but not peripheral, administration BN has a poikilothermic effect in rats (Brown et al., 1977; Avery et al., 1981). Pittman and colleagues (1980) have suggested that this effect may be mediated by the medial preoptic area of the hypothalamus. Under ambient conditions (22 °C), BN induces hypothermia

caused by increased heat loss through the skin (Tache et al., 1980), whereas in cold-stressed animals, the hypothermic effect has been attributed to BN-induced reduction in metabolic rate caused by inhibition of regulatory heat production (Wunder et al., 1980; Brown, 1983). BN has also been shown to have a regionally selective effect on sympathetic efferent activity, interrupting sympathetic systemic innervation during cold exposure trials (Brown et al., 1987). Central injection of BN produces hypothermia at normal ambient temperatures, but only if the animal has been food-deprived or made hypoglycemic with insulin (Babcock et al., 1989). Microinfusion of BN (0.05 μg/1 μl) into the PVN of insulin-pretreated rats produces hypothermia, but not hypophagia, suggesting that the PVN may be a sensitive site for BN-induced hypothermia (Babcock & Barton, 1990).

Cardiovascular function is also altered by central administration of BN. In addition to increasing cardiac parasympathetic activity (Fisher & Brown, 1984), BN has been shown to increase systolic blood pressure and decreases heart rate (Fisher et al., 1985). The BN-induced decrease in heart rate may partially explain the hypothermia observed in cold-stressed rats centrally injected with BN, in that it may prevent the adequate cardiac output needed to provide sufficient nutrient and oxygen supply during periods of increased demand for heat production (Brown et al., 1987). The increased respiration rate elicited by centrally administered BN (Brown & Gillespie, 1988) may be an effort to counterbalance the oxygen deficit under those conditions, or may be due to increased tidal volume (Hedner et al., 1985).

Systemically administered BN also acts as a potent secretagog of endogenous substances, including CCK (Modlin et al., 1980; Jansen & Lamers., 1983), pancreatic glucagon, and pancreatic polypeptide (Bloom et al., 1983).

Pancreatic polypeptide release is also observed after central BN administration (Chowdhury et al., 1985). BN stimulates the release of gastrin from the antral mucosa and CCK from the intestinal mucosa, as well as the secretion of somatostatin, neurotensin, and enteroglucagon (Lehy et al., 1988). It is of interest to note that systemic administration of BN will elicit an increase in gastric acid secretion (Bertaccini et al., 1974), whereas i.c.v. BN decreases the rate of gastric acid secretion (Modlin et al., 1980; Tache et al., 1982; Chowdhury et al., 1985). Central administration of BN elevates blood glucose levels (Brown et al., 1977; Gunion et al., 1989) and decreases blood insulin concentrations (Brown et al., 1979; Woods et al., 1983). BN-induced hyperglycemia has been associated with an elevation of plasma glucagon and concurrent reduction of plasma insulin levels (Brown et al., 1979; Brown et al., 1988). Adrenalectomy prevents the CNS the hyperglycemic effects of BN, as well as BN's effects on insulin and glucagon levels, suggesting that these effects are associated with BN-induced adrenomedullary epinephine secretion (Brown et al., 1977; Brown et al., 1988). Lesions of the LH, similarly, block BN-induced hyperglycemia in rats (Gunion et al., 1984).

Regulation of pituitary hormone secretion is also affected by BN when injected peripherally, inducing lower levels of prolactin and thyroid-stimulating hormone (TSH) after thyrotropin-releasing hormone (TRH) and higher levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) after LH-releasing hormone (LHRH) in both animals and man (Pontiroli et al., 1980; Pontiroli & Scarpignato, 1986).

There has been research to suggest that BN-like peptides may play an important role in cellular proliferation. Sustained BN administration will cause

gastric and duodenal hyperplasia in rats and pancreatic hyperplasia in humans (Carney et al., 1988; Sunday, 1988). In humans, BN-like peptides are present in fetal and newborn lung, are absent in adult lung tissue, and are markedly reduced in infants with respiratory distress syndrome, suggesting a role for BN-like peptides in fetal lung development (Wharton et al., cited in Moody & Korman, 1988; Carney et al., 1988; Giaccone et al., 1992). Since BN-like peptide immunoreactivity, and high-affinity receptors for BN-like peptides have been detected in cell lines of small cell lung carcinoma (SCLC) (Walsh et al., 1979; Sunday, 1988), studies were undertaken to determine if these peptides had a mitogenic role in SCLC proliferation. In cultures of SCLC cell lines, BN- or GRP-stimulated proliferation was 7-150 fold above that seen in control cultures (Carney et al., 1987; Carney et al., 1988). Further, this mitogenesis was specific to SCLC, as cell lines from adenocarcinoma, squamous cell carcinoma, and large cell carcinoma were not affected by BN treatment. The results of these studies have stimulated research towards the development of monoclonal antibodies and inert BN analogs specific to BN-like peptides and their receptors, in the hope that these compounds may slow or arrest the further development of SCLC. Research on that front is currently in progress.

Bombesin: Effects on Food Intake

The definition of a satiety state seems clearly obvious to most who are familiar with the word. Webster's Third New International Dictionary (1986) defines satiety as "the quality or state of being fed to or beyond fullness". Similarly, Dorland's Illustrated Medical Dictionary (1985) defines satiety as the "... full gratification of appetite or thirst, with abolition of the desire to ingest food or liquids". Although the satiety *state* is readily definable, there is less agreement concerning the definition and role of satiety *mechanism(s)*. Satiety mechanisms can be viewed as phasic changes which are initiated at the during ingestion and serve, through various metabolic and physiological indices (such as metabolic rate, gastric load, food-released hormones, etc.), to terminate the meal. If one adheres to this perception of the function of satiety mechanisms, it would then follow that these mechanisms would not be operative during periods when ingestion was not occuring, and cease to operate when a satiety state is achieved. A phasic satiety mechanism, then, would play a role in appetite regulation in an indirect manner through periodic cessation of food or water intake. Tonic satiety mechanisms might regulate ingestion in an alltogether different fashion. This model of satiety would propose that satiety states lie along a continuum and that the mechanisms subserving them are always active to various degrees. The terms "satiety mechanisms" and "appetite mechanisms" would then be interchangeable, and satiety mechanisms would serve, not only to terminate a meal, but also to elicit hunger sensations and to initiate the next meal. This distinction has not seemed, for the most part, to affect the focus or direction of research in the area of ingestive behavior, although the findings that have emerged may indicate that a tonic satiety mechanism may be in place. Anorectic substances infused through

various routes of administration would not resolve the debate, as both models propose an inhibitory role of the satiety process. However, the initiation of food intake in sated animals by administration of antagonists directed at endogenous substances known to have anorectic effects when administered exogenously would clearly refute a phasic satiety process. This has been demonstrated for both CCK (Reidelberger & O'Rourke, 1989) and, more recently, for BN-like peptides (Merali et al., in press).

Although there are presently a large number of substances known to produce an anorectic effect when administered systemically ([glucagon] van der Weele et al., 1980; [calcitonin] Levine et al., 1984; [CCK] Gibbs et al., 1973; [opiate antagonists] Morley et al., 1983; [somatostatin] Lotter et al., 1981; [BN] Gibbs et al., 1979, to name a few), only a select few have been referred to as satiety agents, in reference to the behavioral sequence exhibited post-infusion. The suppression of ingestive behaviors may be the result of general or specific malaise, locomotory depression, or due to elicited behaviors interfering or competing with the behavior of study. Substances which fail to interfere with species-typical postprandial behavioral sequences, and are thus considered not to produce discomfort or depression, are referred to as satiety agents. The best studied of these putative satiety agents include CCK, glucagon, and BN. A description of the postulated mechanism(s) of action of BN-induced feeding suppression will further differentiate BN, not only from other anorectic agents, but from those substances thought also to exhibit induction of satiety effects.

Although systemically infused BN reduces gastric emptying (Bertaccini et al., 1982; Falasco et al., 1986), it has been demonstrated that the peptide-induced satiety effect is not dependent upon the volume within the stomach or changes in

its emptying process (Conover et al., 1989). In fact, satiety produced by BN has proven surprisingly resilient to lesioning of major organs, nuclei and pathways associated with BN effects and/or those functions presently thought to be involved in feeding regulation. Adrenal function has been reported to be altered by BN administration, yet adrenalectomy does not interfere with reduction of food intake (Gibbs et al., 1981). Lesions of the dorsomedial hypothalamus and VMH have, similarly, proven ineffective in blocking the satiating effects of BN (Geary et al., 1986; Bellinger et al., 1984), as has hypophysectomy (Stuckey & Gibbs, 1982). Inasmuch as all of the so-called satiety peptides do not readily cross the blood-brain barrier, it has long since been assumed that these substances had sites of action in the periphery of the organism and that the peripheral action of these substances was communicated to the brain via visceral afferent fibers. What has set BN apart from the other satiety agents is the discovery that abdominal vagotomy abolishes or markedly reduces the satiety effect of CCK (Lorenz & Goldman, 1982; Smith et al., 1981a), somatostatin (Morley et al., 1982) and glucagon (Martin et al., 1978) but fails to alter BN-induced satiety (Smith et al., 1981b). It is worthy to note that one cannot assume that the critical lesions produced by this procedure are solely afferent, since efferent fibers are also lesioned (Smith et al., 1984). However, peripheral anticholinergic blockade with atropine methylnitrate provides a crude mimic of the loss of vagal efferent fibers, and has been shown to have no effect on the satiating properties of CCK (Smith et al., 1981) or glucagon (Geary et al., 1983). This would suggest a vagal afferent dependence of these peptides in their effect. Ladenheim & Ritter (1991) have reported that systemic treatment with capsaicin, a neurotoxin which damages unmyelinated peptide-containing sensory neurons, attenuates BN-induced

suppression of food intake. The authors suggest that the suppression of food intake observed may reflect damage to vagal and/or nonvagal neurons that participate in BN-induced inhibition of feeding. However, the work of others have shown that the mechanisms underlying BN's effects in the periphery may not be directly affected by capsaicin-sensitive neurons. For instance, the response of single neurons in the dorsal vagal complex to BN in gastrically distended animals was unaltered by capsaicin pretreatment (Ewart et al., 1990), and BN-induced suppression of operant response for food reward was similarly unaffected by capsaicin administration (McCoy et al., 1992). Within the CNS, immunohistochemical localisation of BN-like peptides in the area postrema, NTS, and vagal motor nucleus in the brainstem is not depleted by capsaicin pretreatment (King et al., 1989). The complete disconnection of gut from brain (cordotomy, dorsal rhizotomy and total subdiaphragmatic vagotomy) is necessary to abolish BN feeding suppression (Stuckey et al., 1985). Based upon these findings, one can assume that neural communication, whether efferent and/or afferent, must remain to some degree intact in order to produce the reduction in meal size induced by systemic BN. This conclusion is supported by the finding that the effect of systemically administered BN on feeding is significantly attenuated by centrally infused anti-BN antiserum (Merali et al., 1988). This study would suggest that the availability of central BN receptors is a requisite for the afferent and/or efferent transmission of the satiety signal in the proposed polysynaptic pathway activated by systemic BN.

BN also prevents gastric emptying and is present in high concentrations in the mucosa of the stomach and is located exclusively in nerve cells of the GI tract (Dockray et al., 1979). The actions of BN and/or CCK may signal the brain by

stimulating vagal afferents. BN secretion, causing the prevention of gastric emptying through muscular tension would elicit the release of CCK which, in turn, can directly stimulate vagal afferents. This is consistent with two characteristics of abdominal vagal afferent fibers: blood-borne substances have access to the small-terminal portions of the unmyelinated fibers and the abdominal vagus has specific CCK receptors (Smith et al., 1984). This action by CCK would also explain its satiating effect in sham-fed rats (Gibbs et al., 1973). Systemic adminstration of CCK could act at the receptors present on abdominal vagal afferent fibers, eliciting its satiety effect independent of gastric distension.

BN, although independent of vagal afferent fibers in its effect and more persistent than other peptides to lesions of central and peripheral loci, shares a dependence upon an intact PVN. Lesions not affecting BN satiety include adrenalectomy (unlike CCK), hypophysectomy, dorsomedial hypothalamus ablation, spinal cord section at the level of the 6th thoracic vertebra and total subdiaphragmatic vagotomy. Although lesions of the LH and VMH alter feeding in rats, these effects are attributed to destruction of axonal fibers originating from the PVN, and not to the lesioned loci (Leibowitz, cited in McCoy & Avery, 1990). Further, microinfusion of BN directly at either the LH or PVN, as well as the amygdala, elicits a pronounced attenuation of food intake (microinjection at other hypothalamic sites do not) (Stuckey & Gibbs, 1982; Willis et al., 1984; Kyrkouli et al., 1987; Gunion et al., 1989). Although speculative, the signals from the periphery to the brain may be the result of neurocrine projections to these regions and to the NTS , which shows high BLI (Moody & Pert, 1979; Panula et al., 1982) and a high receptor density (Zarbin et al., 1985). Johnson & Merali (1988) have shown that very low doses of BN microinjected into the NTS will significantly

suppress food intake. The involvement of the NTS is also suggested by the finding that lesions of the medial aspect of the NTS will attenuate the satiety response elicited by either centrally or systemically injected BN, indicating that the NTS may be a common neural substrate for both central and peripheral BN-induced suppression of feeding (Ladenheim et al., 1989). Peripheral signals inducing satiety may also be the result of endocrine mechanisms (blood-borne hormonal communication) to target cells, inducing a sensory response (increased gastrin response (Bertaccini et al., 1973); and elevated insulin (Woods et al., 1983), glucagon (Bloom et al., 1983), and pancreatic polypeptide secretion from the pancreas (Modlin et al., 1980)). This contention is supported by research showing that intravenous administration of BN elicits a dose-dependent reduction in food intake (Yen et al., 1989). That study also suggested that local sites of action within the abdomen may not required for the satiety effect of exogenous BN, since, presumably, the intravenous dosages would be diffused systemically and would be present in the abdominal region at doses lower than those previously shown to suppress food intake.

A final proposed mode of action of these peptides upon the CNS is their diffusion into the CNS through a weak area of the blood-brain barrier; namely, the area postrema. Critical receptors on or around the area postrema may serve to carry the signal to satiety-mediating loci. The nausea often reported by subjects systemically infused with CCK, BN, or glucagon supports this suggestion, as the blood-borne peptides may stimulate the chemo-receptor zone of the area postema by penetrating the relatively weak blood-brain barrier below this area. As well, thermal lesions of the area postrema have been reported to attenuate the satiety response of animals to i.c.v. BN (Ladenheim et al., 1989). Finally, BN injected

into the area postrema has been shown to suppress food intake (Willis et al., 1984).

Although exogenous BN suppresses food intake this effect should be considered a pharmacological phenomenon, implying a physiological role for BN-related peptides in the modulation of feeding. Support for the contention that BN-like peptides may mediate endogenous satiety signals was provided by the study in which a specific BN receptor antagonist enhanced food intake in prefed rats (Merali et al., 1990).

It would seem clear, then, that if one wishes to better understand the mechanism(s) by which BN exerts its effects on feeding one could benefit by focusing on changes in endogenous BN-like peptides and their receptors in response to feeding behavior, rather than on the effects on exogenous BN on feeding. In this manner, natural occurrences in the living organism may provide clues to determining the presently obscure relationship between brain and gut as they pertain to BN-induced satiety.

Objectives

The primary objective of this research was to elucidate the changes in BN-like peptides which occur under natural conditions during feeding. Although the effects of central or systemic BN administration on feeding have been extensively investigated and without which this proposal would not be forthcoming, it is crucial to gain a better understanding of if and how the living organism employ these peptidergic systems in the regulation of its own feeding regimen. The internal regulation of BN-like peptides has not been well characterized nor has receptor response been studied within this context. Although completely devoid of pharmacological or surgical manipulations, and for the most part equally devoid of environmental alterations, the proposed research may, in its simplicity, offer foundational information underlying feeding mechanisms by tracing neurochemical changes immediately before and after a meal. Specifically, the objective of this thesis research was to study regional changes in BN-like peptide concentration, and BN-like peptide receptor density change within discrete regions of the brain and GI tract, in response to a single meal. Such an investigation may reveal those regions of central and/or peripheral systems possibly involved in endogenous satiety mechanisms and may also, more globally, help to delineate the relationship between what is known of central feeding regulation sites and those located in the periphery. Prior research has already established that they may work in concert, yet extensive effort to simultaneously monitor both systems has been largely sparse and is in need of further investigation. Should preliminary research reveal peptide and/or receptor changes within healthy, normal animals after a meal, the second phase of the experiment would involve further exploration at varying time points during the

meal. This second phase may offer clues as to the time course of peptidergic changes involved in the satiety process. Such research, if fruitful, would further substantiate prior investigations involving BN, add to the present knowledge of endogenous satiety mechanisms and hopefully fuel investigation of rapidly occurring changes within organisms.

Hypotheses To Be Tested

Exogenous administration of BN-like peptides induces a satiety-like state in several species. The objective of this project will be to test the hypothesis that endogenous mechanisms utilizing BN-like peptides may be involved in the mediation/modulation of satiety.

Specific Objectives

1. The first major objective was to determine whether differences in the concentrations of BN-like peptides within several regions of the GI tract and brain can be detected immediately before (preprandial) and after (postprandial) a test meal.

2. The second major objective was to determine whether changes in the BN system occur at the receptor level. Thus the possible changes in the affinity (K_D) and/or receptor number (B_{max}) for BN-like peptides within the GI tract and brain of preprandial and postprandial rats were quantified.

3. The third major objective was to determine the time course and degree of change in concentration and distribution of BN-like peptides within the GI tract and brain during the course of a meal.

4. The fourth major objective was to determine the degree of change in B_{max} and/or K_D of BN-like peptide receptor sites within the GI tract and brain during a meal.

General Methods

Iodination

Iodination refers to the process of attaching radioactive iodine (in this case, ^{125}I) onto the molecule of investigation. In order to iodinate a peptide, the peptide must contain a tyrosine amino acid in its chain. Thus, Tyr^4-BN, which has been shown to possess identical pharmacological and pharmacokinetic properties to BN (Moody et al., 1981), was used for iodination processes. Tyr^4-BN was iodinated using a modification of the technique of Salacinski et al. (1979). The buffer solution needed for iodination is prepared in the following manner:

- 0.01 M NaH_2PO_4 (monobasic)
- 0.01 M Na_2HPO_4 (dibasic)
- add enough monobasic solution (approximately 180 ml) to dibasic solution to adjust pH to 7.2.

This phosphate-buffered saline solution (PBS), with the addition of bovine serum albumin (BSA), was used to make 1% BSA/PBS and 0.1% BSA/PBS.

On the day before iodination, 3.5 g of Sephadex G-10 (Sigma Chemicals) was left to saturate in 50 ml 1% BSA/PBS overnight at 4 °C. The following day, the Sephadex solution was pipetted into a 10 ml glass pipette containing a small glass wool plug in its tip. This procedure yields a Sephadex-packed column by allowing only the 1% BSA/PBS solution to drip through the glass wool. Before

using the column, approximately 20 ml of elution buffer (0.1% BSA/PBS) is run through the column. A 20 nmol tube of lyophilized (freeze-dried) Tyr^4-BN is taken from the freezer and is reconstituted in 500 µl 0.1M NH_4Ac solution. To the reaction tube, containing a thin film of the reaction catalyst iodogen, is placed 20 µl of NH_4Ac solution followed by 30 µl of reconstituted Tyr^4-BN. The final step involves the addition of 2 mCi of ^{125}I to the reaction tube and allowing the reaction to proceed for 2 min. The reaction mixture is carefully pipetted onto the Sephadex column, followed by elution buffer, and fractions are collected (11 drops per fraction x 40 tubes). Next, 2 µl aliquots of each fraction are counted in the gamma counter. The fraction(s) containing ^{125}I-Tyr^4-BN will be represented by the first radioactive peak counts (generally of a magnitude 500+ times greater than preceding fractions), whereas the second peak represents the free or non-bound ^{125}I. A reproduction of the elution profile is shown in Figure 7. The desired fractions are pooled, processed at 80 °C in the presence of 100 µl of 1M dithiothreitol (DTT), a reducing agent, in order to reverse the oxidation of amino acids. The iodinated peptide is further purified through a disposable Sep-Pak C_{18} column (Waters Associates, Milford, MA), which further separates the pooled fractions and removes any free iodine which may be present in the fraction. The desired fraction is then aliquoted and stored at -70 °C until used (stored for a maximum of 4 weeks). The specific activity, an index of the incorporation of ^{125}I into the Tyr^4-BN, is calculated by the method of Chiang (1987). In our preparation, the specific activity of the ligand is approximately 1200 Ci/mmol.

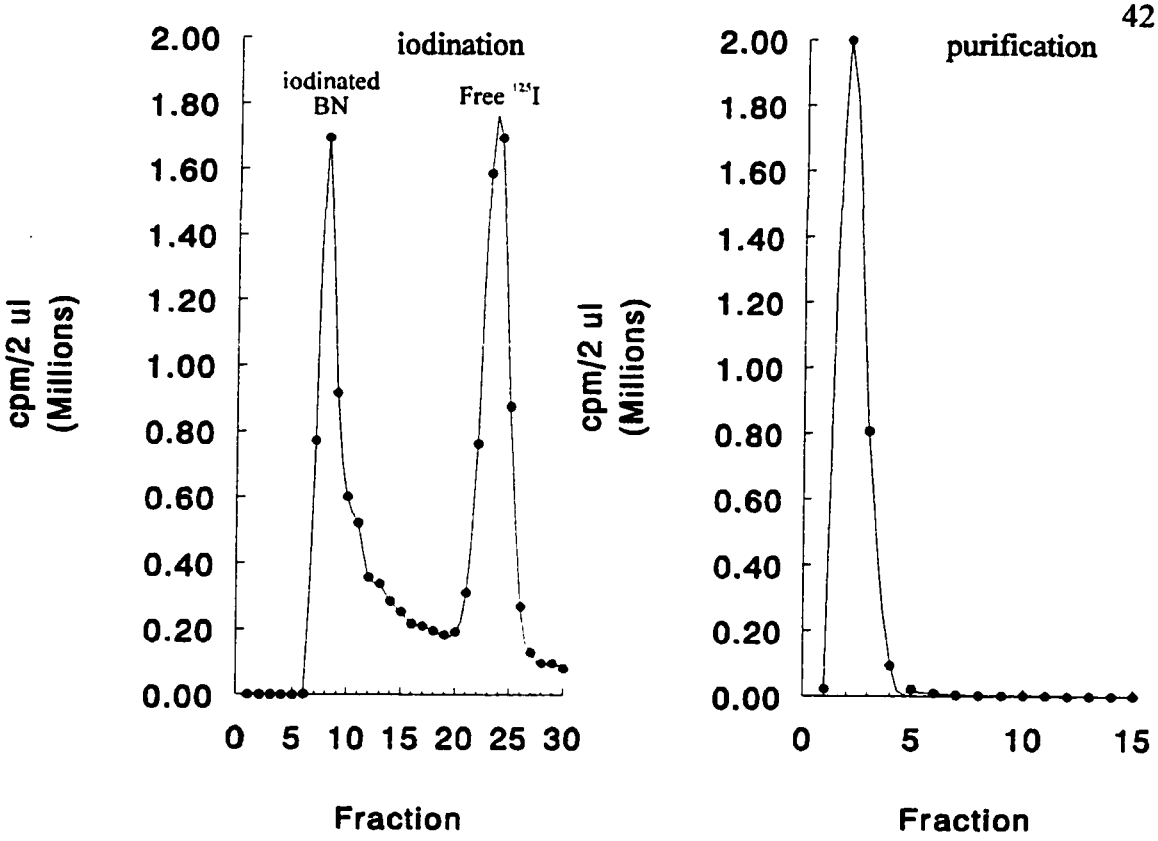

Figure 7. Iodination of Tyr^4-BN and subsequent chromatographic fractionation yields two radioactive peaks. The first peak contains ^{125}I-Tyr^4-BN, while the second peak contains unbound ^{125}I. Further purification using a Sep-Pak C_{18} column concentrates the pooled ^{125}I-Tyr^4-BN fractions into a smaller number of aliquots by separating the peptide from the elution buffer and any free ^{125}I that may have remained in the pooled buffer solution.

Animals

Experiments were conducted with male Sprague Dawley CD rats weighing between 300 and 350 g, obtained from Charles River (Rochefort, Quebec). The

animals were housed individually and maintained on a 12 hr light/dark cycle with the lights on at 6:00 a.m. The room temperature was maintained at 21 to 23 °C with 60% relative humidity.

Sacrifice

All animals were kept in their home cages and were removed just prior to the sacrifice. The animals were rapidly decapitated using a guillotine, in a room adjacent to their normal housing.

Dissection

GI dissection (for RIA & QAR) was performed by placing the decapitated animal on its back on a dissection table, cutting open the abdominal cavity and, beginning with the oesophagus, isolating 2 cm of oesophagus, fundus, antrum, duodenum, jejunum, ileum, and colon (and, for the RIA procedure, the adrenal glands) (see Figure 8). These segments were promptly weighed, as was all food material removed from the entire GI tract. Fundus and antrum segments were measured to approximately 2 cm x 0.5 cm in a cross-sectional orientation of the stomach.

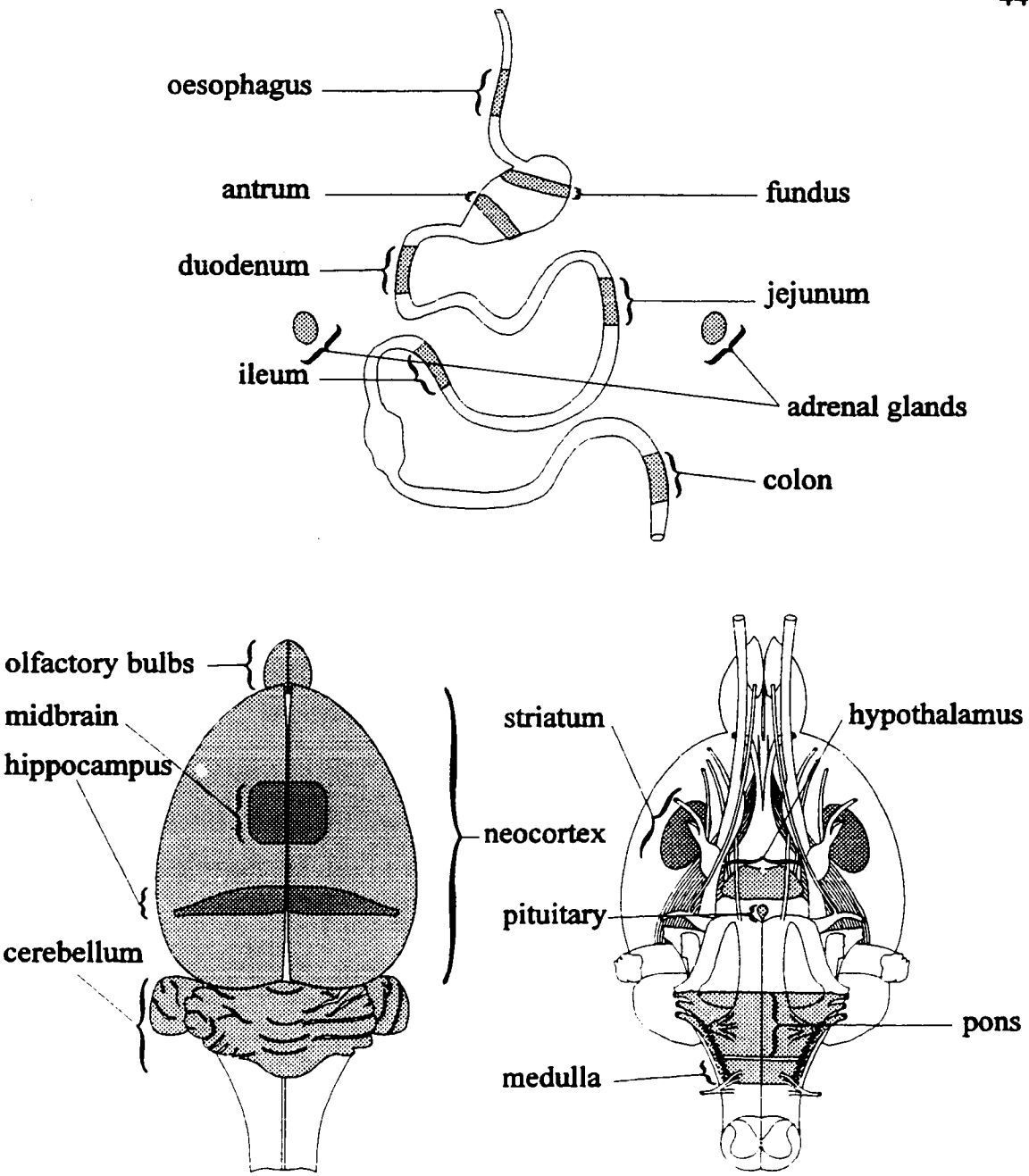

Figure 8. GI and central tissues isolated during dissection procedures. Shaded regions represent tissues harvested.

Dissection of the brain (RIA only) was performed on an ice-supported platform through blunt brain dissection, quickly separating the hypothalamus, cerebellum, pons, medulla, olfactory bulbs, striatum, neocortex, hippocampus, midbrain, and pituitary with 2 cooled spatulae (with the exception of the hypothalamus and pituitary, where cooled scissors were used) (see Figure 8). All areas were then promptly weighed. For QAR, the brains were left intact.

RIA Tissue Processing

Isolated brain or gut segments were placed in separate test tubes containing 2.5 ml of 2M acetic acid at 80 °C for a period of 1 hr. They were then subjected to homogenation and sonication (gut; Polytron PCU-2-110, setting 6 - Brinkman Instruments, Westbury, NY; brain; Kontes Micro-ultrasonic Cell Disrupter, setting 5 - Kontes, Vineland, NJ), and the homogenate was centrifuged at 10,000 x g for 10 min. Two x 1 ml of supernatant from each area were placed in separate cryogenic vials, frozen at -70 °C and lyophilized. The vials were then stored at -70 °C until the day of the assay. Prior to centrifugation, 100 µl of each homogenate was collected and stored at -70 °C for protein analysis using bicinchonimic acid with a BCA Protein Analysis Kit (Pierce Scientific) and a spectrophotometer (Cary 2200 - Varian, Springvale, Australia).

RIA Procedure

Although prior research has shown profound effects of BN on mammalian systems when administered at nanomolar and picomolar concentrations (Merali et al., 1988; Piggins et al., 1989; de Beaurepaire et al., 1988) it must be realized that

the endogenous presence of these peptides are in substantially lower femptomolar concentrations in the living system with specific localizations (Moody et al., 1986; Brown et al., 1978; Decker et al., 1984). The method of RIA involves the determination of endogenous peptide concentrations through the isolation and dissection of discrete brain and/or GI regions.

The BN-like peptides isolated within this supernatant are allowed to actively compete with exogenous ^{125}I-Tyr^{4}-BN for binding sites on the primary antibody (αBN) (see Figure 9). The BN/αBN and ^{125}I-Tyr^{4}-BN/αBN complexes are isolated through addition of a second, magnetic antibody which specifically binds the first antibody. This complex is separated from the solution by applying the assay tubes to magnetic plates and decanting and aspirating the supernatant. What remains in the assay tube is the antibody pellet containing those BN-like peptides, whether radiolabelled or not (see Figure 9). By conducting this procedure with known amounts of unlabelled BN and submitting these standard tubes to measurement by a gamma counter (which measures the presence of gamma emitting particles such as ^{125}I) one can establish a relationship between radioactive counts per minute (cpm) recorded and the actual quantity of unlabelled (in experimental assay tubes, endogenous) BN-like peptides in femptomolar units. In this manner, the precise amounts of these endogenous peptides can be determined by extrapolating from the standard curve, within a variety of central and peripheral sites.

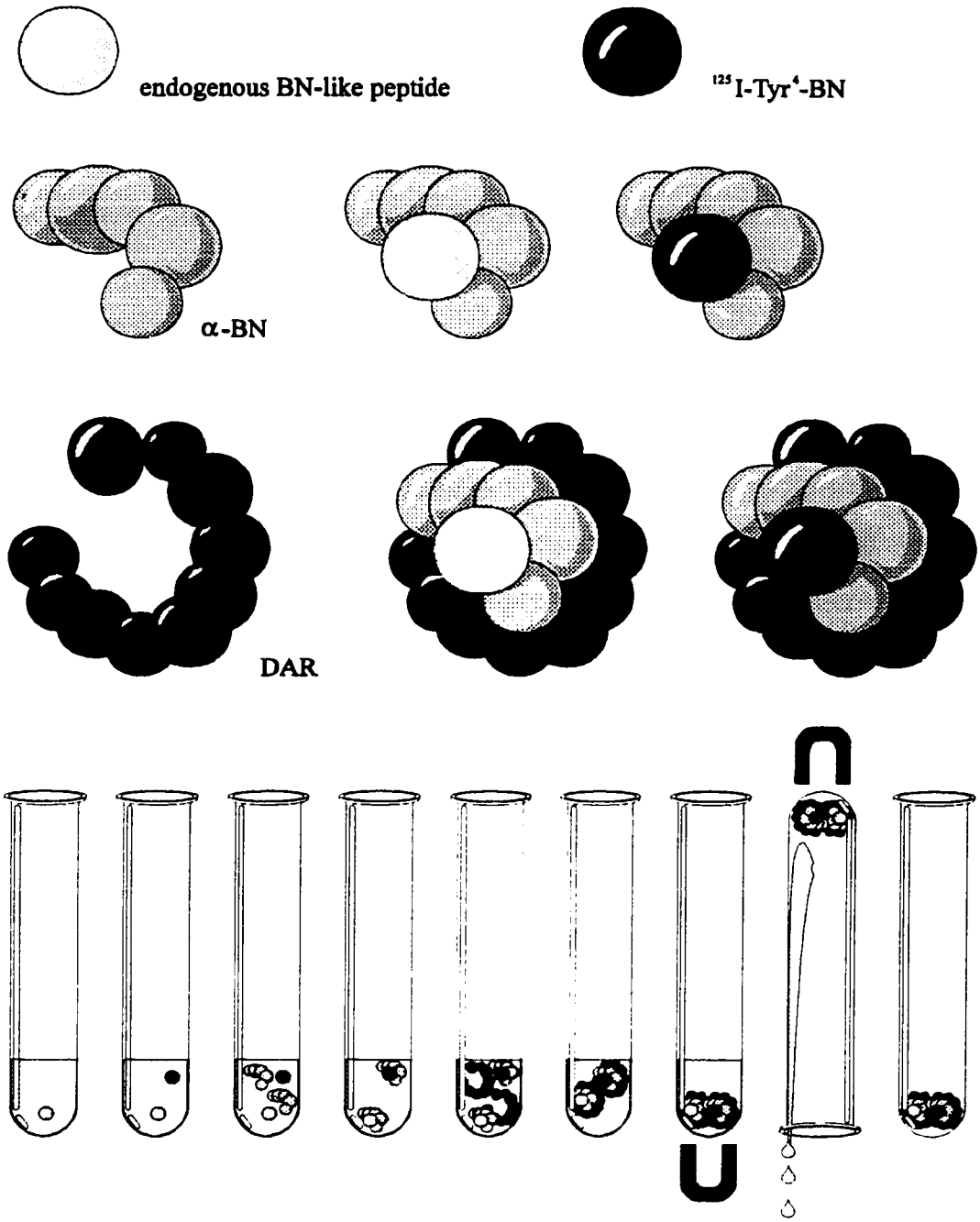

Figure 9. Schematic depicting the elements and procedure of RIA for BN-like peptides.

The RIA buffer solution used in our assay consisted of sodium monobasic (NaH_2PO_4), sodium dibasic (Na_2HPO_4), and BSA. Enough 0.05 M sodium monobasic solution is added to 0.05 M sodium dibasic solution to adjust the pH to 7.4 (as measured by an electronic Corning pH meter), forming the PBS solution. To 1 litre of PBS, 0.25 g of BSA is added to produce the RIA buffer. Lyophilized αBN antibody is reconstituted in 60 ml for use in the assay (final dilution 1:300,000). Lyophilized tissue samples are reconstituted as follows: GI in 2 ml (except adrenal glands - 1 ml), brain in 1 ml (except pituitary - 0.5 ml) RIA buffer. All assay tubes other than reference and blank consist of 200 µl of αBN and a combination of reconstituted tissue sample or standard and RIA buffer, the sum of which equals 200 µl. Different dilutions are needed from each area in order for the measured values to fall within the range of the standard curve. These dilutions have been determined through preliminary studies, and whose values are shown in Table 1.

Gut Regions	Sample Volume (µl)	Buffer Volume (µl)
oesophagus	150	50
fundus	20	180
antrum	20	180
duodenum	100	100
jejunum	100	100
ileum	100	100
colon	30	170
adrenal glands	200	none

Brain Regions	Sample Volume (µl)	Buffer Volume (µl)
hypothalamus	200	none
cerebellum	200	none
medulla	100	100
pons	200	none
striatum	100	100
neocortex	150	50
olfactory bulbs	200	none
hippocampus	100	100
midbrain	100	100
pituitary	200	none

Table 1. Volume of reconstituted tissue sample used per tube in RIA preparation. The sum of sample and buffer volumes totals 200 µl in each instance.

Two sets of tubes, reference and blank, in the standard assay represent total non-competitive ^{125}I-Tyr^{4}-BN binding and non-specific binding, respectively. The reference tubes do not contain cold (unlabelled) BN but contain αBN antibody,

while the blank tubes contain neither. All tubes receive 100 µl (approx. 5000 cpm) of ^{125}I-Tyr^{4}-BN. Every measurement, whether reference, blank, standard, or tissue sample is replicated (2 tubes each) across the assay, and independent standard curves are run before and after the tissue assays. In progressive order, the RIA is run as follows: All assay tubes are placed in an ice bath. Any RIA buffer needed per tube is added (100 µl for standards including reference and blank, see above chart for tissue requisites). All samples, whether standard or tissue sample are added to the respective tubes in necessary volumes (100 µl for standards, see above for tissue requisites). Reference and blank tubes receive 100 µl RIA buffer. To all tubes other than blank, 200 µl of diluted αBN antibody is added (blanks receive 200 µl RIA buffer) and the tubes are allowed to equilibriate for 1 hr at 4 °C. After that, 100 µl (approx. 5000 cpm) of ^{125}I-Tyr^{4}-BN is added to all tubes, which are then incubated at 4 °C for 16 hr.

Following the incubation, 100 µl of donkey anti-rabbit antibody (Amersham Canada, St. Catherines, Ontario) is added to all tubes, the tubes are vortexed and allowed to incubate for 15 min at room temperature. The racks of assay tubes are then placed on magnetic platforms for 15 min, following which the supernatant is discarded. The magnetic platforms, which attract the metallic donkey anti-rabbit antibody, are not removed during the discarding of supernatant. The tubes remain inverted on a section of absorbent paper for 5 min and allowed to fully drain. All tubes, beginning with the first standard run, followed by the tissue assay and ending in the second standard run, are placed in the Beckman gamma counter to be monitored for radioactive counts (1 min per tube). A cpm to femptomolar concentration (fmol) conversion is automatically performed by the gamma counter and printed below the generated standard curve. The standard

curve (represented in Figure 10) is generated by reference tubes (BN concentration = 0; maximal ^{125}I-Tyr^4-BN detection), blank tubes (αBN concentration = 0; non-specific binding), and tubes containing 2, 4, 8, 16, 32, 64, 128, 256 and 512 fmol non-radiolabelled ("cold") BN. Two known concentrations are run against this curve immediately following its generation in order to test its accuracy prior to actual tissue readings. These are known as the low and high controls, which contain 20 and 200 fmol cold BN, respectively.

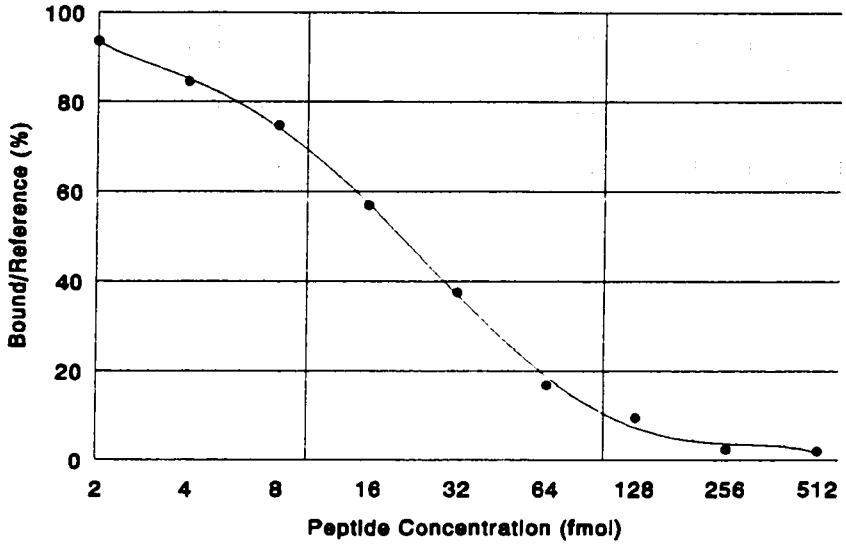

Figure 10. Standard Curve for BN RIA.

Chromatography and RIA of Hypothalamic and Antral Extracts

In order to determine the specificity of the antibody used in the RIA to mammalian BN-like peptides, high-pressure liquid chromatography (HPLC) was used to resolve the components of acid-extracted rat hypothalamus and antrum.

The hypothalami and antral segments were dissected from 3 rats, pooled, and processed as described in the RIA procedure, above. The supernatants were partially purified using a disposable Sep-Pak C_{18} column (Waters Associates, Milford, MA). Samples were loaded on a primed Sep-Pak cartridge, rinsed with 0.1% trifluoroacetic acid (TFA), and eluted with 40% acetonitrile (ACN) in 0.1% TFA. They were then lyophilized and resuspended in 0.1% TFA. The sample was then submitted to reverse-phase HPLC on 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 per min. After injection, 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 elution (Lemaire et al., 1989). Following this procedure, 50 μg of synthetic standards of BN, GRP_{1-27}, GRP_{18-27}, NMB_{1-32}, and NMB_{23-32} were injected in separate runs. BLI peaks were detected by RIA of the 50 collected fractions.

Tissue Preparation for Autoradiography

Following its removal from the cranium, the brain was immediately frozen in powdered dry ice (CO_2) for 5 min. GI segments were suspended in embedding material (Tissue-Tek, Canlab) in a micro-centrifuge tube and also frozen in CO_2, at which point the micro-centrifuge tube was broken away, leaving the embedded tissue. All tissues were then placed on the quick freezing platform (-35 °C) of the Hacker 5030 cryostat and each segment in turn was frozen onto a sectioning block with embedding material and sectioned. In the case of the brain, it was cut with a scalpel at the level of the cerebellum and both pieces, in turn, were frozen onto the

sectioning block. The cerebrum was affixed at its most caudal end, while the cerebellum and more caudal areas of the brain and upper spinal cord were affixed at the most rostral level of the cerebellum.

Sectioning

Sections of both GI tissue and of brain tissue were taken at -16 °C using a Hacker 5030 cryostat. The tissue slices were 16 µm thick and picked up directly from the microtome blade onto the 22 x 22 mm coverslips. They were thaw-mounted onto the coverslips by gently running a finger along the underside of the coverslips. Four sections were taken from each GI area. GI sections were collected in a cross-sectional plane, while brain sections were taken along the coronal plane. The sections were allowed to dry for a period of 1 hr at approximately 20 °C under a stream of cool dry air (provided by the cryostat demist function).

Autoradiography Procedure

Receptor autoradiography is a procedure used to visualize and localize receptors by combining radioligand binding techniques with autoradiography. The most commonly employed methods are those of Young and Kuhar (1979) or Herkenham and Pert (1982). When initially developed, those researchers employing autoradiographic procedures would inject live animals with the large amounts of radiolabelled ligand, making the procedure highly costly and limiting the number of ligands that could be infused *in vivo*. One also had to deal with inherent problems associated with interpreting *in vivo* results (Palacios et al.,

1988). Young and Kuhar's (1979) pioneering work broke through these obstacles when they discovered that *in vitro* receptor interactions in slide-mounted sections were largely comparable to those found in membrane preparations. Receptor binding to slide-mounted tissue sections is performed essentially in the same way as homogenate-based assays. Sample sections between 10 and 20 μm thick are mounted onto microscope slides or coverglass sections, incubated in a buffered solution containing radiolabelled ligand (such as ^{125}I-Tyr^{4}-BN), washed and then allowed to dry. During the incubation, receptors are labelled with the radioactive ligand so that the reaction [free ligand] + [receptor] ⇔ [ligand bound to receptor] proceeds to equilibrium. The radioactivity bound to these sections is the "total binding". A set of consecutive sections must always be incubated in parallel under the same conditions but in the presence of an excess of a displacer unlabelled ligand, in order to determine "non-specific binding". The receptor autoradiograms are produced when the sections are applied to radioisotope-sensitive film and are allowed to expose the film for a predetermined amount of time. Following development of the film, binding sites are visualized as silver grains in nuclear emulsions or as gray images on autoradiographic film. The primary advantage of this method over homogenate-based binding assays is the enhanced anatomical resolution and four to five order of magnitude increased sensitivity reported by researchers (Rogers, 1979). An additional advantage is that the binding sites, shown as patterns of radioactivity, can be related to specific anatomical structures within the animal.

Using film autoradiography, the gray levels associated with receptor localization can be converted into optical densities using an image analysis system. In turn, the optical densities can be converted into molar quantities of receptor-

bound radioligand by comparing the optical density of the tissue sample to a set of calibrated standards of known radioligand concentrations. By including a set of calibration standards (which can be prepared or are commercially available) alongside the tissue sections during film exposure, one obtains a series of reference standards of increasing optical densities and known radioactive content, against which the optical densities of the tissue sections can be compared. Subtraction of non-specific binding values from total binding values yield the amount of specific binding attributable to the preparation. Thus, changes in distribution of receptor sites can be visually documented whilst alterations in local receptor density can be quantified. Complete details of the procedure employed follow.

As with the RIA procedure, a buffer solution is necessary to conduct an autoradiography. One litre of QAR buffer consisted of:

- 130 mM NaCl
- 4.7 mM KCl
- 5.0 mM $MgCl_2 * 6H_20$
- 1.0 mM EGTA
- 10.0 mM HEPES, pH 7.4

This mixture was titrated with 5.0 M NaOH to a pH of 7.65. One g of $MnCl_2$ (which returns the pH to 7.4), 1.0 g BSA and 0.1 g bacitracin were added to the solution to complete the buffer preparation. The QAR buffer was stored at 4 °C until use.

There were five baths in which the brain or gut slices had to be incubated. The first bath was the pre-incubation bath, consisting solely of QAR buffer, but without the BSA or bacitracin. The tissue slices were allowed to soak in this bath for 20 min, in order to allow the dissociation of any endogenous ligands which may have been occupying BN-like peptide receptors. The second bath consisted of QAR buffer, to which was added enough ^{125}I-Tyr^{4}-BN to bring its final concentration to approximately 3 nM. The sections were incubated for 60 min at room temperature (21 to 23 °C). This stage of the procedure was performed directly on the coverslips containing each section, rather than in the incubation baths. Specifically, 700 µl of incubation buffer was pipetted onto each coverslip, so as to cover the entire surface containing the section. The sections were then drained 60 min later, placed momentarily on absorptive tissue and placed into the third bath for 4 min. The third and fourth baths in this procedure consisted exclusively of QAR buffer at 4 °C. After the 4 min, the sections were again drained, placed on absorptive tissue and placed in the fourth bath for 4 min. Finally, after drainage and partial drying on absorptive tissue, the sections were momentarily dipped in deionized H_2O (about 3 seconds) at 4 °C and the coverslips blotted and aspirated at the edges to remove excess liquid. In order to determine non-specific binding, the identical protocol was used in parallel on alternate sections in the presence of excess cold BN (1 µM non-radiolabelled BN). All sections were dried immediately under cool rapid air flow and left overnight at room temperature.

Film Application

The following morning, all sections were placed on a sheet of black cardboard and placed inside a film cassette. Standards containing different concentrations of ^{125}I ([^{125}I] micro-scales RPA 523 Amersham) were also affixed to the cardboard. The cassette was brought into a darkroom and, under safelight conditions, a sheet of Hyperfilm βmax (Amersham) was apposed to the sections and standards. The cassette remained closed and in the dark for a period of 3 days, at which point it was developed.

Film Development

The first step in film development was, under safelight conditions, removal of the exposed film from the film cassette and its immersion in Kodak D-19 Developer solution for a period of 4 min. This was followed by a 30 sec immersion in water (which acted as a stop bath) and a 9 min fixing stage in Kodak Rapid Fixer. After fixation, the film was placed in a running water bath for 20 min and allowed to air dry. All solutions were kept at room temperature, except the water, which was kept at 16 °C. In normal light conditions, the autoradiograms would be fully visible and ready for image analysis.

Staining

The sections used for autoradiographic exposure were then stained with neutral red in order to facilitate structural identification during image analysis. The staining solution comprised of 3 parts neutral red stock solution (0.5 g/L H_2O) and 2 parts 0.1M acetate buffer, pH 5.6. The staining procedure, a modification of

the Lockhard & Reers (1962) protocol (in Clark, 1984), involved immersion of the tissue sections for 5 min in the staining solution and a 10 sec rinse in deionized H_2O. After staining, Permount solution was applied and the sections were covered with a coverslip, being careful not to trap air bubbles during the mounting procedure.

Image Analysis

The image analysis system supports the MCID Computer Image Analysis System program (Imaging Research, St. Catherines, Ontario), which receives an image of the autoradiogram through a video camera and displays it on an Electrohome Multisync high resolution monitor for user analysis. The image is displayed through 256 levels of gray and the analysis itself is based on the optical density (a function of the gray level) of the area(s) sampled. Histological overlay of the stained section onto the autoradiogram facilitated identification of anatomical loci. QAR necessitates the establishment of the relationship between film opacity and tissue radioactivity. This is often done using radioactive standards. Conversion of optical density to nCi/mg was performed by the system through the availability of standard autoradiograms of known nCi/mg content (as was provided by the ^{125}I standards exposed along with the sections). Manual conversion from nCi/mg to fmol/mg protein was then conducted.

Experimental Protocols and Results

Determination of Behavioral Profile During Ingestion

These experiments measured the frequency of eating, drinking, grooming, exploring, and resting exhibited by the rats for 1 hr, following test meal (regular Purina Rat Chow) presentation. These preliminary experiments were conducted to determine the timepoint at which 12 hr food deprived animals would end a meal, and could thus be considered in a state of satiety.

Animals

All animals were deprived of food, but not water, for a 12 hr period (N=8). The rats were given their regular diet of Purina Rat Chow and behaviorally monitored for 60 min. They were monitored using a time-sampling porcedure such that each animal was observed for 2 sec every 20 sec interval, for a period of 60 min. The behaviors observed were operationally defined as follows:

- Eating: Biting, chewing, and/or ingesting the rat chow.
- Drinking: Licking and swallowing water from the water bottle nozzle.
- Exploring: Moving around the cage, and/or engaging in behaviors other than eating, drinking, grooming, or resting.
- Grooming: Licking, or scratching with the fore- or hind-paws, of any part of the animal's face or body.
- Resting: Awake but not moving, or sleeping.

Results

Results are presented as mean ± standard error of the mean (S.E.M.), and are shown in Figure 11. All behavioral measurements are presented as frequency of behavior within the indicated 10 min time frame (30 observations). During the first 20 min following food presentation, the animals' behavior comprised almost exclusively of eating and exploring. Eating behavior accounts for approximately 74% of the total responses during the first 20 min. After 30 min, only about 22% of total behavior is attributable to food intake, while resting and exploring combine to represent approximately 65%. The animals reduced their initial rate of food intake by 86% by the 40 min timepoint, with eating activity representing 11%, and resting and exploratory behavior accounting for 80% of total measurements. By 50 min, the animals had completely stopped eating.

Cumulative indices of consumed chow was collected throughout the 60 min period. After 10 min, 1.41 ± 0.52 g was eaten. At the 30 min timepoint, 6.51 ± 0.70 g had been consumed, whereas after 60 min, a total of 6.7 ± 0.97 g had been ingested. The 30 and 60 min timepoints differed significantly for the 10 min measure ($p<0.01$), but not from each other. These measures further validate the assumption that the animals had reached a satiety state after 30 min of food access.

In terms of behavioral sequence, it appears that 12 hr food deprived animals, when presented with solid food, will eat and explore during the first 30-40 min, with eating behavior decreasing in frequency as exploratory and resting activity increase. After 40 min, exploratory behavior diminishes, and resting activity becomes the predominant behavior. Drinking is not frequent during the course of the meal, but seems more pronounced as eating diminishes. One can

observe a mild increase in grooming behavior during the course of ingestion, with a modest peak observed at the 50 min time point.

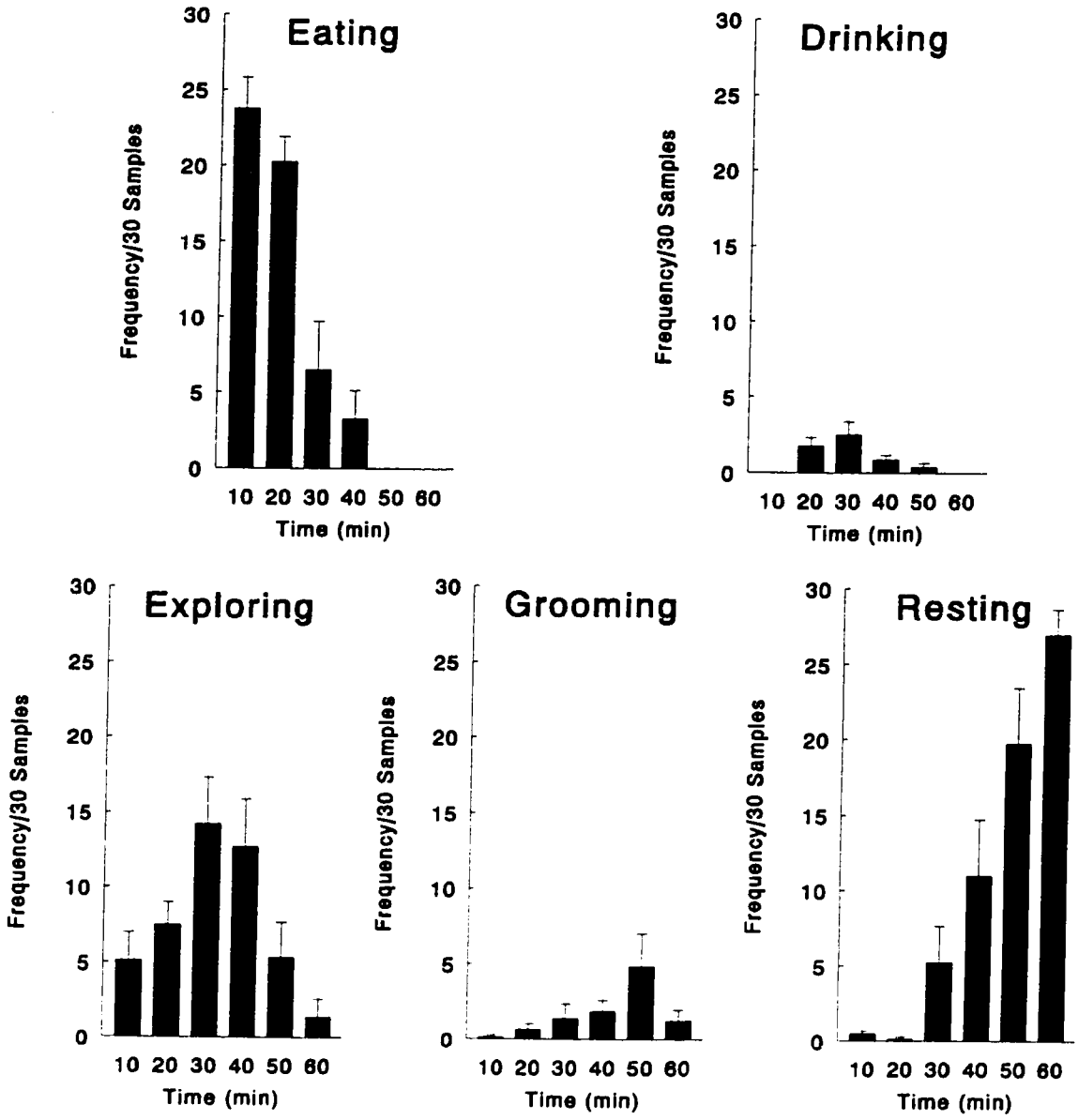

Figure 11. Behavioral profile: 12 hr food deprived rats during food access.

Discussion

The primary purpose of this experiment was to determine a timepoint at which we could reliably conclude that animals were at the initial phase of the satiety state. Following the ingestion of a meal, hunger is replaced by an increasing quiescent state. The satiety sequence in rats initially replaces ingestive behavior with exploratory and grooming activity, and then resting behavior (often sleep) (Smith & Gibbs, 1979). Our data seem to indicate that, in 12 hr food deprived rats, the satiety sequence begins at approximately 20-30 min. Although grooming behavior was not markedly increased until 40-50 min into the experiment, one can observe a sharp increase in exploratory activity between 20-40 min, coupled with a steady increase in resting behavior beginning at 20 min and becoming the predominant activity by 40-50 min after food presentation. If endogenous BN-like peptides are involved in the termination of a meal, and if postprandial satiety is initially signaled by a decrease in ingestion and concurrent increase in exploration, our data would suggest a timepoint of between 30 and 40 min to most accurately reflect the onset of postprandial satiety. Acknowledging that hunger-satiety states lie along a continuum, a 30-40 min timepoint would most plausibly indicate the temporal window in which one might expect the role of endogenous BN-like peptides to be greatest. In keeping with this notion, we have chosen a timepoint of 35 min after food presentation to represent postprandial animals in further experiments.

Gut and Brain Bombesin Levels in Pre- and Postprandial Rats

These experiments were designed to determine the regional levels of BN-like peptides within central and gastrointestinal systems in pre- and postprandial animals. The objective of these experiments was to determine whether changes in BN-like peptide immunoreactivity occured in response to feeding. We reasoned that if the release of BN-like peptide(s) signal the initiation or maintenance of satiety then the peptide levels should change at the relevant site(s) in response to food deprivation and food intake. Thus, it was hypothesized that higher levels of BN-like peptides would be detected in postprandial tissue segments in comparison to their preprandial counterparts. This could indicate an increased turnover (synthesis and release) of BN-like peptides in response to food ingestion, hence supporting its involvement in endogenous satiety mechanisms. Such results could also, however, reflect decreased release of BN-like peptides, challenging their proposed role as satiety agents. Hence, the results of this set of experiments cannot be conclusive, as we do not have the necessary techniques to measure peptide levels at presynaptic and synaptic sites at our disposal. We could, however, determine if endogenous BN-like peptide levels are altered with feeding status, and the loci where these changes may be apparent.

Animals

All animals were deprived of food, but not water, for a 12 hr period. Some rats were given food for 35 min. Animals were then sacrificed in a pairwise manner, such that half the number of animals were in the food-deprived (preprandial) state (n=7), whilst the other half were fed, forming the postprandial

group (n=7). All tissues harvested from both conditions were analyzed in a single radioimmunoassay.

Tissue Processing

Rats were killed by decapitation, and their brains and gastrointestinal systems rapidly dissected. Food material was removed from the entire GI tract and the following segments were rapidly dissected: oesophagus, fundus, antrum, duodenum, jejunum, ileum, and colon. Dissection of the brain involved the rapid isolation of the hypothalamus, cerebellum, pons, medulla, olfactory bulbs, striatum, neocortex, hippocampus, midbrain and pituitary. The adrenal glands were also removed.

Statistical Analysis

Results are presented as mean ± S.E.M. BN-like peptide concentrations are expressed in fmol/mg protein. Data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey post-hoc comparison.

Results

The highest BLI concentration was detected in the GI tract. In context of regional distribution, the antrum of the stomach had the highest BLI levels. Of the other peripheral tissues studied, comparatively high BLI levels were found in the colon, fundus, and jejunum while moderate levels were present in the duodenum and ileum. The oesophagus and adrenal glands contained low BLI levels.

Brain areas demonstrating the greatest BLI were, in descending order, the pituitary, the medulla, the striatum, and the hippocampus. All other areas had

comparatively lower levels of BLI, with the neocortex and cerebellum demonstrating the lowest values.

Significant differences in the BLI between the pre- and postprandial conditions were detected in both central and peripheral tissues. ANOVA of BN-like peptide levels in the brain revealed a significant increase in peptide concentration after a meal, $F(1, 8) = 5.2196$, $p = 0.0228$. Tukey post-hoc comparison indicated significant elevation of measurable peptide levels within the hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$) (Figure 12).

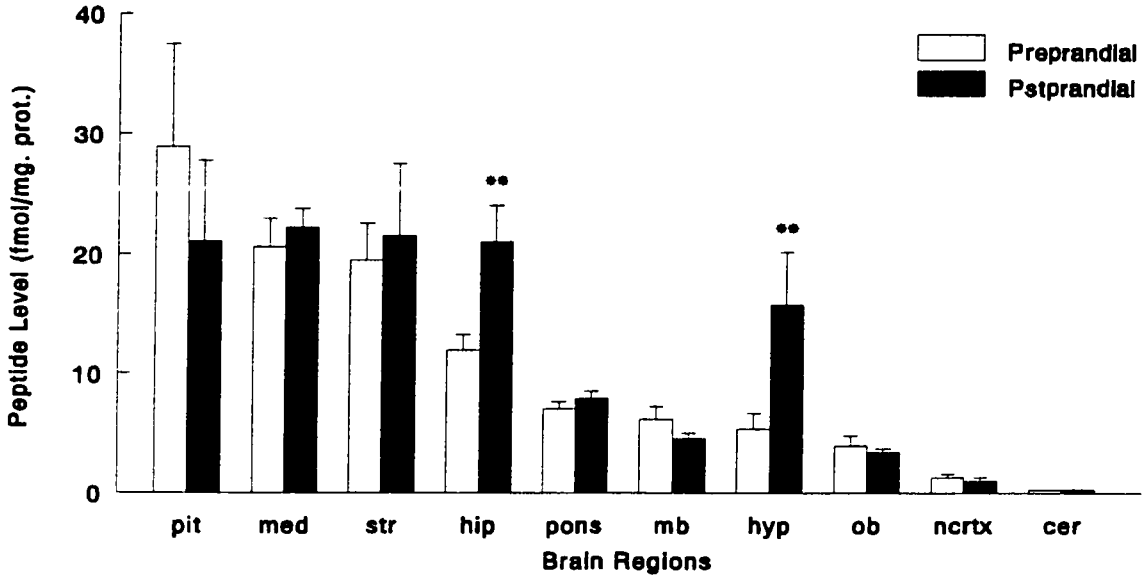

Figure 12. Changes in BN-like peptide levels in brain regions of 12 hr food deprived animals after 35 min food access. pit, pituitary; med, medulla; str, striatum; hip, hippocampus; pons, pons; mb, midbrain; hyp; hypothalamus; ob, olfactory bulbs; ncrtx, neocortex; cer, cerebellum. (** $p < 0.01$)

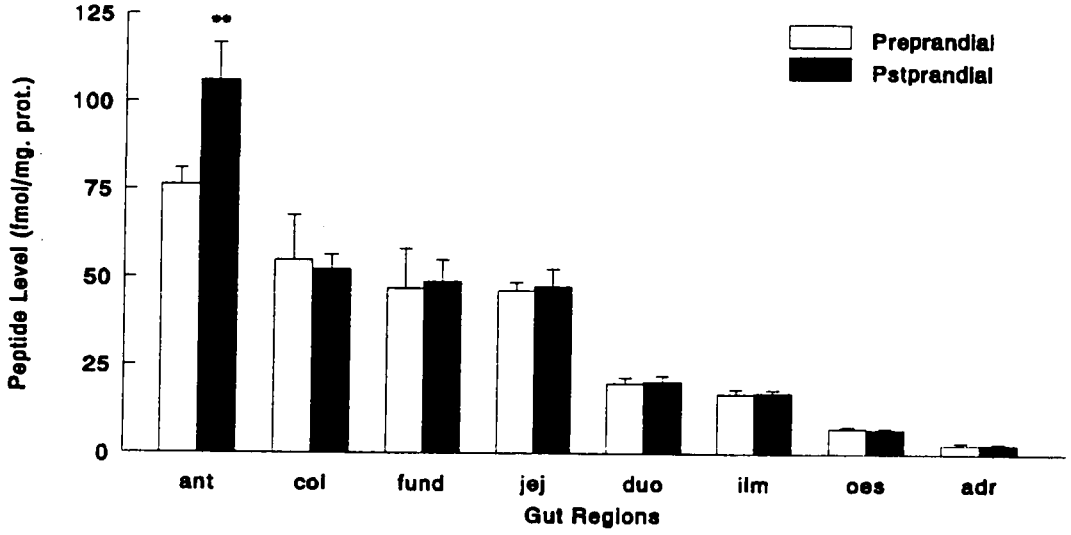

Figure 13. Changes in BN-like peptide levels in gut regions in 12 food deprived animals after 35 min food access. ant, antrum; col, colon; fund, fundus; jej, jejunum; duo, duodenum; ilm, ileum; oes, oesophagus; adr, adrenal glands. (** $p < 0.01$).

In the gut, ANOVA revealed no significant difference between the two experimental conditions, $F(1, 6) = 1.8543$, $p = 0.1736$. *A priori* prediction that the stomach would be the most likely gut region to reveal a change in BN-like peptide levels after a meal prompted Tukey post-hoc comparison of the stomach regions. Prior studies have shown the fundus and antrum of the stomach to contain the highest levels of BN-like peptides in the GI tract, and the antral mucosa has been indicated as the source of BN-like peptide production in the gut. Post-hoc comparison revealed a significant increase in the presence of BN-like peptides in a single region of the GI tract - the antrum ($p < 0.01$) (Figure 13).

Analysis of the two endocrine glands studied, the pituitary and adrenal glands, showed no significant difference between the pre- and postprandial conditions, $F(1, 1) = 0.5088$, $p = 0.5109$.

HPLC of GRP_{18-27}, NMB_{23-32}, BN, GRP_{1-27}, and NMB_{1-32} standards revealed that these peptides elute at fractions 30, 35, 37, 39, and 45, respectively.

Figure 14 shows that after injection of the hypothalamic extract into the HPLC, two peaks of BLI eluted at the 30th and 38th fractions, respectively. Injection of the antral extract revealed BLI peaks at the same fractions as did the hypothalamus, as well as a minor peak at 44th fraction.

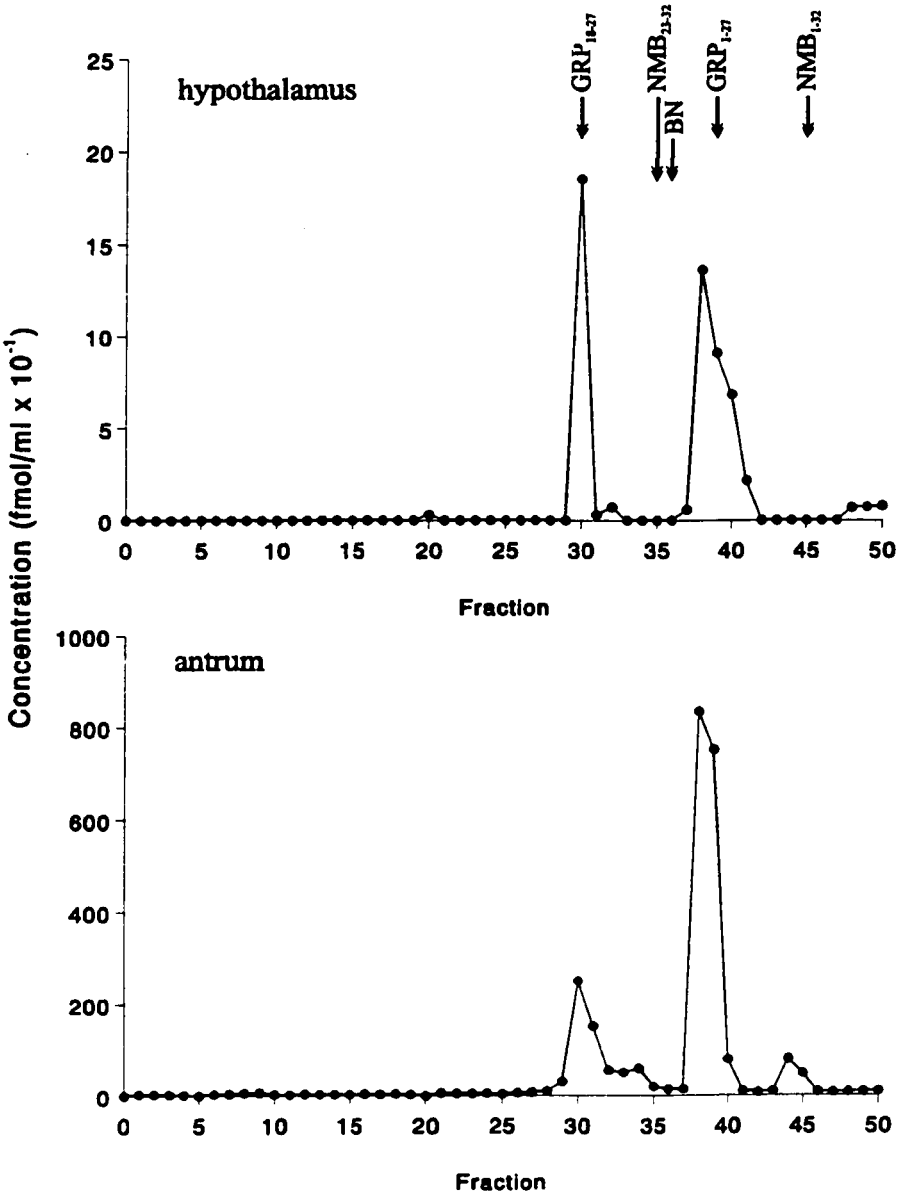

Figure 14. Chromatographic Resolution of BN-like peptide detection and concentration in the hypothalamus and antrum of *ad libitum* fed animals.

Discussion

The purpose of this experiment was to determine whether changes in the concentration of endogenous BN-like peptides were evident as a result of the ingestion of a meal. Our results indicate that these changes occur in specific regions of the brain and GI tract. Interpretation of these results must be, at the present time, done cautiously. Demonstrating an increased concentration of BN-like peptides in the postprandial condition may reflect one of two occurrences; an increase in presynaptic or intrasynaptic availability of these peptides after a meal, or a decrease in the peptide level in the preprandial condition as compared to the *ad libitum* fed condition. In the case of CCK, the latter phenomenon has been demonstrated in the GI tract of food deprived rats (Koop et al., 1987). Both interpretations, however, support the contention that endogenous BN-like peptides may play a role in the control of food intake. If an increased release (without concurrent change in synthesis) of BN-like peptides occurred after a meal, one might have expected a reduction in BLI postprandially, since the peptide molecules are rapidly catabolized following release. Thus, the increases we observed may indicate either a reduced release or an increased synthesis and release of these peptides. The locus specificity of the changes we observed suggests that these are not generalized or broadcast effects but rather the response of specific central and peripheral sites which may play particularly significant roles in the elicitation or mediation of BN effects in the regulation of food intake.

The hypothalamus, one of the two central areas shown to produce an increase in BN-like peptides in the postprandial state, has been shown to contain numerous BN-immunoreactive terminals, particularly in the PVN, with lesser numbers within the VMH and LH (Chronwall et al., 1985; Panula et al., 1982).

Further, high doses of BN (1 μg) into the PVN and VMH, as well as low doses of BN (5 ng) into the LH have both been shown to inhibit food intake (Kyrkouli et al., 1987; Stuckey & Gibbs, 1982). Hence, our observation that, after a meal, BLI is found in higher concentrations in the hypothalamus is consistent with prior research demonstrating an inhibition of food intake following exogenous administration of BN at the level of the hypothalamus.

Although it has previously been shown that the fundus of the stomach produces the greatest amount of BN-like peptides in the periphery, our results indicate that the antral portion of the stomach exhibits the meal-dependent alterations of these peptides. An increased presence of BLI in the stomach at the end of a meal is concordant with prior observations that BN stimulates the secretion of gastrin, gastric acid and pepsin in the stomach (Bertaccini et al., 1973; Modlin et al., 1980), and supports its involvement in an endocrine mechanism triggering a central satiety response via the stimulated release of plasma CCK from the small intestines, vagal afferent transmission or by neurohumoral interaction at the area postrema during the course of food ingestion. Speculatively, BN-like peptides released in the pyloric antrum, which communicates directly with the duodenum, may serve to augment the release of CCK from the CCK-producing cells within the duodenum and thus enhance the satiety response.

The observed increase in the levels of BLI in the hippocampus under the postprandial condition may have less to do with the satiety mechanisms and be more related to affective qualities of food, such as memories of particular taste inputs or other relevant memories associated with the timing, duration or presentation of a meal. Peripheral administration of BN has been shown to enhance memory processing (Flood & Morley, 1988). The measurable presence of

these peptides in the hippocampus may implicate a role for these peptides in memory function, although the mechanism(s) by which this process occurs remains as yet unknown. It would appear, however, that the memory enhancing effect of systemic BN is vagally dependent, as vagotomy abolishes this effect (Flood & Morley, 1988).

The antibody used in these RIAs (generously donated by Dr. T.W. Moody) recognizes the C-terminal fragment of BN and has been previously shown to cross-react strongly with the mammalian peptide GRP_{1-27} (110%), BN (100%), and GRP_{18-27} (82%), but not with NMB_{23-32} (<0.1%) or ranatensin (<0.1%) (Moody et al., 1988). Our present data with this antibody indicate a strong cross-reactivity with GRP_{1-27} and GRP_{18-27}. In the antrum, a small peak was detected at the 44th fraction, corresponding to the NMB_{1-32} standard elution. It is possible, however, that this BLI is also due to the small cross-reactivity which exists between BN and substance P (0.1%), reported elsewhere (Moody et al., 1981). A replication of the present study using antibodies raised specifically against NMB and substance P would determine the extent to which these peptides are involved in the animals' response to ingestion. Although the specific BN-like peptide(s) involved is (are) not known, it seems evident that endogenous peptides do indeed respond in a site specific manner to the ingestion of a meal. Our chromatography results indicate similar ratios of GRP_{18-27} and GRP_{1-27} in the hypothalamus, whereas a 2-3 fold greater concentration of GRP_{1-27} as compared to GRP_{18-27} was detected in the antrum. These results are consistent with those found by Hernanz (1990), and suggest that, in this study, the changes in BLI observed between meal conditions are attributable to GRP_{1-27} and its fragment, GRP_{18-27}.

It is important to note that, where we find significant increases in the concentrations of BN-like peptides from the pre- to the postprandial condition, we will also find receptors for BN-like peptides (Moran et al., 1988; Moody et al., 1978). This would suggest a functional significance to the observed changes.

Peptide Content of Central Tissues Under Various Feeding States

These experiments were intended to determine the levels of BN-like peptides within central regions across the time course of a meal. The objective was to characterize the pattern of change in BN-like peptide immunoreactivity evident as a result of feeding. It was hypothesized that, where we had detected significant differences between pre- and postprandial groups, we would now observe a positive correlation between the degree of change and the time elapsed from meal onset.

The experimental protocol was identical to that of the preceding experiment, with the exception that only those areas having shown significant differences between the pre- and postprandial states were studied. Also, an area which had failed to show significant differences between pre- and post-meal states, the medulla, was included as a control. Baseline data was also collected from *ad libitum* fed rats in order to provide a control sample for the study. This data will provide a baseline to which we can compare the effects of the 12 hr deprivation used in other aspects of our studies.

Animals

Four groups of animals were used; 1) an *ad libitum* fed control group, and three groups of animals that were deprived of food, but not water, for a 12 hr period (9:00 pm - 9:00 am) and then 2) left unfed, 3) given food for 10 min, or 4) given food for 35 min. The food presented was their regular diet of Purina Rat Chow. Animals were then sacrificed. One animal from each group was sacrificed and processed for radioimmunoassay on each day of the experiment, and the order in which they were sacrificed was randomized for each day. We used 8 animals in

each group. Subsequently, a single animal in the postprandial group failed to eat after food deprivation and was dropped from the study, reducing the number of animals in that group to 7. All tissues harvested from each of the four conditions were analyzed in a single RIA.

Tissue Processing

Rats were killed by decapitation, and their brains rapidly removed. Dissection of the brain was performed, quickly separating the hypothalamus, medulla, and hippocampus.

Statistical Analysis

Results are presented as mean ± S.E.M. BN-like peptide concentrations are expressed in fmol/mg protein. Data were analyzed by two-way ANOVA followed by Tukey post-hoc comparison.

Results

The brain regions differed significantly in BLI content, $F(2,80) = 33.32$, $p < 0.0001$, with highest overall levels being detected at the hypothalamus. There was also a significant main effect of the feeding status on regional BLI levels, $F(3, 80) = 3.83$, $p = 0.012$). The analysis of the simple main effects revealed that the differences were mainly attributable to the meal-related changes at the hypothalamus and the hippocampus. Tukey post-hoc comparison revealed significant differences between those animals food deprived for 12 hr, and those that had been similarly deprived and then fed for 35 min (Figure 15).

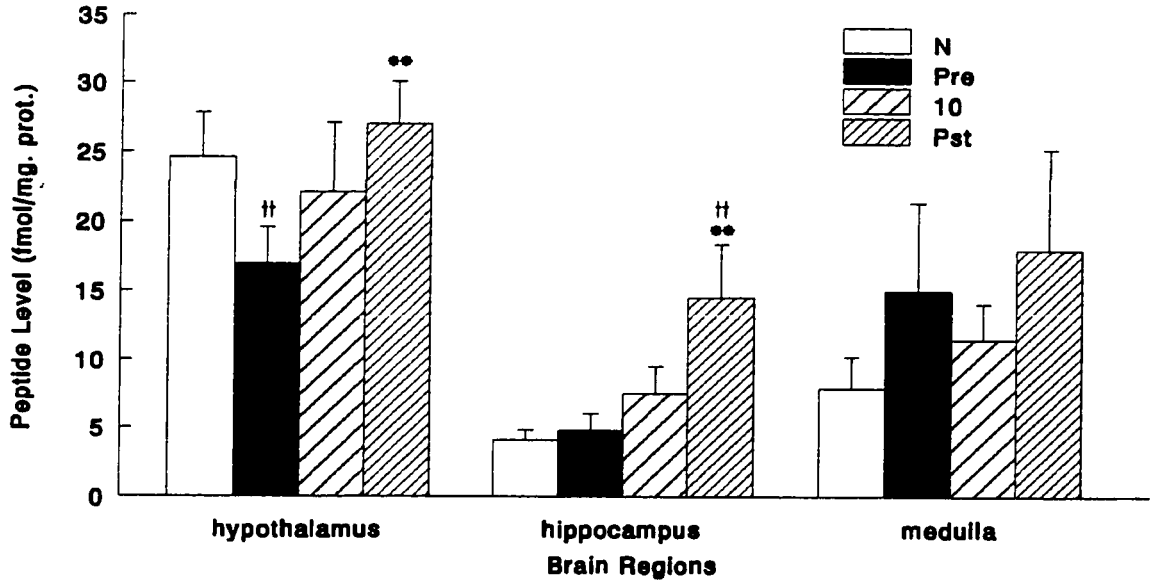

Figure 15. BN-like peptide concentrations in brain regions of free feeding (N), preprandial (Pre), 10 min fed (10), and 35 min fed or postprandial (Pst) animals. (†† $p < 0.01$ from N; ** $p < 0.01$ from Pre).

In the hypothalamus, the BLI content increased significantly as the food deprived animals (preprandial group) became fully satiated (35 min food access; postprandial group), $p < 0.01$. Similarly, in the hippocampus, a significant increase in BLI was observed from the pre- to the postprandial condition, $p < 0.05$. The medullary levels of BLI remained unaffected across the various timepoints.

When compared to the *ad libitum* fed condition, hypothalamic BLI content was significantly lower in the 12 hr food deprived animals, $p < 0.01$. However, after 35 min of food ingestion, the peptide levels climbed back up and were no longer significantly different from those of the *ad libitum* fed animals. Within the hippocampus, food deprivation failed to alter the BLI content. Furthermore, the

BLI levels increased significantly as the animals became satiated (postprandial group), $p < 0.01$. The group of animals that were partially satiated (fed for 10 min) were not significantly different from any of the other conditions within the hypothalamus or hippocampus. However, there seemed to be a time- and/or meal size-dependent increasing trend in BLI.

Discussion

The objective of this experiment was to try to establish whether endogenous levels of BLI change in response to food ingestion, and to characterize the pattern of alteration during the course of a meal. Our results indicate that, in animals that have been deprived of food for a 12 hr period, there is a steady increase in the levels of BLI during the course of a meal, with the highest levels being detected at the end of the meal. This trend was seen in both the hypothalamus and hippocampus. At the level of the medulla, no change was observed; a finding consistent with our previous report (Kateb & Merali, 1992). Thus, these alterations in peptide level seem to be region-specific and not the result of a generalized upregulation of central BN-like peptide system(s). It is important to note that, when comparing this data to our previous experiments, the focus lies predominantly on the direction of change across conditions and not on the absolute values detected. Each RIA series has its own standard curves and, as such, can generate considerably different final values across experiments.

The increased levels of BLI in the postprandial condition could be due to 1) an increase in the synaptic availability of these peptides after a meal or, 2) a decrease in the levels of BLI in the preprandial condition. Since exogenous administration of BN or GRP consistently induces a satiety-like state, the observed

reduction in endogenous hypothalamic BLI in the preprandial condition could be interpreted as reflecting reduced availability of the peptide and consequently a diminution in the 'satiety signal'. Conversely, the postprandial return of BLI to *ad libitum* range could reflect increased availability of the BN-like peptides and the reinstatement or maintenance of the satiety-like state. Although this interpretation fits well with the BN-satiety hypothesis, alternate interpretations of the results are also possible. For instance, if one assumes that the rate of peptide synthesis is constant, the reduction of the peptide levels could reflect an increased rate of peptide release and metabolism in the preprandial state and the converse during the postprandial condition. These possibilities need to be tested directly, using techniques such as push-pull perfusion or microdialysis combined with RIA, during different feeding states. In the hypothalamus then, the levels of BLI do not seem to increase after a meal but, rather, decrease during fasting. Hippocampal BLI revealed quite a different pattern; the levels of BLI in ad libitum fed animals were similar to those of the postprandial group. In the hippocampus, it would appear that food ingestion results in an elevation of BLI, and that a 12 hr food deprivation or fasting does not alter the endogenous stores of these peptides. As hypothalamic levels of BN-like peptides seem to respond to fasting, hippocampal peptide levels change in response to ingestion.

Our previous chromatography results (Kateb & Merali, 1992) indicate similar proportions of GRP_{1-27} and GRP_{18-27} in the hypothalamus, a finding concordant with those of Hernanz (1990). These findings suggest that the observed meal-related changes may be primarily attributable to GRP_{1-27} and its fragment, GRP_{18-27}. Since the antibody used in this study does not detect NMB_{23-32} or NMB_{1-32}, our findings do not rule out the possibility that these

peptides are also affected by food ingestion or fasting. In the hypothalamus, there is a high concentration of GRP_{1-27} and GRP_{18-27} receptor mRNA (Moody et al., 1981; Panula, 1986; Wada et al., 1990), as well as moderate grain densities of ^{125}I-Tyr^4-BN binding and high density of GRP nerve terminals and cell bodies (Panula, 1986; Roth et al., 1982). In contrast, moderate to low grain densities of ^{125}I-NMB_{1-32} have been observed in the hypothalamus, as well as low levels of NMB_{1-32} receptor mRNA (Moody et al., in press). In terms of immunoreactive content, some report high GRP/NMB ratio (Moody et al., 1988), whereas others report a low ratio (Chronwall et al., 1985; Minamino et al., 1988). In terms of mRNAs within the rat hypothalamus, there is a greater preponderance of the GRP_{1-27} mRNA than the NMB_{1-32} mRNA, within most nuclei (Wada et al., 1990). In light of the literature, our data would suggest that the meal-dependent changes seen in the hypothalamus are related more to GRP-like than the NMB-like peptides.

In the hippocampal formation, the mRNA for GRP_{1-27} is more preponderant than that for NMB_{1-32}. However, hippocampal NMB immunoreactive content has been reported to be relatively high by some (Chronwall et al., 1985; Minamino et al., 1988) and low by others (Moody et al., 1988). GRP_{1-27} receptor mRNA within the dentate gyrus and Ammon's horn have been reported to be low, whereas that of NMB_{1-32} are high in hippocampal tissue (Wada et al., 1991; Moody et al., in press). Binding studies using ^{125}I-NMB_{1-32} and ^{125}I-Tyr^4-BN have demonstrated moderate and high grain densities in the hippocampus, respectively (Moody et al., in press). It would thus appear that in this region of the brain, both NMB-like and GRP-like peptides may serve a functional role. A similar study

assessing the specific changes in NMB-like peptides would help elucidate their relative importance in ingestive behavior.

Because the hypothalamic levels were similar in *ad libitum* fed and postprandial animals, it would appear that, after a meal, the levels of BN-like peptide within this region return to those observed in non-deprived satiated rats. This finding supports the contention that the changes observed are directly related to the ingestion of a meal, and not to other behaviors associated with feeding such as increased locomotion or temperature alteration. The gradual, meal-dependent increase in BLI from the preprandial to the postprandial state, to a level closely approximating that of *ad libitum* fed animals, strongly suggests that we are observing a satiety signal within the hypothalamus.

Although this gradual increase was also detected in the hippocampus, the significant difference between the *ad libitum* fed and postprandial groups may indicate that this observation is not directly related to satiety, but to a concomitant or alternate process. As has been discussed in the previous experiment, the observed increase in the levels of hippocampal BLI in postprandial animals may be associated with 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 meal.

Central and Peripheral Receptor Status of Pre- and Postprandial Rats

The objective of these studies was to demonstrate an endogenous response by BN-like peptide receptors in the brain and/or periphery as a direct result of the ingestion of a meal. It was hypothesized that postprandial rats would exhibit higher density of BN receptor sites than would preprandial animals, reflecting an upregulation of the BN-mediated satiety response. The design was broad based in order to scan as much of the central and GI systems as possible, and was intended as a preliminary study. The results of this experiment would determine whether we would continue to investigate potential receptor changes resulting from ingestion of food, and the direction of further studies, should they be warranted.

Animals

All animals were deprived of food, but not water, for a 12 hr period. Some rats were given food for 35 min. Animals were then sacrificed in a pairwise manner, such that half the number of animals were in the food-deprived (preprandial) state (n=8), whilst the other half were fed, forming the postprandial group (n=8).

Tissue Processing

Rats were killed by decapitation, their GI systems were rapidly dissected, and their brains were removed. Food material was removed from the entire GI tract and the following segments were harvested: oesophagus, fundus, antrum, duodenum, jejunum, ileum, and colon. Brain sections were taken between the following coordinates (Paxinos & Watson, 1982): Bregma 6.2 mm to Bregma -5.3 mm, with a single section taken at every 160 μm. All visible binding

was measured, so as to obtain the broadest possible sampling of central ^{125}I-Tyr^{4}-BN receptor binding under both feeding conditions. Only those central areas where BN binding was detected in at least 5 of the 8 animals in each group are reported. A total of 22 areas satisfied this criterion. These areas are presented below:

central amygdaloid nucleus	centromedial thalamic nucleus
medial amygdaloid nucleus	paraventricular thalamic nucleus
paraventricular hypothalamic nucleus	hippocampus
cingulate cortex	hippocampus CA4
frontal cortex	dentate gyrus
rhinal cortex	septohippocampal nucleus
medial preoptic area	anterior olfactory nucleus
nucleus, vertical limb of the diagonal band	olfactory tubercle
suprachiasmatic nucleus	nucleus accumbens
stria terminalis	indusium griseum
fundus striatum	reuniens

Table 2. Central Regions Exhibiting Binding Sites for BN-Like Peptides

The autoradiography procedure for this first experiment was adopted from Wolf et al (1983). The concentration of radiolabelled BN employed was initially estimated as falling between 0.1 and 1.0 nM, a concentration range demonstrated in the literature to produce satisfactory results (Zarbin et al., 1985; Vigna et al., 1987; Pert et al., 1980; Moran et al., 1988). The incubation time for brain sections

has consistently been reported to be 1 hr for brain sections (Zarbin et al., 1985; Wolf et al., 1983; Moody et al., 1988). Within the gut, however, this period has ranged from 1 to 2 hr (Vigna et al., 1987; Moran et al., 1988) and has not been as well represented in prior investigations. Hence, we decided that, prior to conducting this experiment, we would establish the optimum incubation time (when the specific/non-specific binding ratio is maximal) for GI tract sections in our laboratory.

Incubation of fundal and antral sections for 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 hr revealed that equilibrium was reached by 3.0 hr (Figure 16). In the antrum, as well as in the fundus, a 3 hr incubation period seemed optimal. Although total binding in the fundus of the stomach was an order of magnitude higher than that detected in the antrum, non-specific binding was approximately 5 times higher. The difference in specific binding was relatively small (about 300 cpm/section).

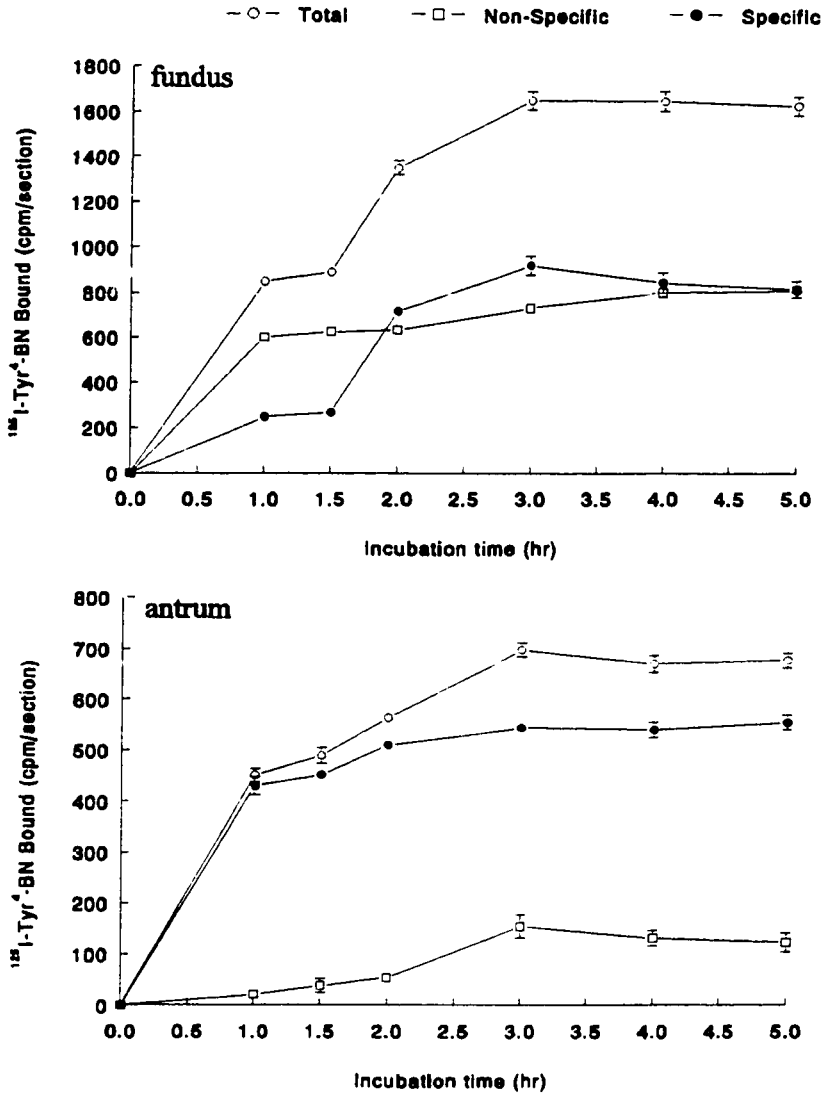

Figure 16. Determination of optimal incubation time of fundal and antral sections. Specific binding becomes optimal after 3 hr, for both tissues.

Statistical Analysis

Results are presented as mean ± S.E.M. BN-like peptide receptor densities are expressed in nCi/mg. Data were analyzed by two-way ANOVA followed by Tukey post-hoc comparison.

Results

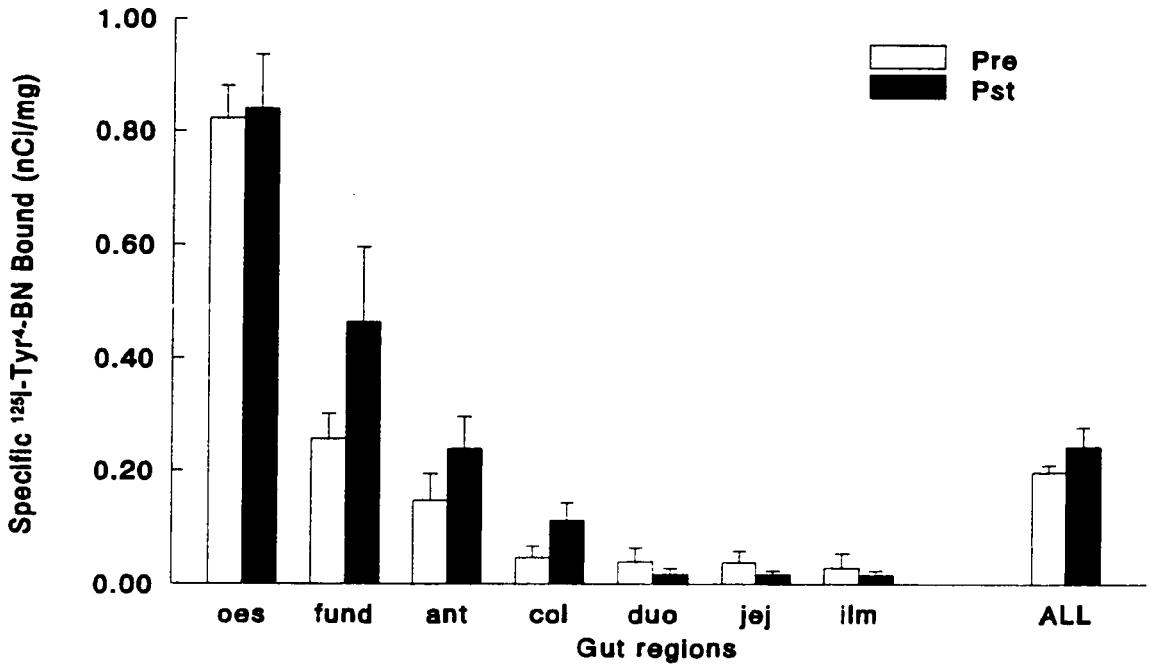

Figure 17. BN-like peptide receptor binding in GI tract of preprandial (Pre) and postprandial (Pst) animals. oes, oesophagus; fund, fundus; ant, antrum; col, colon; duo, duodenum; jej, jejunum; ilm, ileum.

As can be seen in Figure 17, the highest binding density observed in the GI tract was found in the oesophagus. Comparatively moderate binding was found in

the fundal and antral areas of the stomach, while receptor binding along the intestinal portion of the digestive tract was comparably low. Generally, BN-like peptide receptor binding decreased from the uppermost to lowermost portions of the digestive tract. Within the intestinal regions, however, the colon appeared to possess greater binding capacity when compared to the duodenum, jejunum, or ileum. Within the GI tract, our data do not suggest a response by BN-like peptide receptors as a result of food intake. Analysis of overall GI tract receptor binding showed no significant differences between pre- and postprandial groups ($F(1, 12) = 0.084$, $p = 0.773$). Further, analysis of feeding status by GI region similarly revealed no significant differences ($F(1, 6) = 2.572$, $p = 0.108$).

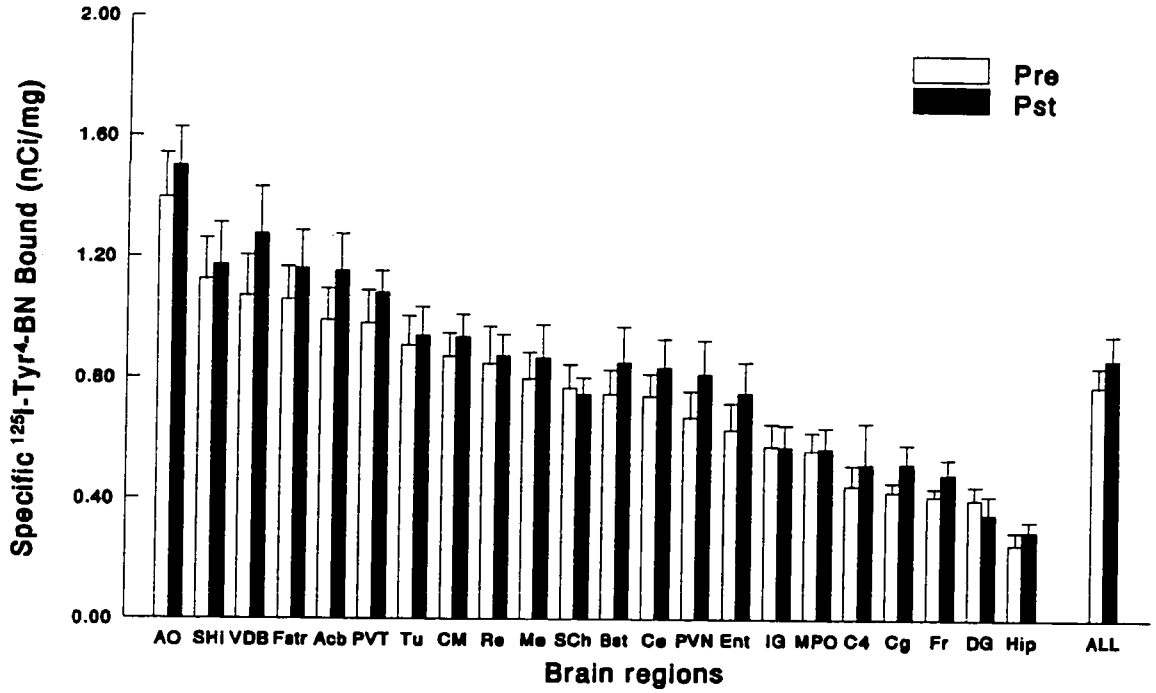

Figure 18. BN-like peptide receptor binding in brain regions of preprandial (Pre) and postprandial (Pst) animals. AO, anterior olfactory nucleus; SHi, septohippocampal nucleus; VDB, vertical limb of the diagonal band; Fstr, fundus striatum; Acb, nucleus accumbens; Tu, olfactory tubercle; PVT, paraventricular thalamic nucleus; CM, central medial thalamic nucleus; Re, reuniens; Me, medial amygdala; SCh, suprachiasmatic nucleus; Bst, bed nucleus, stria terminalis; Ce, central amygdala; PVN, paraventricular hypothalamus; Ent, entorhinal cortex; IG, indusiem griseum; MPO, medial preoptic area; C4, CA4 region of Ammon's horn; Cg, cingulate cortex; Fr, frontal cortex; DG, dentate gyrus; hip, hippocampus.

With a few exceptions, one can observe within the brain a gradual decrease in receptor binding as one proceeds caudally from the anterior olfactory nucleus to the level of the hippocampus (Figure 18). The notable exceptions include the medial amygdala, bed nucleus of stria terminalis, and frontal cortex. Central data did not reveal an effect of feeding condition on receptor binding when an overall analysis of brain regions was conducted ($F(1, 42) = 0.057$, $p = 0.566$. However, two-way ANOVA of feeding status by brain region demonstrated a significant difference between pre- and postprandial conditions ($F(1, 21) = 5.807$, $p = 0.016$). Further analysis of this result through Tukey post-hoc comparison did not show a region-specific effect of feeding status on central BN-like peptide receptors.

Discussion

These experiments were primarily designed to indicate the status of central and peripheral BN-like peptide receptors during food intake. Within the GI tract, our results were clear. There were no observed changes in BN-like peptide receptor availability as a result of ingestion, whether GI tract receptor binding was compared as a whole between feeding states, or compared as a function of specific GI regions. The receptor binding profile observed was similar to that reported in the literature (Moran et al., 1988). In terms of receptor distribution, Figure 17 illustrates that, under either feeding condition, the highest levels of $^{125}Tyr^{4}$-BN binding were found, in decreasing order of magnitude, in the oesophagus, fundus, and antrum. Within the oesophagus, it has been determined that both iodinated BN and NMB_{1-32} bind exclusively to the muscularis mucosae with differing affinities (Von Schrenck et al., 1989). Their study revealed that, although both peptides demonstrated similar relative potencies for causing contraction of muscle

strips from whole oesophagus and from isolated muscularis mucosae, receptors present in the oesophagus were primarily NMB_{1-32}-preferring receptors. Since ^{125}I-Tyr^4-BN will bind to both receptor sub-types we were not able, in our experiment, to determine the percentage of binding observed attributable to NMB-preferring or to BN-preferring receptors. Differentiation between these two BN-like peptide receptor subtypes has yet to be described in the other GI areas we have studied. However, receptors binding ^{125}I-Tyr^4-BN have been localized primarily in the circular muscle layer of the fundus and antrum of the stomach, in the submucosal layer and circular muscle layer of the duodenum, the submucosa of the jejunum and ileum, and the longitudinal and circular muscle layers of the colon (Moran et al., 1988). Our observation that feeding status had no discernible effect on receptor binding in the GI tract led us to decide not to include GI tract regions in further autoradiographic studies.

Analysis of central receptor binding data indicated an effect of feeding status, although this effect could not be attributed to a specific brain region or regions. Being a preliminary study, we had to this point relied on previously reported incubation parameters and protocols for brain receptor autoradiography. Although the results of this experiment were not entirely clear, what seemed clear was the indication of an effect of feeding condition on central BN-like peptide receptors. We therefore decided to continue our investigation of the central receptors, but not before confirming optimum binding parameters in our laboratory. The only conclusions that can be made at this stage is that a central response to ingestion by BN-like peptide receptors supports the contention that BN's effects on feeding have central sites of action (Gibbs et al., 1981; Morley & Levine, 1981; Kulkosky et al., 1982; Yao et al., 1989), and the fact that we found

an effect of feeding status centrally but not peripherally does not negate the hypothesis that BN's effects on feeding are centrally mediated and independent of a gastric site of action (von Schrenck et al., 1989).

Establishment of Optimal Binding Parameters

When using a radioactive ligand for autoradiography, the incubation conditions have to be established. In the case of ^{125}I-Tyr^4-BN, these binding parameters have been previously reported (Moody et al., 1978; Pert et al., 1980; Moran et al., 1988), and include concentration of radiolabelled ligand and incubation time. These measures were reassessed in our laboratory, in order to ensure that, under our conditions, the experiments were conducted using optimal parameters. The incubation temperature and wash times were kept constant and in keeping with previous studies.

It is also important to determine the pharmacological specificity of the ligand employed. However, these data have been previously reported in a highly consistent manner (Moran et al., 1988; Moody et al., 1982; Panula, 1986; Moody et al., 1988; Moody et al., in press), and were not reassessed in these studies.

Concentration of ^{125}I-Tyr^4-BN

To select the optimal incubation concentration of radiolabelled ligand, it is necessary to calculate the ratio of specific to non-specific binding at equilibrium. A concentration that occupies half of the total number of receptors is recommended (Palacios et al., 1988). Serial tissue sections are incubated with increasing concentrations of the radiolabelled ligand (total binding), and blanks (non-specific binding) have to be generated for each concentration of the ligand. In this experiment, 5 concentrations of ^{125}I-Tyr^4-BN were used: 0.18, 0.21, 0.42, 0.86, and 1.72 nM. Duplicate sections containing the Acb were taken from 3 animals for each concentration at every timepoint. Sections were incubated for

15, 30, 45, 60, and 90 min, and radolabelled binding was assessed by crushing and counting the sections in a gamma counter.

Results

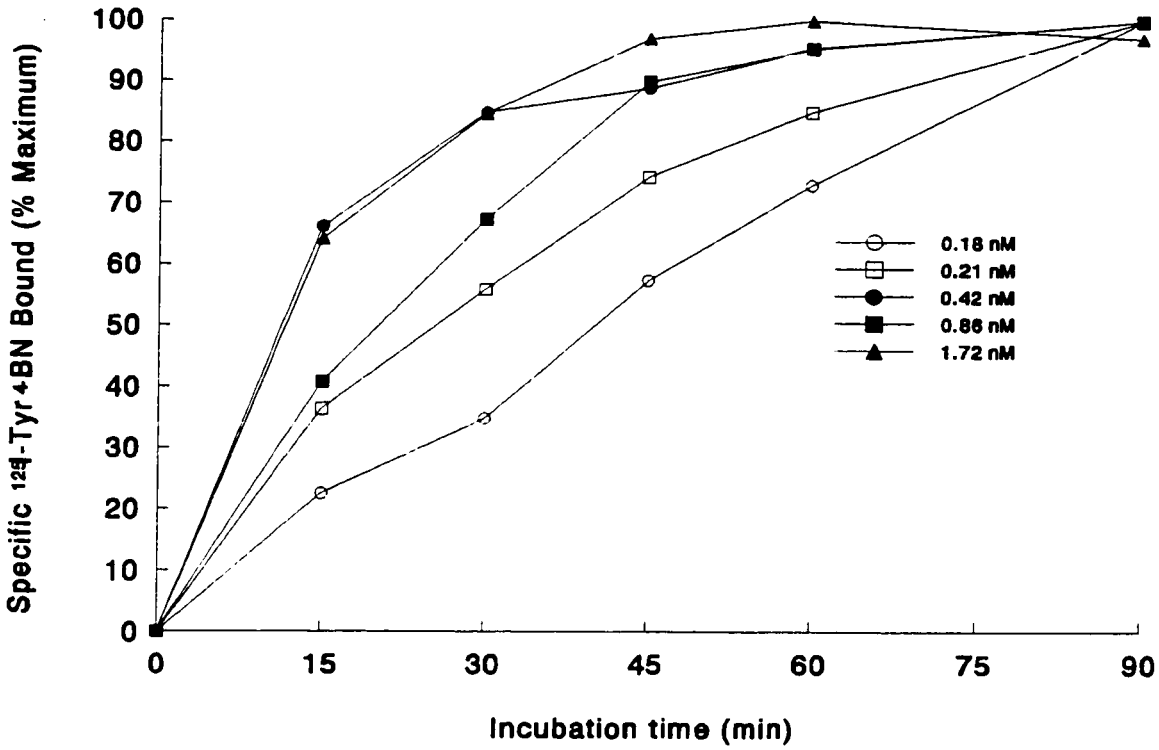

Figure 19. Binding profile of ^{125}I-Tyr^{4}-BN at 0.18 - 1.72 nM concentrations in the Acb. Binding is expressed as the percentage of maximal binding observed for each concentration, in order that plots may be comparable.

In this experiment, we found that the lowest concentration to reach equilibrium before 90 min was 0.42 nM (Figure 19). Concentrations 0.42 through

1.72 nM all reached equilibrium by 60 min incubation time. At the 60 min timepoint, the specific/non-specific ratios for each concentration were calculated and are shown below.

^{125}I-Tyr^4-BN Concentration	Specific/Non-Specific Ratio (60 min)
0.18 nM	3.90
0.21 nM	3.96
0.42 nM	8.125
0.86 nM	6.65
1.72 nM	7.30

Discussion

From our results, it was determined that a 0.42 nM concentration of ^{125}I-Tyr^4-BN yielded optimum specific/non-specific binding ratios. This value corresponds to the concentration used in receptor binding studies by other researchers (von Schrenck et al., 1989; Vigna et al., 1987). Therefore, all autoradiographic procedures to follow will utilized a concentration of 0.42 nM ^{125}I-Tyr^4-BN during incubation.

Incubation Time

It is important that sections be left in the incubation medium long enough to reach an equilibrium, such that [free ligand] + [receptor] $\Leftrightarrow$ [ligand bound to receptor]. Sections are incubated for increasing periods of time while keeping other variables constant. This procedure allows the investigator to choose the incubation time at which equilibrium is attained and specific binding is maximal.

Equilibrium data was acquired from brain sections at the Acb. Sections were taken in duplicate from 3 *ad libitum* fed animals. Acb sections were incubated in 0.42 nM radiolabelled BN for 15, 30, 45, 60, and 90 min.

Results

Our results (Figure 20) indicate that, in sections at the level of the Acb, total binding increases with incubation time and equilibrium is attained at the 1 hr incubation timepoint. Non-specific binding seems to increase rapidly to the 30 min incubation period, at which point a very gradual increase can be seen over time. Maximal specific binding is attained after 1 hr of incubation.

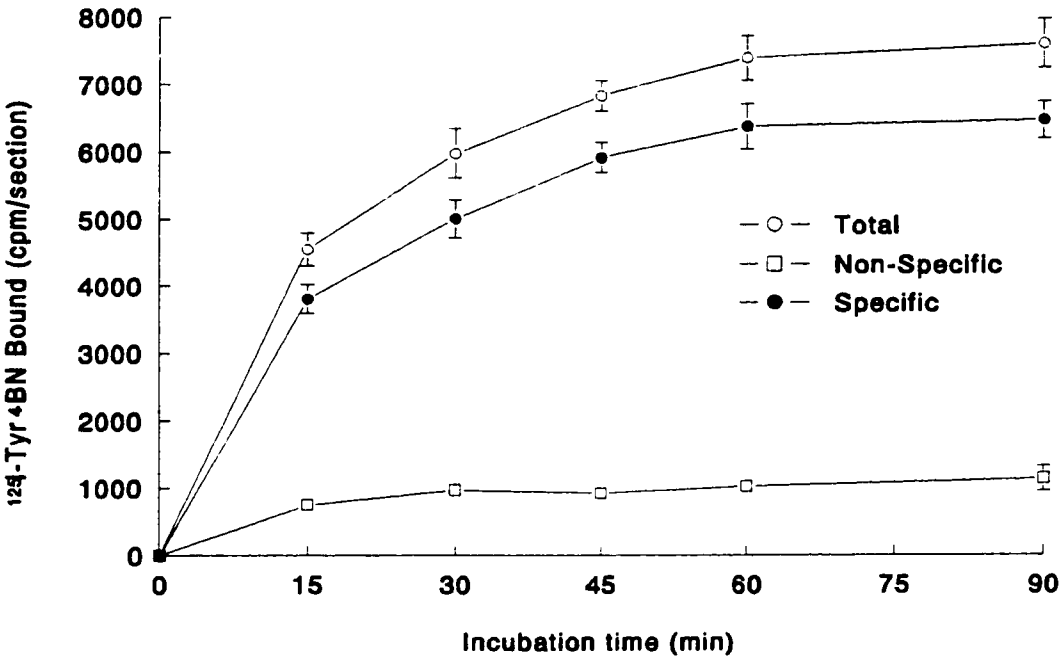

Figure 20. Binding profile of 0.42 nM ^{125}I-Tyr^{4}-BN to Acb sections. Maximal specific binding is attained after 60 min incubation.

Discussion

This experiment revealed the optimum incubation period within the Acb of the brain to be 60 min in the presence of 0.42 nM ^{125}I-Tyr^{4}-BN. As a result, autoradiography of brain sections will henceforth be performed using a 1 hr incubation period.

Receptor Autoradiography of Central Sections Under Various Feeding States

The purpose of this series of experiments was to quantify the density of BN-like peptide receptor binding sites in specific brain regions at different timepoints of a meal. Our objective was to ascertain whether there are changes in central BN-like peptide receptor binding during food ingestion and, if so, to describe the pattern of this change. We hypothesized that, should differences be observed in specific loci, they would be represented by increased receptor binding at the end of a meal (postprandially) or decrease during a food deprived state (preprandially), and that postprandial binding would not significantly differ from that observed in *ad libitum* fed animals. Our reasoning was that, if BN-like peptides act as satiety-inducing peptides, and this role is partially mediated through central receptor regulation, an upregulation in binding after ingestion would potentiate this effect. Conversely, a downregulation of receptors during food deprivation would suppress the satiety state. Finally, it was expected that non-deprived (*ad libitum* fed) animals should exhibit similar receptor binding to those animals in the postprandial condition, since both groups are presumably in a satiated state.

Having decided to pursue our investigation of central BN-like peptide receptors during ingestion, practical considerations forced us to choose a greatly reduced number of brain regions for the study. For this next set of autoradiographic studies, we chose amongst only those areas for which 8 of 8 animals in both pre- and postprandial groups showed adequate receptor density in the previous experiment. Our final choices were based on 1) those areas known to subserve mechanisms affected by BN, such as locomotion, thermoregulation, feeding, and memory, and 2) those areas having shown the greatest potential for

change in receptor binding between food states in our preliminary scan, indicated by Tukey post-hoc comparison. In this series of experiments, we also included the NTS as an area of investigation, as its implication in feeding regulation is well documented (Norgren, 1978; Powley & Laughton, 1981; Crawley, 1985b; de Beaurepaire & Suaudeau, 1988; Johnson & Merali, 1988; Ladenheim & Ritter, 1989).

Animals

Four groups of animals were used; 1) an *ad libitum* fed control group, and three experimental groups of animals that were deprived of food, but not water, for a 12 hr period and then 2) left unfed (preprandial), 3) given food for 10 min, or 4) given food for 35 min (postprandial). The order of sacrifice was randomized for each of the 8 experimental days, and the sections taken from each group were all processed for receptor binding and applied to a single autoradiography film on each day of the study.

Tissue Processing

Rats were killed by decapitation, and their brains were removed and frozen. Triplicate coronal sections were taken of areas containing the Acb, fundus striatum (Fstr), Bst, medial preoptic area, PVN, hippocampus, and NTS. The coordinates representing the sections were taken from Paxinos & Watson (1982), and are shown below.

- Acb & Fstr - Bregma 1.2 mm
- medial preoptic area - Bregma -0.8 mm
- PVN - Bregma -1.8 mm
- hippocampus - Bregma -2.8 mm
- NTS - Bregma -12.8 mm

Statistical Analysis

Results are presented as mean ± S.E.M. BN-like peptide receptor binding densities are expressed in nCi/mg. Data were analyzed using two-way ANOVA, followed by Tukey post-hoc comparison.

Results

In *ad libitum* fed animals, the highest levels of BN-like peptide receptor binding were observed in the Fstr and Acb (Figure 21). Relatively moderate receptor binding was detected in the central amygdala, medial preoptic area, and Bst. The PVN, hippocampus, and NTS revealed comparatively low receptor densities. With the exception of the Fstr and Acb, comparison of the *ad libitum* fed control group to all of the experimental conditions revealed no significant differences at any of the other brain sites studied. An overall comparison of all the brain areas of control animals to those of the experimental groups, similarly, showed no significant differences from the control condition. At the level of the Acb, a significant increase in receptor density was detected from the control to the preprandial condition ($p < 0.01$). In contrast, a decrease in receptor binding was observed when comparing control to postprandial animals, in the Fstr ($p < 0.01$).

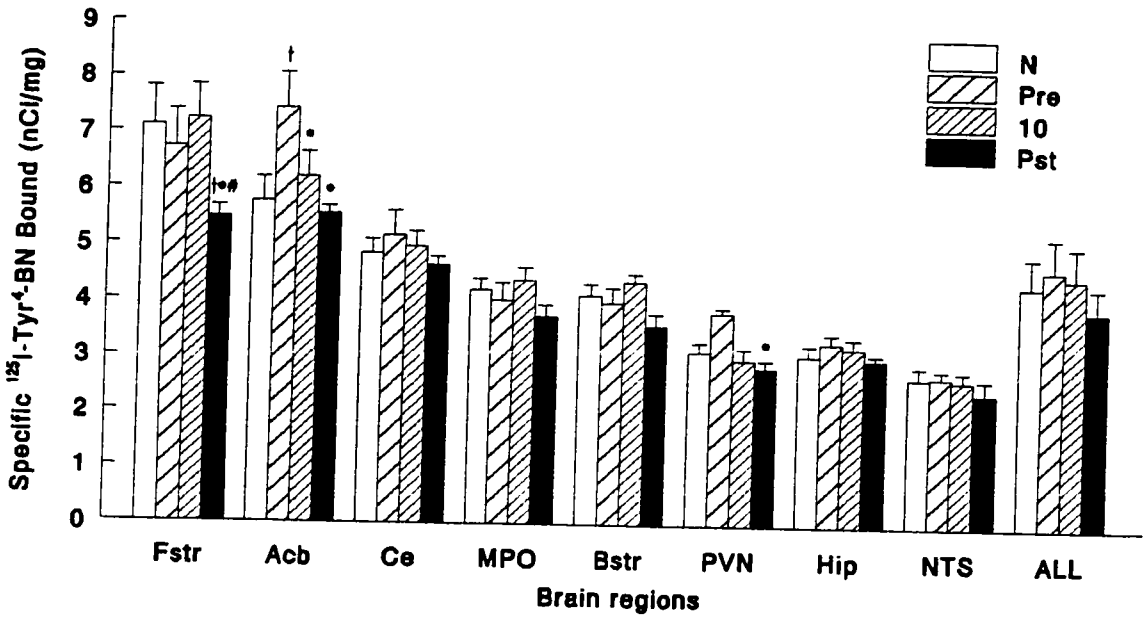

Figure 21. Specific receptor binding of ^{125}I-Tyr^{4}-BN to brain regions under various feeding conditions. († $p < 0.01$ from N; * $p < 0.01$ from Pre; # $p < 0.01$ from 10). N, *ad libitum* fed; Pre, preprandial; 10, fed for 10 min; Pst, postprandial.

Figure 22. Representative autoradiographs of BN-like peptide receptor binding in Acb, Fstr, and PVN of Pre- and Postprandial animals. Darker areas reflect greater binding. Compared to the preprandial group, postprandial rats showed significantly less BN-like peptide receptor binding in those three areas ($p < 0.01$).

Data analysis using two-way ANOVA revealed significant differences between the different feeding states across different brain regions ($F(3, 7) = 7.574$, $p < 0.0002$). Tukey post-hoc comparison revealed significant differences between those animals that had been food deprived for 12 hr (preprandial), and those that had been similarly deprived but fed for 35 min (postprandial), in the Fstr ($p < 0.01$), Acb ($p < 0.01$), and PVN ($p < 0.05$). Those animals that had been permitted to eat for 35 min showed a significant decrease in the receptor density of BN-like peptide receptors in all three areas, when compared to the preprandial group. Further, in the Fstr and Acb, a significant decrease ($p < 0.01$) in receptor binding was also observed in 10 min fed animals when compared to their preprandial counterparts. In other brain regions no significant differences were observed. Overall one-way ANOVA of ingestive state for mean brain receptor density revealed no significant effects of the different meal states, ($F(3, 28) = 0.362$, $p = 0.784$).

Discussion

This series of experiments were aimed at following the pattern of change in BN-like peptide receptor binding during the course of a single meal. It was our intention to demonstrate that a dynamic relationship exists between feeding states and receptor availability of BN-like peptides in the brain. Our results seem to indicate that this is, indeed, the case.

In order to assess the effect of acute food deprivation, we compared receptor binding in preprandial animals to binding in the *ad libitum* fed control group. With the exception of receptors located in the Acb, it appears that 12 hr food deprivation during the active (overnight) phase does not significantly alter the

availability of BN-like peptide receptors in the areas studied. In the Acb, however, we observed a significant increase in receptor binding in the 12 hr food deprived group. In fact, this increase was so pronounced that receptor binding in preprandial Acb slices was significantly greater than all other conditions. This result was unexpected and difficult to explain. The Acb has not been extensively investigated in relation to BN-like peptide function except in its involvement in BN-induced locomotor activity, implicating BN in the activation of the mesolimbic dopaminergic system (Pert et al., 1980; Merali et al., 1983; Schultz et al., 1984; Merali et al., 1985). A similar link has been established between potentiation of dopaminergically activated locomotion and another satiety peptide, CCK, in the Acb (Crawley, 1985a; Fink & Ott, 1992). DA agonist activity at DA receptors is believed to contribute to the reduction of food intake (Heffner et al., 1977; Leibowitz & Rossakis, 1979; Clifton et al., 1989).

Interestingly, CCK alone does not cause an increase in locomotor activity when directly injected into the Acb (Crawley, 1985a; Gower & Broekkamp, 1985) and inhibits DA turnover when infused into the anterior Acb (Hökfelt et al., 1980). In contrast, Merali et al. (1989) have demonstrated, through microdialysis, a release of intra-accumbens DA in response to locally infused BN. The results of Manzanares and colleagues (1991) have challenged this finding, reporting that i.c.v. injection of BN, does not activate dopaminergic neurons in the Acb at doses up to 10 ng. The results of these studies may indicate a modulatory role of these peptides on mesolimbic system function, although their precise roles and mechanisms are, as yet, unclear. In any event, it is clear that in order to better understand the significance of BN-like peptide receptor changes in the Acb during

food deprivation, it is useful to explore these events in term of mesolimbic system response to different feeding states.

Recently, researchers have reported significant release of DA in the Acb of either feeding or satiated animals, when compared to food deprived rats (Yoshida et al., 1992; Mark et al., 1992; McCullough & Salamone, 1992). It is important to note that, in these studies, food deprived animals served as controls. These findings are inversely correlated with our demonstrated binding profile of ^{125}I-Tyr^{4}-BN in the Acb. It would appear that, during a meal, DA is released in the Acb and BN-like peptide receptor availability is diminished. Should BN-like peptides serve a modulatory role in the mesolimbic system, then our results support this contention. Hypothetically, an increase in DA release during ingestion may be accompanied by a concurrent release of BN, and consequent receptor binding, in the Acb. BN, after binding to its receptors, has been shown to be internalized, and the degradation products released from the cell. In the same study, exposure to BN also resulted in a subsequent decrease in cell surface binding (Zhu et al., 1991). This study, however, was conducted using pancreatic acini and not brain tissue. If the same process occurs at central loci, then an observed decrease in receptor binding in our present study may represent the internalization of these receptors in response to the release of BN-like peptides during ingestion. Further research will be needed to confirm or refute this hypothesis. The functional role of BN-like peptide release and/or BN-like peptide receptor internalization during the course of ingestion in the Acb is presently suggested to correspond to behavioral activation, and specifically to the grooming response and increased locomotion observed with central BN administration and seen in feeding animals. This suggestion is speculative, but based on the literature

indicating that 1) BN may induce intra-accumbens DA neuron activation (Merali et al., 1989), 2) DA release in the Acb is associated with hyperlocomotor activity (Costall et al., 1977), 3) DA-induced hyperlocomotor activity is associated with D2 receptor subtypes in the mesolimbic system (Fuxe et al., 1985), 4) Researchers have reported that specific D1 agonists induce grooming (Molloy & Waddington, 1987), whilst specific D2 agonists elicits exploratory activity (Longini et al., 1987), and 5) BN-induced behaviors such as grooming and locomotion have been associated with D1 and D2 mesolimbic receptor subtypes, respectively (Piggins & Merali, 1989).

Our data also indicate a significant effect of ingestion at the end of a meal on receptor binding in the Fstr and PVN. The Fstr is believed to be generally continuous with the central nucleus of the amygdala (de Olmos et al., 1985). Although both the striatum and the central amygdaloid group send projections to DA neurons in the VTA, Bolam et al. (cited in de Olmos et al., 1985) noted that only in the Fstr were unusual (for the striatum) projection neurons with spine poor dendrites found (de Olmos et al., 1985). The features of these neurons are consistent with the large spine poor neurons in the medial division of the central amygdala (McDonald, cited in de Olmos et al., 1985). The pool of literature relevant to the understanding of the role of the Fstr in ingestive processes is sparse, and in BN-like peptide function, non-existent. A clue to its possible function, with regards to ingestion, may lie in its projection. The Fstr send neurons to the areas surrounding the SCP (Jackson & Crossman, cited in Paxinos, 1985). These projections may be indicative of an indirect role of the Fstr in reinforcing mechanisms associated with feeding behavior. As discussed previously in this document, studies have shown that axons passing through the SCP play a crucial

role in classical conditioning responses and reinforcement. Therefore, a tentative explanation of the Fstr's involvement in BN-like peptide receptor regulation during a meal might relate to the reinforcing quality of ingestion in animals that were in a preprandial state. As in the Acb, a release in BN-like peptides during ingestion in the area of the Fstr may result in receptor internalization and, hence a decrease in observed binding post-ingestion. Although our preliminary RIA studies included the striatum, our dissections are not discrete enough to isolate specific loci within the striatum. Therefore, our RIA results of striatal regulation of BN-like peptide levels during ingestion lack the anatomical resolution to support or refute the present hypothesis concerning BN-like peptide receptor response in the Fstr.

The PVN receives a number of inputs from structures conveying information about the peripheral, physiological state of the organism. It receives input from various circumventricular organs as the subfornical organ, organum vasculosum, lamina terminalis and area postrema (Steffens et al., 1988; Sawchenko & Swanson, 1983). A significant source of input to the hypothalamus in general, and to the PVN in particular, is revealed through immunocytochemical staining of fibers reactive to NE. The significance of NE innervation of hypothalamic nuclei is indicated by the notable increases in feeding and body weight elicited by chemical stimulation with NE (Lichtenstein et al., 1985; Leibowitz et al., 1984). Noradrenergic neurons in the NTS and dorsal motor vagus nucleus are the major source of noradrenergic innervation of the hypothalamus via the ventral noradrenergic pathway (Steffens et al., 1988). The PVN has been shown, through retrograde tracing, to project fibers to the dorsal vagal complex and has also been shown to contain BN immunoreactive cells (Costello et al., 1991). In that study, 15 to 37 % of the total BN-immunoreactive cells in the PVN

were labelled with retrograde tracer injected into the dorsal vagal complex. NTS projections to the hypothalamus are very dense in the PVN, moderately dense in the LH but virtually absent in the VMH. The functional significance of these inputs as they relate to feeding may be indicated by the observation that the NTS is the recipient of chemical sensory input from the GI tract and related organs, carried primarily through the sensory branches of the facial, glossopharyngeal and vagal nerves (Hamilton & Norgren, 1984). The involvement of the NTS is also suggested by Johnston and Merali's (1988) finding that BN will suppress feeding at extremely low doses when administered in the NTS. Furthermore, lesions of the medial aspect of the NTS attenuate the satiety response elicited by either centrally or systemically injected BN, indicating that the NTS may be a common neural substrate for both central and peripheral BN-induced suppression of feeding (Ladenheim & Ritter, 1989).

The PVN appears to be the locus of NE-induced feeding (Leibowitz, 1978). This effect seems to occur through a disinhibitory action of NE on the PVN, which normally suppresses feeding (McCoy & Avery, 1990). BN has been shown to suppress NE-induced feeding when peripherally administered and may, in this respect, suggest that peripheral BN acts as a secondary central satiety agent. The presence of BN-like peptides in the periphery may act as systemic modulators of the central satiety process, possibly communicating via vagal afferents and endocrine interaction at the area postrema, which has been shown to possess receptors for BN-like peptides and to which project fibers of the solitary tract. Thus, both vagal and endocrine signals may be transmitted to the NTS and on to other brain sites involved in the central satiety response.

The observed reduction in receptor binding post-ingestion in the PVN may indicate, as was suggested in the discussion of the Acb data, the internalization of BN-like peptide receptors in response to BN-like peptide release.

Microinfusion of BN into the PVN, an area rich in BN-like peptides and BN-like peptide receptors, results in a significant attenuation of feeding (Willis et al., 1984; Gunion et al., 1989). Destruction of axonal fibers originating in the PVN has been shown to block the satiety response to BN (Leibowitz, 1978). An increase in the availability of BN-like peptides in the hypothalamus, although not specifically attributable to the PVN, as a result of ingestion has been demonstrated in the above-described RIA experiments (Kateb & Merali, 1992; Merali & Kateb, in press), but has yet to be investigated by other researchers.

The observed changes in receptor binding in satiated and/or feeding animals, when compared to food-deprived animals, has largely been explained, on the basis of receptor internalization in response to BN-like peptide release. The possibility remains that BN-like peptide receptors in those loci may be responding to food intake through the regulation of their affinity for the peptides, and/or an actual change in receptor number. Our next experiment addressed this question, and so discussion of these possibilities is reserved to the following section.

Saturation Analysis of Pre- and Postprandial Acb Sections

The objective of these experiments was to determine whether the changes observed in the previous experiment were attributable to a change in receptor number (B_{max}) or receptor affinity (K_D). Our receptor studies have demonstrated a decreased binding capacity in postprandial animals, when compared to animals that had been not been fed. We proposed, in the previous section, that this decrease may be explained by receptor internalization in response to endogenous release of BN-like peptides. We thus predicted that, in this saturation experiment, we would observe a decrease in the maximum binding capacity (B_{max}) after food intake, since a number of receptors would have been internalized during the meal and would not be available to bind with the radiolabelled BN.

Procedure

Saturation analysis of separate preprandial (n=4) and postprandial (n=4) groups was performed in order to determine the K_D and B_{max} of receptors under both conditions. The saturation experiment included triplicate coronal sections of the Acb. In order to determine the equilibrium dissociation constant (K_D) serial sections were incubated in increasing concentrations of the radiolabelled BN. Sections were taken from the Acb of preprandial and postprandial animals. Ten concentrations of ^{125}I-Tyr^4-BN were used; 0.05, 0.07, 0.10, 0.13, 0.17, 0.23, 0.30, 0.41, 0.55, and 0.74 nM. We could not use concentrations higher than 0.74 nM, as the film was approaching saturation at the level of the Acb. All tissues were processed in accordance with the autoradiography procedure described earlier, and applied to film for 3 days. A parallel preparation contained 1μM unlabelled BN, in order to determine non-specific binding, and was applied to the same films.

Non-specific binding was determined at the highest concentration of ^{125}I-Tyr^4-BN, and linear regression was used to assess non-specific binding at the lower concentrations. Scatchard-Rosenthal graphs were plotted to reveal the K_D and B_{max} of the tissues under both preprandial and postprandial conditions.

Statistical Analysis

Results are presented as mean ± S.E.M. The K_D values are expressed in nanomolar concentrations (nM), and the B_{max} are reported as femptomoles per mg wet weight (fmol/mg wet wt.). Data were analyzed using two-way ANOVA.

Results

The Scatchard-Rosenthal plots representing the receptor characteristics of Acb ^{125}I-Tyr^4-BN binding in pre- and postprandial animals are shown in Figure 23. For each Scatchard-Rosenthal plot, the K_D is calculated as the negative inverse of the slope $\left[\frac{-1}{slope}\right]$ of the linear regression, while the B_{max} is represented by the intercept of the regression at the X-axis $\left[X=\frac{-(Y\text{-}intercept)}{slope};\ Y=0\right]$. The results of individual analyses is shown in Table 3. Our result do not indicate a significant difference in either the K_D or B_{max} when comparing pre- and postprandial groups.

	Preprandial		Postprandial	
Rat	K_D	B_{max}	K_D	B_{max}
1	1.9	11.9	0.9	6.2
2	1.0	8.2	1.6	19.9
3	4.9	57.1	3.7	48.0
4	1.4	21.5	1.3	23.8
Mean ± S.E.M.	2.3 ± 0.88	24.7 ± 11.17	1.9 ± 0.62	24.5 ± 8.71

Table 3. Pre- and Postprandial K_D and B_{max} values for ^{125}I-Tyr^4-BN Binding at the Acb.

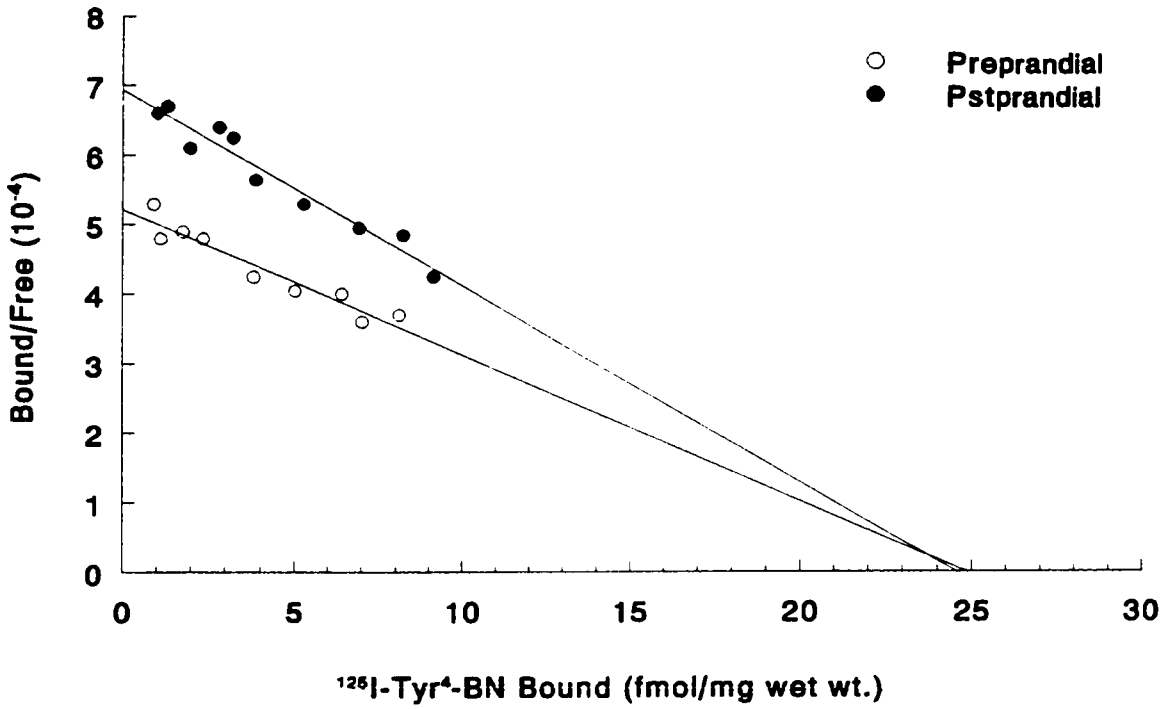

Figure 23. Representative Scatchard-Rosenthal plots of ^{125}I-Tyr^4-BN receptor binding to Acb in pre- and postprandial animals.

Discussion

This experiment was performed in order to determine whether there were measurable differences in the relative affinities and/or relative number of BN-like peptide receptors present in the Acb before and after a meal. We had previously shown decreased receptor binding at the Fstr, Acb, and PVN of postprandial animals. Our saturation experiment data would suggest that the decreased binding we had observed in the prior study cannot be attributed to concurrent changes in affinity or receptor number during food intake. By performing the saturation study using autoradiography on brain sections rather than homogenates, we encountered a trade-off. In order to monitor binding changes and distribution in a specific nuclei, namely the Acb, we chose to visualize our data through autoradiography. However, our technique was limited by two factors. The first factor involved the saturation limit of the autoradiography film itself. ^{125}I-Tyr^{4}-BN concentrations higher than those we employed would have saturated the film at the Acb and thus rendered data at those concentrations uninterpretable. As a result, the extrapolation of the regression curve was necessary in order to determine B_{max} under both feeding states. Conceivably, a small change in data values would only slightly alter the slope of the curve, but would result in a disproportionate change in B_{max}. This is illustrated in Table 3. Although K_D values appear consistent in both groups of animals, the B_{max} values derived from the Scatchard-Rosenthal plots are considerably varied. The second factor is the resolution of the technique itself. Homogenate-based assays are an order of magnitude more resolved than similar autoradiographic assays, although they lack the anatomical resolution of the data. We can, however, conclude that K_D values under either feeding condition are not significantly different. In the final analysis, we can only

conclude that although we have not ruled out the possibility of receptor internalization due to BN-like peptide release during ingestion, our data and, specifically, our B_{max} values do not support this contention.

Conclusions

In 1973, Gibbs and colleagues hypothesized that one role of gut peptides that are peripherally released during food ingestion may be to signal postprandial satiety. According to Geary (1990), there are five criteria that a peptide must fulfill in order to be considered a satiety signal. These criteria are stipulated below.

Criteria For A Peptide Satiety Signal

1. The concentration of the endogenous peptide must change at the active site during a meal.
2. Exogenous administration of the peptide must produce a specific satiety effect.
3. Antagonism (or potentiation) of prandial changes in peptide concentration must have desatiating (or satiating) effects.
4. The change in endogenous peptide during a meal must be sufficient for its satiety effect.
5. The peptide's satiety effect must occur under ecological conditions.

Criterion 1 has been directly addressed during the course of our studies, and the results seem to support a functional role for BN-like peptides as a satiety signal. We have reported, in the RIA experiments, that a significant increase in endogenous BN-like peptides, specifically GRP_{1-27} and GRP_{18-27}, can be detected peripherally in the antrum of the stomach and centrally in the hypothalamus and hippocampus after food ingestion. However, the site(s) of action of endogenous

BN-like peptides have not been identified. The task of identifying these sites is made more difficult by the fact that BN-like peptides exert many physiological effects other than the suppression of food intake. Differentiation of these effects in a locus-specific manner has yet to be achieved.

In an attempt to identify such a site, Kirkham et al. (1991) have recently demonstrated that BN administered through the celiac artery, which directly perfuses the stomach, pancreas, spleen, and proximal duodenum, is significantly more effective at inhibiting food intake through this route than through the mesenteric artery (which perfuses the entire length of the small intestine except the proximal duodenum, as well as the cæcum, colon, and rectum). These results strongly suggest an upper abdominal gastric site of action for peripheral BN, and are corroborated by our findings that endogenous levels of BN-like peptides are elevated in the antrum after a meal. Similarly, several previously cited studies have shown the hypothalamus to be a strong candidate as a site of action for BN-like peptides of feeding. The hippocampus has not been studied in this regard, but has been implicated in the enhanced memory capacity demonstrated in rats peripherally injected with BN or $GRP_{1\text{-}27}$. The fact that this effect is vagally dependent may support the contention that it is a phenomenon related to feeding-related stimuli.

Without question, the second criterion has been satisfied with respect to BN-like peptides, although most convincingly with BN itself. Although endogenous the BN-like peptides $GRP_{1\text{-}27}$, $GRP_{18\text{-}27}$, $NMB_{1\text{-}32}$, and $NMB_{23\text{-}32}$ have been shown to suppress food intake and elicit a satiety response, their effects are not as potent as those of exogenous BN. The relative potencies are $BN > GRP_{1\text{-}27} \geq NMB_{1\text{-}32} > GRP_{18\text{-}27} > NMB_{23\text{-}32}$, but their satiety effects are

certainly demonstrable in all cases (Stein & Woods, 1982; DiPoala & Gibbs, 1985; Piggins et al., 1989).

Blockade of central BN-like peptide receptors, as well as antagonism of endogenous BN-like peptides, have been shown to have a desatiating effect (Merali et al., 1988; Merali et al., 1990), fulfilling the requirements for the third criterion. As there are presently no compounds which specifically potentiate endogenous BN-like peptide activity, a potentiation of the satiety effect of these peptides has yet to be demonstrated.

Ideally, the changes in endogenous BN-like peptide concentrations we have detected during a meal would have been great enough to equal or surpass the lowest dose of exogenous BN shown to have suppressed feeding in those sites. This is not the case. Although the fourth criterion is, theoretically, a justifiable requisite, there have been no reported instances of any proposed satiety peptide having a suppressive effect on feeding behavior when exogenously administered at physiological concentrations. This is not an uncommon finding, not only for peptides, but for a number of substances known to elicit behavioral responses at specific sites. It is also possible that the changes we observed, for instance in the hypothalamus, may be attributable to a circumscribed region of this tissue, acting on a specific nerve bundle. Although purely speculative, the example is offered, not as an explanation or hypothesis, but to illustrate how a comparatively concentrated, diffuse infusion of BN-like peptide into the hypothalamus may not mimic the increased concentrations of endogenous BN-like peptide at specific synaptic regions within the same tissue. Despite the sensitivity of the RIA procedure, our technique lacks the resolution necessary to address this concern.

Perhaps the most significant merit of these investigations is that they were designed to be without surgical or pharmacological invasion of the animals' normal physiology. The manipulations were restricted to a single 12 hr food-deprivation period in some animals and, unavoidably, decapitation. The food deprivation period restricts our conclusions to animals similarly deprived, and does not allow us to make statements about spontaneously feeding animals without a certain degree of caution. Although automatic infusion of glucagon during spontaneous food ingestion has been demonstrated to suppress food intake, a similar study has yet to be performed with BN (Geary & Le Sauter, 1989). Certainly, one must consider the effects of decapitation on the endogenous levels of the peptides we wish to measure and receptor sites we hope to quantify, but assessing this effect is beyond the scope of our abilities. If there is an effect, we would hope that it is constant across our experimental groups and would, hence, conserve the validity of our results. We have no reason to believe that this is not the case. We would like to, therefore, suggest that our experiments approached ecological conditions but, given the intrinsic restrictions imposed by our measurement procedures, fell short of the mark. Having stated this, it would appear that the fifth and last criterion refers more to the effects of exogenously administered peptide, rather than to endogenous release of said peptide, and may thus not be directly relevant to our set of experiments.

An overview of our results reveal several interesting findings. When we initially measured BN-like peptide levels in hungry and fed animals, we discovered that hypothalamic, hippocampal, and antral peptide levels were significantly elevated in the postprandial state. HPLC studies revealed that what we were actually measuring in the RIAs were GRP-like peptides, specifically

GRP_{1-27} and GRP_{18-27}. In our next RIA study, we were able to reproduce these results in the hypothalamus and hippocampus (the antrum was not studied). However, since this experiment included an *ad libitum* fed control group as well as a 10 min fed group, we were able to establish that the differences we had observed in the hypothalamus were a result of fasting, while feeding produced those changes seen in the hippocampus. Hence, although we saw comparatively higher levels of GRP-like peptide levels in the hypothalami and hippocampi of fed as opposed to food deprived animals, comparing *ad libitum* fed animals to both conditions revealed that food deprivation reduced hypothalamic GRP-like peptide levels, and feeding increased hippocampal concentrations of these peptides. We suggested that at the hippocampal level, since a significant difference could be observed between the *ad libitum* fed and postprandial conditions, this phenomenon was not related to satiety but to an alternate process more closely associated with the affective qualities of ingestion. In the hypothalamus, however, we maintained that our observations were indicative of an attenuated satiety state mediated by lower levels of BN-like peptides in hungry animals.

Receptor binding studies using radiolabelled BN under various feeding states revealed a reduction in binding in the PVN, as well as in the Acb and Fstr, in postprandial animals when compared to their preprandial counterparts. We hypothesized that BN-like peptide receptors in those areas may have internalized during food ingestion in response to endogenously released BN-like peptides. This contention is supported by our evidence that there are lower levels of BN-like peptides in hungry, as opposed to satiated, animals.

The next set of experiments were designed to further understand what receptor properties were different between pre- and postprandial animals. If, as

we had hypothesized, receptors had been internalized in fed animals, we should have observed a comparative increase in receptor number (B_{max}) in hungry rats. This was not observed. In fact, we saw no significant changes in either receptor affinity or number as a result of food intake. As previously discussed, these results may have been due to the implicit limitations of our technique and cannot be considered to be conclusive. At the present time, our experiments in receptor binding remain phenomenological, without compelling evidence pointing to a mechanism of action.

It also remains possible that these changes may be related to a number of metabolic and ingestive events known to occur during the ingestive process. Temperature alteration induced by food intake could result in the stimulation BN-mediated pathways, a suggestion supported by BN's pharmacological effect on thermoregulation (Brown et al., 1977). The concentrations of BN-like peptides could also change in response to the increased locomotor activity during and following a meal. BN has been shown to increase locomotor activity when administered i.c.v. (Pert et al., 1980). Further, the release of other gastrointestinal peptides and pancreatic hormones, such as CCK, glucagon, and insulin, may modulate the release of BN-like peptides. Conversely, the changes in BN-like peptide concentrations observed may reflect their involvement in the release of gastrointestinal peptides, such as CCK, which is released by peripheral infusion of BN (Jansen & Lamers, 1983), or hormones such as glucagon and insulin, which can be stimulated by parenteral BN (Kaneto et al., 1978). Also, BN may be involved in the normal secretion of gastrin, gastric acid, and/or pepsin during food intake, since exogenous BN has been shown to stimulate secretion of these substances (Bertaccini et al., 1973; Basso et al., 1976). Thus, the functional

significance of the observed changes remains open to debate. Proponents of BN's role in the satiety process, whether direct or indirect, may choose to interpret these findings as further proof of its involvement in the ingestive process. Other researchers may suggest that they are the result of behaviors or metabolic changes unrelated to satiety. Further studies are required to resolve this question. At present, we can only say that the changes in concentration of BN-like peptides were locus specific in both central and peripheral systems, were seen when comparing hungry to satiated animals, and were the result of food intake.

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