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Peptidergic Mediation of Response to Stress: Focus on Bombesin-like Peptides

A Doctoral Thesis Dissertation

By

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Submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy to the School of Graduate Studies,

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ABSTRACT

Peptidergic Mediation of Response to Stress: Focus on Bombesin-like Peptides

In addition to the relatively well established role of corticotropin-releasing hormone (CRH), and arginine-vasopressin (AVP) in the mediation of the stress response, there is reason to believe that the bombesin (BN) family of peptides may also contribute to the integration of these responses. Indeed, central administration of BN-like peptides produces endocrine, autonomic and behavioral effects, resembling those elicited by stressor exposure as well as by exogenously administered CRH and/or AVP. These observations led us to hypothesize that BN-like peptides may be important mediators of the stress response and that this family of peptides may mediate their stress-relevant effects via activation of CRH and/or AVP circuits. Using physiological approaches we demonstrated that stressor exposure is associated with changes in post-mortem tissue levels of BN-like peptides, BN-like peptide receptor densities and in the *in vivo* release of BN-like peptides from selected brain sites. Using pharmacological approaches we showed that 1) blockade of central CRH receptors with αh-CRF attenuated BN-induced neuroendocrine and autonomic activation and 2) central BN administration elicited site-specific alterations in endogenous levels of CRH and AVP as well as the release of CRH and AVP from selected brain sites. Together, these data provide evidence for the involvement of BN-like peptides in the mediation and/or modulation of the stress response via activation of CRH and/or AVP neurons.

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Table of Contents

List of Figures

Introduction

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

General Discussion

List of Tables

Introduction

Chapter II

Summary

Bombesin (BN), an amphibian derived tetradecapeptide, and its mammalian counterparts, gastrin-releasing peptide (GRP) and neuromedin B (NMB), and related receptors are widely distributed throughout the central and peripheral nervous systems and are thought to participate in a wide range of biological activities. Amongst the physiological actions of this family of peptides is their ability to activate the hypothalamic-pituitary-adrenal axis and sympathetic nervous system. Moreover, central administration of BN-like peptides elicits a number of behavioral responses which resemble those induced by stressor exposure. Together, these findings suggest that the BN-related peptides may be involved in the mediation and/or modulation of the stress response. Thus, the overall objectives of the studies presented in this thesis were to determine whether BN-like peptides 1) play a functional role in the stress response and 2) mediate their effects through other stress-relevant peptides, namely corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP). Together, the physiological and pharmacological findings presented here provide strong evidence indicating that the BN family of peptides are involved in the mediation and/or modulation of the stress response, partly through interactions with CRH and/or AVP neurons. This summary highlights our main findings.

1. If BN-related peptides are involved in mediation of the stress response, then acute stressor exposure should be associated with fluctuations in endogenous levels of BN-like peptides and/or relevant receptor based changes. To attain these objectives, rats

were subjected to acute immobilization stress for 10, 30 or 120 min and compared to non-stressed controls. Our findings indicated that levels of immunoreactive BN-like peptides (ir-BN) increased significantly at the hypothalamus and medulla within 30 min and returned to basal levels by 120 min after stressor exposure. Autoradiographic analyses revealed a significant increase in the density of BN binding sites at the nucleus of the solitary tract (NTS), and at the paraventricular and arcuate hypothalamic nuclei. These findings support the notion that the BN family of peptides may play a functional role in the mediation and/or modulation of the stress response.

2. A multitude of factors including organismic variables (such as genetic loading) as well as the nature of the stressor influence the response(s) mounted by the challenged organism. In order to gain further insight into the nature of the role of BN-like peptides in the stress response, the present experiment assessed 1) the effects of exposure (15 min) to a pure psychogenic stressor (predator (ferret) exposure) or a mixed neurogenic/psychogenic stressor (restraint) on plasma levels of ACTH and corticosterone, as well as on regional levels of ir-BN and ir-CRH and 2) whether the endocrine and/or peptidergic responses varied differentially in two rat lines with differential proneness to stressors (Fast and Slow seizing rats). Results revealed that the Slow rats showed a greater elevation of plasma ACTH and corticosterone levels in response to ferret exposure, whereas the Fast rats exhibited a more pronounced rise in plasma ACTH levels in response to restraint stress. In terms of peptidergic alterations, the Slow animals showed increased levels of ir-BN at the anterior

hypothalamus and increased levels of ir-CRH at the median eminence/arcuate nucleus (Me/Arc), paraventricular hypothalamic nucleus (PVN) and pituitary (Pit) in response to both ferret and restraint exposure, whereas decreased levels of ir-BN were found at the NTS following exposure to both stressors. The Fast rats showed decreased levels of ir-BN at the NTS as well as decreased levels of ir-CRH at the Me/Arc, PVN and Pit in response to both ferret and restraint exposure. Furthermore, restraint stress elevated levels of ir-CRH at the central amygdaloid nucleus (CeA) in Fast rats. A subsequent microdialysis experiment revealed the *in vivo* release of CRH from the CeA in Fast rats in response to restraint exposure and in the Slow rats in response to ferret exposure. Taken together, the current results demonstrate that these two rat lines show differential endocrine and neurochemical response patterns to psychogenic and neurogenic stressors.

3. There is evidence suggesting that stressor exposure can have long-lasting effects such that the subsequent response(s) to stressors would be influenced by the stress history of the organism. In the preceding post-mortem experiments we showed that stressor exposure is associated with site-specific alterations in endogenous levels of ir-BN. In order to determine whether stressor-induced alternations in ir-BN represent fluctuations in peptide release, synthesis or metabolism, in the present experiment we assessed (using push pull perfusion) the dynamics of the *in vivo* release of the BN-like peptides, GRP and NMB (as well as other stress relevant peptides, namely CRH and AVP) at the anterior pituitary, in response to acute stressor (air-puff) exposure. We also examined whether the subject's prior stress-history (previous exposure to

acute or chronic restraint) influenced the stressor-induced release of GRP, NMB, CRH and AVP at the anterior pituitary. Results showed that basal levels of interstitial CRH, AVP, GRP and NMB at the anterior pituitary were significantly elevated in animals previously exposed to either acute or repeated restraint as compared to naive control animals. In response to an acute novel stressor (air-puff), the naïve control rats showed a more rapid and in general, a more pronounced enhancement of peptide levels at the anterior pituitary as compared to rats previously subjected to restraint stress. Taken together these findings suggest that 1) the availability of interstitial levels of GRP, NMB (as well as CRH, AVP) is increased at the anterior pituitary in response to an acute stressor and 2) the role of the BN family of peptides in HPA activation may be influenced by the prior stress history of an organism.

4. In light of the similarities between BN-like peptides and CRH in terms of anatomical distribution, behavioral and endocrine effects, the next set of experiments investigated whether BN-like peptides mediated their effects via activation of CRH neurons. The initial study revealed that like CRH, central BN administration (0.25 and 0.5 μg) significantly increased plasma ACTH, corticosterone, epinephrine, norepinephrine and glucose levels. Blockade of CRH receptors with αh-CRF (10 μg) attenuated the BN (0.25 μg)-induced rise in plasma ACTH, epinephrine, norepinephrine and blood glucose levels, but had no effect on plasma corticosterone levels, seen 30 min after BN administration. The subsequent time-course study revealed that central BN (0.2 μg) increased corticosterone and glucose concentrations above baseline

levels at 15, 30 60 and 120 min relative to saline administration. Pretreatment with αh-CRF (10 μg) attenuated the BN-induced rise in corticosterone and glucose levels at all time points. These findings support the notion that BN-induced effects are mediated, at least in part, via CRH neurons.

5. Since BN-like peptides seem to mediate some of their effects through the CRH system, we next attempted to determine the potential locus (or loci) of such peptidergic interactions. Initially, a mapping experiment was carried out where regional levels of ir-CRH (and ir-vasopressin (AVP)) were assessed following central injection of BN (0.25 and 0.5 μg). Administration of BN decreased ir-CRH at the NTS, ventromedial and anterior hypothalamic nuclei, and CeA. Furthermore, BN treatment decreased ir-AVP at the ventromedial hypothalamic nucleus, but elevated its levels at the PVN and Me/Arc. Subsequent, more direct release experiments (using *in vivo* push-pull perfusion) revealed that central BN administration markedly enhanced the release of CRH and AVP at the Me/Arc translating into an increased availability of these ACTH secretagogues at the anterior pituitary.

In conclusion, this multidisciplinary investigation, using physiological and pharmacological approaches provides novel and strong support for our working hypotheses that i) BN-like peptides may be important in the mediation and/or integration of the stress response and ii) that BN-like peptides may in part, mediate their stress-relevant effects via activation of CRH and/or AVP circuits.

Introduction

What is stress?

"Stress" is an ill-defined term generally referring to a state of threatened homeostasis or equilibrium.[185] Much of the ambiguity surrounding the definition of stress stems from confusion between related concepts such as "stressors", referring to the factors or stimuli that disrupt homeostasis, and "response to stress" which describes the forces put forth to counteract stressor exposure in order to restore homeostasis.[185] The modern definitions of "stress" and "response to stress" can be traced to the early 19th century. It was at this time that the French physiologist, Claude Bernard, introduced a theory suggesting that a relatively constant 'milieu intérieur' or internal environment was necessary to sustain life.[185,206] Bernard's theory was elaborated during the early 20th century by Walter Canon, who showed that physical or emotional disturbances activate the sympathoadrenal system.[185,206] Canon hypothesized that the sympathoadrenal system was responsible for coordinating what he described as the 'fight or flight' response, a series of behavioral and physiological responses activated by stressor exposure. In 1936, Hans Selye expanded Canon's notion of stressor-induced sympathoadrenal activation to include responses by the hypothalamic-adrenal-pituitary (HPA) axis and other hormonal systems as well. Selye presented what he called the "General Adaptation Syndrome (GAS)". The GAS represented a reaction to stressors, which was composed, of three main stages.[372] The first stage was the "alarm reaction" characterized by sympathoadrenal activation; this was followed by "the resistance stage" characterized by activation of the HPA axis, culminating in the final stage of "exhaustion". Selye's GAS provided the basis for contemporary theories on the response to stress.

Individual responses to stressors are dependent on a multitude of factors including the severity (e.g. weak or strong), duration (e.g. acute or chronic), or quality (e.g. physical or psychological) of the stressor, as well as on experiential (e.g. early life experiences or previous adult stressor exposure) and individual (or organismic) variables (e.g. species, genetics, age or gender)(see Fig. 1). It is a complex interplay of these variables that will influence the overall success of the response to stress. Whereas a successful response to stress will help ensure the survival of the organism, an unsuccessful or maladaptive response will threaten the health of an organism ultimately resulting in pathology and/or death.[14,185,381]

It is generally agreed that in addition to limbic factors, a typical response to stress is largely mediated by two interrelated systems; namely the HPA axis and the sympathetic division of the autonomic nervous system.[185,218,286] Upon activation, these two systems trigger a complex cascade of behavioral and physiological changes, which function to help the organism cope successfully with the stressful stimulus. The behavioral changes include increased arousal and alertness, heightened attention, selective memory enhancement, altered cognition and attention span, suppression of feeding and reproductive behaviors and the provocation of analgesia.[185,286] The physiological changes include a redirection of energy within the body to where it is most needed (the release of glucose, stimulation of amino acids and free fatty acids), cardiovascular changes (increased heart rate, blood pressure and respiration), suppression of anabolic processes (digestion, growth, reproductive and immune functions), and a concomitant increase of catabolic processes.[185,286]

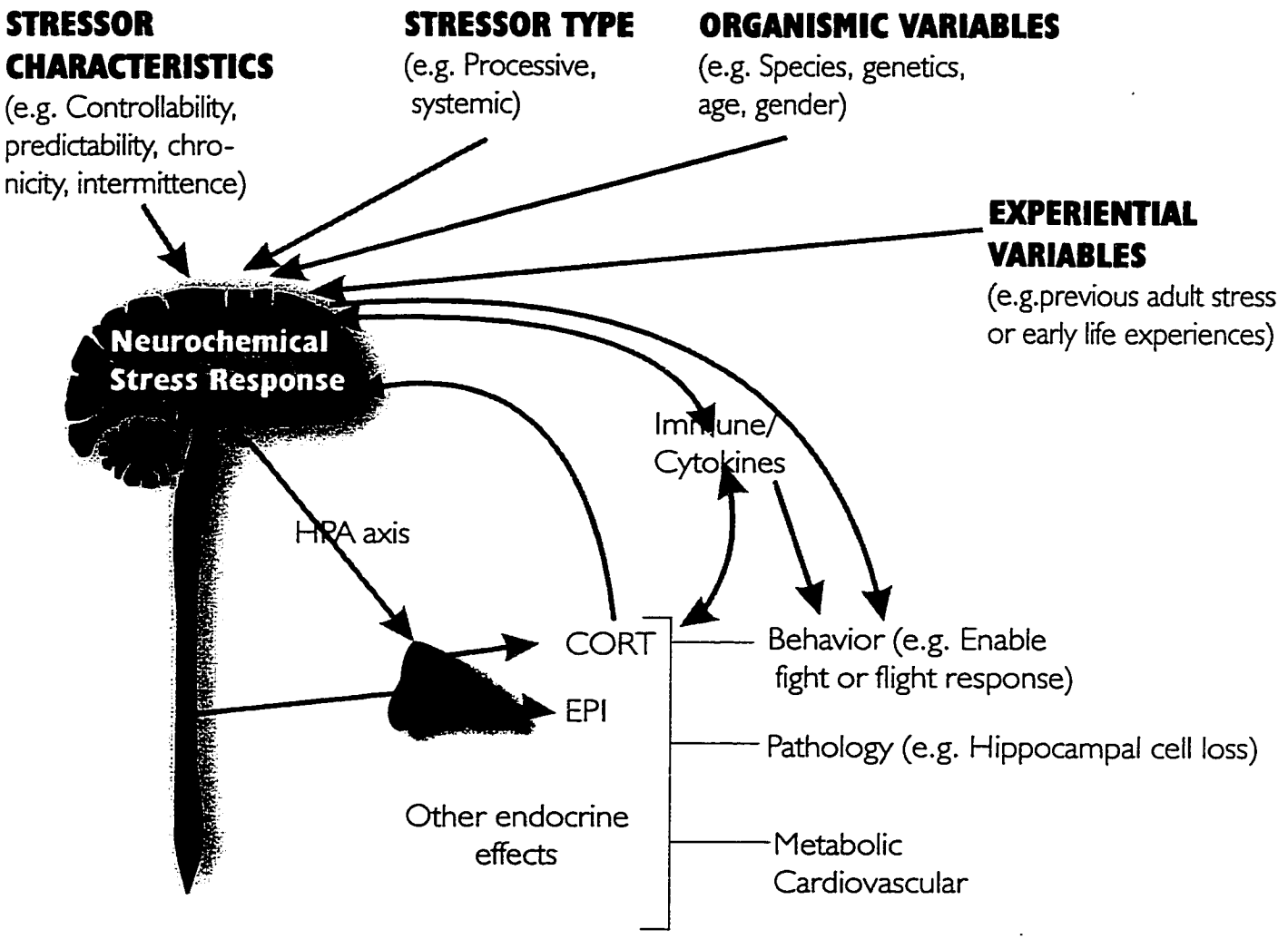

Fig. 1 Factors that influence the neurochemical stress response

The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA axis, comprised largely of corticotropin-releasing hormone (CRH) neurons in the hypothalamus that control ACTH and glucocorticoid secretion from the pituitary and adrenal cortex respectively, is a complex system which appears to function under both normal (basal) and stressful conditions. During basal conditions, the HPA axis has a permissive role, and the activity of which fluctuates in a circadian fashion.[286] However, during stressful conditions, the HPA axis becomes activated and sets into motion a cascade of events, which result in the maximization of the body's ability to respond effectively and efficiently to the homeostatic threat.

Activation of the HPA axis occurs when neurons of the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN), projecting to the external zone of the median eminence (ME), become activated and secrete a cocktail of adrenocorticotropic hormone (ACTH) secretagogues, the most important of which are CRH and arginine-vasopressin (AVP) (see Fig. 2). These secreted peptides are then transported by the portal blood system, to the anterior portion of the pituitary gland, where they synergistically stimulate the release of ACTH.[160,185,286,381] ACTH enters the general circulation to reach the adrenal cortex, and evokes the release of glucocorticoids from this site.[160,185,286,381] The glucocorticoids are the final effectors of the HPA axis and exert many different effects which are aimed at 1) the redirection of energy, and 2) providing a negative feedback signal to prevent stressor-activated defense mechanisms from overshooting or overcompensating (to the detriment of the organism) the demands of the system.[286] More specifically, some of the effects of glucocorticoids include 1)

elevation of blood glucose levels by stimulating gluconeogenesis in the liver and lipolysis, as well as by inhibiting insulin secretion, 2) suppressing immune and inflammatory activity, and 3) terminating the stress response by inhibiting the stressor-induced secretion of CRH and ACTH via multiple inhibitory feedback loops involving pituitary, hypothalamic and extrahypothalamic sites.[286]

The mechanism(s) by which the negative feedback regulation of glucocorticoids is accomplished, remains poorly understood. Glucocorticoids interact with two distinct types of receptors in the brain; type I or mineralcorticoid receptors and type II or glucocorticoid receptors.[286] Mineralcorticoid receptors are found primarily in the hippocampus and limbic structures, whereas glucocorticoid receptors are expressed throughout the brain but are present in high concentrations in the hypothalamus. It has been suggested that the negative feedback action of glucocorticoids during a response to stress occurs primarily through activation of the type II or glucocorticoid receptors. Mineralcorticoid receptors, which have a higher affinity for glucocorticoids, are thought to operate at low corticosterone concentrations (during basal activity of the HPA axis and circadian rhythms), whereas glucocorticoid receptors become more critically involved when glucocorticoid levels are high (e.g. during a stress response). Therefore, as levels of glucocorticoids increase, mineralcorticoid receptors become saturated, and the glucocorticoids progressively occupy the glucocorticoid receptors in addition to the mineralcorticoid receptors.[286] Furthermore, the potential role of other neural systems that influence the activity of the parvocellular neurons of the PVN in the negative feedback regulation remain relatively unexplored.

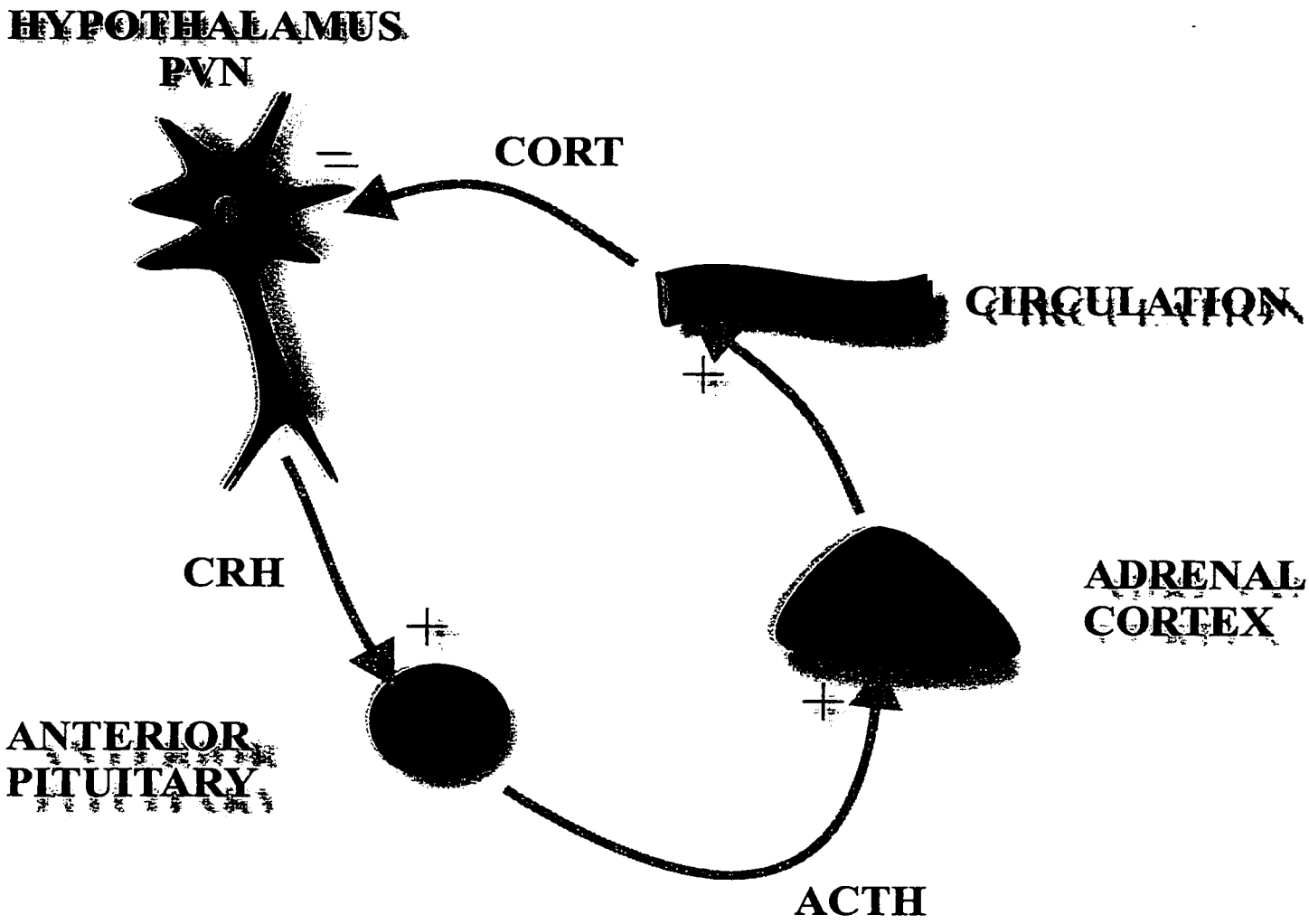

Fig. 2 Schematic diagram of the hypothalamic-pituitary-adrenal axis

Neural Circuits Influencing the HPA axis

While glucocorticoid secretion is immediately preceded by activation of the relevant hypothalamic PVN neurons, the diverse sets of afferent neural circuits that regulate the activity of PVN neurons remain less well characterized, and are the subject of much recent interest. One of the most extensively investigated neuronal circuits contributing to HPA activation is the brainstem catecholaminergic system[77,131,233,319], which has direct projections to the CRH-containing neurons of the PVN. It has repeatedly been demonstrated that stressors elicit a rapid induction in the expression of the immediate early gene (c-fos) as well as an increase of noradrenaline/adrenaline metabolism in several brainstem regions.[65,77,233,319,326] Interestingly, deafferentation of ascending catecholaminergic fibers by neurochemical lesions or mechanical interruption has been shown to cause a reduction in stressor-induced CRH levels in portal blood as well as in plasma ACTH and corticosterone levels, suggesting involvement of the catecholaminergic projections in the provocation of the neuropeptide changes.[131,148,241,348] A key structure of the brainstem catecholaminergic system which is thought to influence HPA activity is the nucleus of the solitary tract (NTS). The NTS serves as a primary relay for information as it receives direct sensory input from the vagal and glossopharyngeal nerves and gives rise to both adrenergic and noradrenergic projections extending to the PVN.[347] The NTS also has projections extending to other brainstem regions including the A1 and C1 cell groups of the ventrolateral medulla and the parabrachial nucleus.[306,363] These regions innervate the PVN and may serve as alternate or indirect routes through which stress-relevant information may reach the HPA axis. Another brainstem nucleus which influences HPA activity is the locus coeruleus (LC).

Increased firing as well as a dramatic induction of c-fos at the LC neurons has been observed following exposure to various types of stressors, including footshock, restraint, tail pinch, social stress and swim stress.[18,40,76,77,82,202] Moreover, bilateral LC lesions blunted the ACTH and corticosterone responses to acute restraint exposure.[435] Although the LC is one of the most stress-responsive nuclei in the brain, the precise role of this structure in HPA axis regulation has yet to be determined as the projections from the LC to the PVN are very sparse.[80,160,376] It has been suggested, however, that the LC may exert its effects on HPA activity indirectly through its dense projections to neocortical, limbic or hypothalamic structures.[160]

There is considerable evidence suggesting that limbic structures including the hippocampus, amygdala, septum and medial prefrontal cortex influence HPA activity.[78,102,120,364,405,426] Moreover, a dramatic induction of c-fos expression has been observed in many limbic structures, particularly the ones mentioned earlier, following acute stressor exposure.[61,77] However, the precise pathways by which these structures convey HPA-excitatory information remain unclear. The central and medial amygdaloid nuclei are the only stress-relevant limbic structures with direct projections to the PVN suggesting that these may be important sites to transmit information to the HPA axis.[63,145] Supporting this notion, it has been demonstrated that ablation of the central (CeA) or medial amygdaloid nuclei blocks the HPA response to certain stressors and electrical stimulation of amygdaloid nuclei elicits the secretion of corticosterone.[27,109,119,405] In addition to the amygdala, the bed nucleus of the stria terminalis (BNST) may serve to relay limbic information to the HPA axis. This nucleus has direct projections to the

PVN, in addition to being interconnected to several stress-relevant limbic structures, including the amygdala, hippocampus, and lateral septum.[78,290,420] In support of this contention, it was shown that lesions to the BNST cause a reduction in immunoreactive (ir)-CRH mRNA at the PVN as well as corticosterone secretion, in response to conditioned fear.[144,161]

It appears that the HPA axis is influenced by several hypothalamic nuclei as well. In addition to the central PVN-ME circuit, retrograde tracing experiments indicated that the PVN receives inputs from virtually all hypothalamic nuclei with the exception of the medial and lateral mammillary nuclei[374,387], and there is evidence that many of these hypothalamic nuclei alter the secretory function of the PVN.[62,69] Moreover, *in situ* hybridization studies have consistently shown that acute stressor exposure is associated with a dramatic induction of c-fos mRNA expression at various hypothalamic sites including the PVN, anterior hypothalamus, dorsomedial hypothalamus, lateral hypothalamus and arcuate nucleus.[77,166,174,175,373]

Autonomic Nervous System

While the HPA axis represents an integral component of the stress response, equally important is the sympathetic division of the autonomic nervous system (often referred to as the locus coeruleus-norepinephrine (LC-NE)/sympathetic system). As the name implies, the LC-NE/sympathetic system consists largely of the NE cell bodies located in the LC, as well as some NE cell bodies located in the lateral tegmental area.[41] The noradrenergic fibers originating from the LC project to various regions throughout

the brain (including the cerebral cortex, cerebellum, cingulate gyrus, thalamus, hippocampus, hypothalamus, amygdala, BNST and nucleus accumbens) and also down through the thoracic and lumbar regions of the spinal cord.[74,128] Activation of the NE-LC/sympathetic system results in 1) the central release of NE from the dense fiber network mentioned above, and 2) the peripheral release of NE and epinephrine from the adrenal medulla.[185] The main actions of NE (and epinephrine) release are to increase vigilance and arousal in order to help an organism respond more efficiently to a stressful situation.[41] Peripherally, the effects of NE and epinephrine release include 1) redirection of energy to where it is most needed (mobilization of blood glucose), 2) shunting of blood flow temporarily from splanchnic and renal regions to active muscle groups, 3) increased blood pressure and heart rate, 4) dilation of pupils and 5) constriction of skin vasculature. Within the CNS the release of NE can have both inhibitory as well as excitatory effects on neuronal function, depending on the postsynaptic receptors upon which it is acting.[41] For example, NE may have an inhibitory effect (acting through β-receptors) on spontaneous neuronal activity in the hippocampus, cerebellum, and auditory cortex.[114,129,130,371] It has been hypothesized that this leads to an increase in the signal-to-noise ratio of neuronal activity, resulting in an increased ability of the organism to respond to relevant stimuli.[252] In contrast, NE acting through α-receptors within the cerebral cortex has been shown to have excitatory effects on neuronal activity.[41] In this context, excitation of α-noradrenergic receptors results in a shift in the firing pattern of neurons from spontaneous bursting to single-spike activity.[416] Such a shift has been shown to be associated with increased responsiveness to excitatory potentials (resulting in an enhanced transmission of sensory inputs) and also to mediate the transition between

sleep and wakefulness.[416] Both of these functions would be considered highly adaptive for an organism faced with a need to respond to a stressful situation.

Neuropeptides Involved in the stress Response

The past three decades have been particularly active from the perspective of neuropeptide research, as over 70 'new' neuropeptides have been discovered in the mammalian central nervous system. Not only do neuropeptides act via specific receptors to directly affect neurons, but they also affect non neuronal tissue and organs as well.[380] Based on these facts, it has become increasingly clear that a primary role of neuropeptides is to integrate the functions of the brain with the various systems of the body. These substances, acting as neurohormones (chemicals released by endocrine glands that act on target cell via the blood stream), neuromodulators (chemicals that diffuses through the extracellular fluid to modulate the activity of many neurons) or neurotransmitters (chemicals that communicate with specific neurons a short distance away) are involved in a multitude of functions including, regulation of food and water intake, gastrointestinal activity, cardiovascular activity, respiratory activity, thermoregulation, reproduction, growth, nerve development, nerve regeneration, learning, memory and affective state. In addition, neuropeptides are involved in the regulation of the neuroendocrine, autonomic and behavioral components of the stress response. Evidence points to a number of different peptides which could be considered 'stress-relevant' or 'stress peptides'. It is however, important to stipulate that while the label "stress peptide" suggests an exclusive action, peptides, as a rule rather than an exception, are involved in multifunctional systems.

Characteristics of a stress peptide

What are the characteristics of a peptide, which permits one to define it as a 'stress peptide'?

1. The putative 'stress peptide' should be present in the brain, particularly at brain sites known to be affected by stressors with markers such as c-fos.
2. Exogenous peptide administration should mimic the endocrine, autonomic and behavioral effects elicited by stressors.
3. Antagonism of peptide action (e.g. using peptide receptor antagonists) should attenuate or block the behavioral and/or neurochemical effects of stressors or of exogenously administered peptide.
4. Stressor exposure should alter peptide release, levels and/or turnover, mRNA expression, and/or receptor function.
5. Stressor-elicited peptidergic alterations should be attenuated by treatments believed to reduce stressor effects (e.g. benzodiazepines).

These characteristics are by no means an exhaustive list of criteria, but rather represent a list of testable characteristics that can serve as a first step towards defining and characterizing 'stress peptides'. Of course, it is important to consider that the extent and possible direction of the peptidergic alteration may also be dependent upon the type of stressor to which an organism is exposed, as well as its intensity or duration. For instance, it was proposed that the neural pathways or circuits that are activated will vary as a function of the type of stressor to which an animal is exposed.[160] Stressors involving an immediate physiological threat (systemic stressor), such as an immune challenge or

respiratory stressor seem to directly activate the PVN without activating limbic structures. In contrast, stressors which require interpretation by higher brain structures (processive stressor), such as psychological stressors, seem to first activate limbic forebrain structures before filtering and transmitting relevant information to the PVN.[160] In addition to the type of stressor, a peptide's role in the stress response may change depending upon the duration and intensity of the stressor (acute vs chronic; weak vs strong) or on individual (organismic) differences (genetic factors or life experiences).[14,185] While these other factors are indeed relevant and should not be disregarded, the focus of this thesis will be on the five major characteristics presented above.

Corticotropin-Releasing Hormone (CRH)

Two teams of researchers, Guillemin & Rosenberg (1955)[149] and Saffran & Schally (1955)[358] independently demonstrated the existence of a hypothalamic factor that could induce the release of ACTH from the anterior pituitary. This substance was coined 'corticotropin-releasing factor (CRF)' due to its ability to stimulate ACTH secretion. Although CRF was named in 1955, technical difficulties prevented the elucidation of the exact chemical structure of this factor(s) for the next 25 years. Finally in 1981, Vale and his colleagues isolated and characterized the 41 amino acid peptide from extracts of ovine hypothalamus and termed it 'corticotropin releasing hormone or (CRH)'.[402] Since that time, substantial evidence has accumulated suggesting the CRH is one of the primary mediators of the stress response, at least as it pertains to the activation of the HPA axis. Aside from CRH, two non-mammalian CRH-related peptides, sauvine and urotesin I, are

known to exhibit CRH-like activity.[398] Both of these peptides share 50% homology with CRH. More recently, a novel CRH-related peptide, named urocortin, was identified.[412] Like the other members of the CRH family of peptides, urocortin is both structurally (shares 45% homology) and pharmacologically similar to CRH.

Does CRH meet the stipulated characteristics of a stress peptide?

The first characteristic of a 'stress peptide' is that its anatomical distribution should include brain sites activated by stressor exposure. Immunohistochemical and radioimmunoassay studies have revealed that CRH-containing neurons are widely distributed throughout the central nervous system, particularly at brain regions thought to be involved in the stress response.[79,321] The primary source of CRH (and the most widely studied population of CRH neurons) is located in the parvocellular region of the PVN. CRH-containing PVN neurons project to the ME, where CRH is released into the hypothalamo-hypophysial portal system.[36,365,388] Many other hypothalamic nuclei also contain CRH immunoreactivity (ir-CRH) (although to a lesser extent than the PVN and ME) including the supraoptic, suprachiasmatic, preoptic, premammillary, arcuate, periventricular, anterior, lateral, dorsomedial and ventromedial hypothalamic nuclei.[79,388] CRH-containing cells are located in many extrahypothalamic sites as well including the cerebral cortex, limbic and brainstem regions.[79,188,283] Briefly, the highest concentrations of extrahypothalamic ir-CRH are found in the cerebral cortex (within the prefrontal, insular and cingulate cortices), amygdaloid complex, BNST, central gray area, dorsal vagal complex, LC, parabrachial nucleus and inferior olive. High to moderate levels of ir-CRH have also been demonstrated in the olfactory bulb, NTS, certain thalamic nuclei and the hippocampus.[79,188,283] Although generally thought of as a brain peptide, ir-CRH

has been also been observed in the pituitary gland as well as in many peripheral tissues, including the adrenal medulla.[153,183]

Using both quantitative autoradiography and cell-membrane binding techniques, a high degree of correspondence was observed between the distribution of binding sites for CRH and the relative distribution of ir-CRH within the CNS. Briefly, the highest density of CRH receptors was found in the external zone of the ME, cerebral cortex, olfactory bulb, several cranial nerve nuclei and the cerebellar cortex. Moderate density of CRH receptors was found in the PVN, nucleus accumbens, caudate-putamen, basolateral amydgadoid nucleus, superior and inferior olives and the NTS. Finally, low levels of CRH binding sites were observed at the BNST, medial and central amygdaloid nuclei, hippocampus and regions of the thalamus.[87,88,430] In addition, CRH receptors have also been localized in the pituitary as well as peripheral tissues including the adrenal medulla and sympathetic ganglia.[88,89,401,430]

More recent studies have revealed that there are at least 2 subtypes of CRH receptors, designated as CRH_1 and CRH_2. Using an *in situ* hybridization approach, mRNA for the two receptor subtypes has been identified within the brain and pituitary.[101] According to these examinations, CRH_1 receptor mRNA expression is most prominent in the cerebral and cerebellar cortices as well as in some subcortical structures, such as the medial septum, amygdala, and CA3 region of the hippocampus. In addition, the CRH_1 receptor is the prevailing subtype in the anterior and intermediate lobes of the pituitary. In contrast, CRH_2 receptor expression in confined to subcortical regions including the

lateral septal nucleus, ventromedial hypothalamus and the choroid plexus. Moderate levels of CRF_2 receptor mRNA are also present in the PVN, supraoptic nucleus of the hypothalamus and the amygdala. The heterogeneous distribution of CRF_1 and CRF_2 receptor mRNA suggests distinct functional roles for each of these subtypes. Based on the preponderance of CRH_1 receptors in the pituitary and cortical regions, it has been suggested that this receptor subtype is responsible the CRH-induced ACTH release as well as the mediation of cortical and sensory actions of CRH. In contrast, the distribution pattern of CRH_2 receptors suggests its involvement in the mediation of the hypothalamic neuroendocrine, autonomic and behavioral actions of CRH.[101] Interestingly, CRH binds with higher affinity to CRH_1 than CRH_2 receptors, whereas other CRH-related peptides, including urocortin, bind with high affinity to both CRH_1 and CRH_2 receptors.[101] While these findings suggest that urocortin may play a role in the mediation of endocrine, autonomic and behavioral responses to stressor exposure, at present this contention is speculative as information about the functional role of urocortin (and other putative endogenous ligands) as well as CRH_1 versus CRH_2 receptor subtypes, is rather limited and controversial.[101,421]

Taken together, the anatomical distribution of ir-CRH, CRH binding sites and CRH receptor mRNA within several hypothalamic, limbic and brainstem structures, as well as within the pituitary gland and adrenal medulla, is anatomically consistent with the notion that this peptide is involved in the mediation of the endocrine, autonomic and behavioral responses to stressor exposure.

In accordance with the second and third characteristics of a 'stress peptide' there is indeed an abundance of evidence showing that exogenous CRH administration produces endocrine, autonomic, and behavioral effects that resemble those observed following stressor exposure.[107,317] Conversely, treatment with CRH antisera or CRH receptor antagonists attenuate the neurochemical and behavioral effects of exogenous CRH administration or stressor exposure.[107,317] In terms of endocrine effects, *in vitro* studies have shown that CRH is a potent stimulator of ACTH and β-endorphin secretion in rat anterior pituitary cell cultures.[402] In *vivo* systemic CRH administration dose-dependently increases plasma ACTH and corticosterone levels.[105,350] These effects can be blocked by pretreatment with either CRH antagonists or CRH antiserum suggesting that CRH plays a physiological role in ACTH release.[350,356] Paralleling the effects of systemic treatments, central CRH administration has also been shown to potently stimulate the HPA axis as reflected by increased plasma ACTH and corticosterone (or cortisol) levels in a variety of species including rats, sheep, primates and man.[105,179,228,350] In sum, these findings suggest that central CRH administration may directly or indirectly (by promoting endogenous CRH release) elicit ACTH secretion from the anterior pituitary. A physiological role for CRH in the regulation of pituitary-adrenal function is further emphasized by the demonstration that CRH antisera or CRH receptor antagonists can attenuate or abolish ACTH and corticosterone elevations elicited by a variety of stressor including; ether, hemorrhage, immobilization/restraint, cold-water swim and bone fracture-related trauma.[245,303,315,337]

Aside from its ability to stimulate ACTH secretion, CRH has also been shown to effect the release of other hormones from the anterior pituitary. One of the better documented endocrine responses to stressor exposure in rodents is the inhibition of growth hormone secretion.[317] Supporting a role for CRH in this respect, it was demonstrated that central administration of CRH dose-dependently reduced plasma growth hormone secretion[314,353], whereas pretreatment with αh-CRF blocked both CRH- and stressor-induced decreases of plasma growth hormone levels.[354] The ability of CRH to inhibit elevations of plasma growth hormone appears to involve CRH-induced stimulation of somatostatin release, as *in vivo* and *in vitro* studies indicated that CRH can stimulate the release of somatostatin from the ME.[2,289] In addition, pretreatment with somatostatin antiserum prevented the CRH-induced inhibition of plasma growth hormone secretion.[354]

In addition to the endocrine actions of CRH, this peptide promotes many of the autonomic responses typically associated with the stress response. For example, central CRH administration dose-dependently increases plasma NE and epinephrine levels[55,216], elicits elevations of plasma glucose and glucagon, and a decrease of plasma insulin concentrations.[55,216] These autonomic effects are prevented by pretreatment with central blockade of CRH receptors with αh-CRF, but not with systemically administered CRH antiserum or αh-CRF, suggesting the CRH-induced autonomic effects are mediated via sites within the CNS.[54] Moreover, central administration of αh-CRF can abolish the rise in plasma NE observed following exposure to various stressors, including ether, insulin-induced hyperglycemia and hemorrhage[54,56], implicating CRH participation in stressor-

induced autonomic activation. Indeed, central CRH administration has been shown to increase mean arterial blood pressure and heart rate, and once again these effects are attenuated by pretreatment with αh-CRF.[299] Treatment with chlorisondamine (an autonomic ganglionic blocker), can also block the CRH-induced autonomic effects.[54,55,124] However, these effects cannot be blocked by hypophysectomy or adrenalectomy, suggesting that CRH's involvement in autonomic regulation is centrally mediated and independent of the HPA axis.[55,124] In an attempt to localize the site(s) of action for the autonomic effects of CRH, Brown and his colleagues (1986)[51] microinjected this peptide into 50 different brain sites and then measured plasma catecholamine levels. Results showed that several sites were responsive to CRH-induced elevations in plasma NE and epinephrine levels, suggesting that multiple rather than single site(s) may be involved in the mediation of the autonomic effects of CRH.

Commensurate with the notion that CRH involvement is underlying the stress response, central CRH administration was shown to elicit a wide range of behavioral responses, some resembling those observed in response to stressors. Central infusion of exogenous CRH (i.c.v.) induces locomotor changes, which appear to be situation specific.[157,205] For example, the same i.c.v. dose of CRH that enhances behavioral activation in a familiar environment, suppressed exploratory activity in a novel (presumably stressful) environment.[157,205] It has been shown that central infusion of CRH produces behavioral inhibition in an open-field, multi-compartment chamber, or an elevated-plus maze, findings consistent with increased "emotionality".[45,157,204,386] In an attempt to determine the anatomical loci concerning the locomotor effects of CRH, Butler

et al. (1990)[60] demonstrated that bilateral microinfusion of CRH into the LC increased spontaneous motor activity in a familiar environment and decreased exploration in a novel test situation (open field). In addition, microinjections of CRH into the LC increased the concentration of the NE metabolite, 3,4-dihydroxyphenylglycol, at the amygdala and posterior hypothalamus (forebrain projection areas of the LC).[60] Taken together, these findings suggest that CRH may produce its locomotor effects, at least in part, by causing an increase in the activity of LC noradrenergic neurons.

The PVN may be another site of action for CRH-induced locomotor effects. For example, direct microinjection of CRH into the PVN (but not into the LH or outside the PVN) caused an increase in locomotor activity (in a familiar environment).[294] However, at the highest dose of CRH, locomotor activity was greatly reduced and freezing behavior was observed, suggesting that the PVN may be involved in both CRH-induced behavioral activation and anxiogenic effects. The PVN also appears to be involved in the CRH-induced behavioral response to novelty, as lesions to the CRH-containing neurons in the PVN (with a cellular toxin) produced an increase of locomotor activity during exposure to a novel environment.[274] In effect, it appeared that the impairment of the CRH neurons in the PVN reduced the emotionality and fear/anxiety related to the environmental stimuli.

Finally, the central amygdaloid nucleus (CeA) also appears to be important in mediating some of the effects of CRH on exploratory behavior. Microinjections of CRH into the CeA increased locomotor activity in a familiar environment and reduced exploratory activity in a novel environment (open field).[242,392] Furthermore, infusion of

αh-CRF into the CeA reversed the decreased exploratory behavior (observed on the elevated-plus maze) following exposure to a social stressor.[158] Interestingly, the dose of αh-CRF used to reverse these behavioral effects failed to alter the stressor-induced increase of ACTH and corticosterone, providing further support that the observed behavioral effects were independent of the activation of the HPA axis.

As already indicated, central CRH administration produces a dose-dependent increase of locomotor activity among animals tested in a familiar environment.[48,204,386] This locomotor activation is not observed following peripheral administration of CRH, and it is not blocked by hypophysectomy or pretreatment with dexamethasone (a synthetic glucocorticoid) at doses that block HPA activation.[46,110] Thus, it appears that CRH acts as a central neurotransmitter (to produce these behavioral effects) independent of the HPA axis. CRH-induced locomotor activation is also not inhibited by pretreatment with the opiate antagonist, naloxone, or a dopamine receptor antagonist suggesting that CRH acts independently from the opioid and dopaminergic systems to mediate this behavioral response.[204] However, it has been reported that the CRH-induced locomotor activation can be partially attenuated with ganglionic blocking drugs suggesting that this effect may involve sympathetic activation.[44] The ability of CRH to increase locomotor activity in a familiar environment can also be blocked by pretreatment with αh-CRH suggesting that CRH mediates this behavioral effect via CRH receptors.[48] As well, it has recently been shown that the increase of locomotor activity induced by a psychological stressor was reversed by pretreatment with either αh-CRH (a non-selective CRH receptor

antagonist) or a selective CRH_1 antagonist, suggesting that CRF_1 receptors mediate, at least in part, the increase of locomotor activity elicited by a psychogenic stressor.[167]

Central administration of CRH has been shown to induce still other behavioral actions that resemble those ordinarily elicited by stressors. In particular, CRH is a potent anorexic agent, as central administration of this peptide suppresses food intake in both novel or familiar environments.[43,45,142,212,300] In an attempt to localize the site(s) of action for CRH-induced suppression of food intake, Krahn and his colleagues (1988)[213] demonstrated that microinjection of CRH decreased food intake only when injected into the PVN and not when injected into several other brain regions including the lateral and ventromedial hypothalamic nuclei or the globus pallidus. Similar to the CRH-induced effects on locomotor activity, the effects of CRH on food intake are not prevented by hypophysectomy or pretreatment with dexamethasone, providing further support that CRH-induced behavioral responses are independent of the HPA axis.[48,300] The inhibitory effect of CRH on food intake is similar to that observed following 1 hour of restraint stress.[212] Moreover, pretreatment with αh-CRF partially reversed the restraint stress-induced suppression of food intake, suggesting that endogenous CRH may play a role in stressor-induced anorexia.[212] Likewise, Hotta et al. (1999)[167] recently demonstrated that pretreatment with either αh-CRH or a selective CRF_1 receptor antagonist reversed the inhibition of food intake induced by an emotional stressor, suggesting that the CRF_1 receptor mediates stressor-induced suppression of food intake.

Animals, particularly rodents, often engage in grooming behavior when confronted with a stressful condition. It is thought that this so-called displacement behavior is a coping behavior.[1,14,121] As such, it is of interest that exogenously administered CRH also increases grooming behavior. Several studies have shown that central injection of CRH dose-dependently increased grooming in both novel and familiar environments.[45,48,108,204,300,386] In contrast, systemically administered CRH failed to affect grooming, suggesting that central mechanisms mediate this CRH-elicited behavior.[386] Furthermore, CRH-induced grooming is not dependent on the HPA axis as hypophysectomy and dexamethasone treatment did not block this effect.[48,300] One potential anatomical locus for CRH-induced grooming is the PVN, as direct microinjection of CRH into this nucleus increased grooming behavior.[294] In addition, the hippocampus has also been implicated as being important in mediating CRH-induced grooming, as microinfusion of antisense oligonucleotide (directed against the CRH gene) into the hippocampus markedly decreased grooming in rats.[429]

Like moderate stressor exposure, central CRH administration has been shown to improve learning and/or memory function in rats.[204,270] In this context, central CRH administration improves acquisition and retention of a visual discrimination task.[204] While the neural mechanism(s) mediating this effect are not known, some investigators have attempted to pin point anatomical sites responsible for this CRH-induced behavioral effect. For example, Lee and his colleagues[234,236,242] demonstrated that microinjection of CRH into the amygdala, hippocampus or lateral septum produced a dose-dependent enhancement of retention in an inhibitory avoidance learning paradigm. Moreover, Wu

et al. (1997)[429] showed that injection of a CRH antisense oligonucleotide into the hippocampus (that decreased CRH mRNA at this site) significantly impaired the retention of a visual discrimination task in rats. The LC has also been shown to be involved in CRH-induced memory enhancing effects, as microinjection of CRH into the LC markedly improved retention in a passive avoidance learning paradigm.[67] Given that decreased levels of NE were observed at the amygdala and hippocampus (projection sites of the LC) in this same experiment, it was suggested that CRH may mediate its memory enhancing effects via activation of NE neurons.

A fourth characteristic of a 'stress peptide' is to demonstrate alterations in peptide release, levels and/or turnover, mRNA expression, and/or receptor function in response to stressor exposure. Using push-pull perfusion, investigators have consistently demonstrated that the *in vivo* release of CRH from the ME is enhanced in response to a variety of stressors including ether, injections of adrenaline, interleukin-1ß, or tumor necrosis factor.[22,181,417-419] Using *in vivo* microdialysis, the release of CRH at the mediobasal hypothalamus has been observed following both restraint and hypertonic saline stress.[327] Moreover, the release of CRH in response to restraint stress and ethanol withdrawal has also been demonstrated at the CeA.[278,328] Interestingly, in addition to aversive stimuli, we have shown that appetitive events can also evoke the release of CRH at the CeA, suggesting that this peptidergic system may prepare organisms to deal with biologically significant events (rather than just fearful or threatening events).[278]

In addition to the demonstration of stressor-induced release of CRH, there are a multitude of studies showing alterations in endogenous levels of CRH and CRH mNRA

expression in response to stressor exposure. Specifically, Moldow et al. (1987)[293] reported a significant decrease in hypothalamic ir-CRH up to 30 min after onset of restraint, which was followed by a significant increase at the 60 min time point. It was suggested that the initial decreased ir-CRH content reflected increased release or utilization of CRH, whereas the increase measured at 60 min reflected increased peptide synthesis (to compensate for the preceding increased release). In another study, Chappel et al. (1986)[66] examined the effects of both acute (3 hour cold immobilization) and chronic stressor exposure (14-day unpredictable stressor exposure) on ir-CRH levels in various brain regions. Increases in ir-CRH were apparent at the LC (following both acute and chronic stressor exposure) and at the periventricular and anterior hypothalamic nucleus (following chronic stressor exposure). Significant decreases in ir-CRH were observed at the ME (following both acute and chronic stressor exposure), medial preoptic area (following acute stressor exposure) and dorsal vagal complex (following chronic stressor exposure). Interestingly, changes of ir-CRH levels were not observed at the PVN. However, it should be noted that measurement of ir-CRH levels at a single time point is dependent upon the rates of synthesis, transport and release of the peptide, and therefore may not capture an accurate profile of CRH fluctuations. In order to circumvent interpretation difficulties, some studies have measured CRH transcriptional activity following stressor exposure, as this technique provides a more sensitive measure of CRH neuron responsiveness. Indeed, a significant increase in CRH mRNA at the PVN was observed following acute exposure to a variety of different stressors (restraint, hypertonic saline injection, swim stress or ether exposure) and following various chronic stressor exposures as well (restraint, hypertonic saline injections,

footshock).[25,152,210,243,250,251,375] There is only limited information available concerning stressor-induced alterations of CRH mRNA in extrahypothalamic regions. In one study, Imaki et al. (1991)[172] reported an increase of CRH mRNA expression at Barrington's nucleus and a decrease of CRH mRNA expression at the olfactory bulb following both acute and chronic stressor exposure.

In terms of receptor-based changes, a number of studies have shown changes of CRH receptor density in various brain regions following stressor exposure. For example, prolonged immobilization or chronic intermittent footshock was associated with decreased CRH receptor density at the anterior pituitary.[3,7,154,155,190] The CRH receptor down-regulation at the anterior pituitary is consistent with a hypersecretion of CRH into the portal system resulting from stressor exposure. An up-regulation of CRH receptor mRNA has been observed at the PVN following immobilization, systemic endotoxin treatment or central CRH injection.[200,248,258] In addition, a similar stressor-induced up-regulation of CRH receptor mRNA was observed at the supraoptic nucleus.[248] The up-regulation of CRH receptor mRNA at the PVN and supraoptic nucleus suggests that receptor protein levels in these regions may be increased following stressor exposure. Consistent with this notion, an increase of stressor-induced radiolabeled CRH binding was observed at the PVN and supraoptic nucleus.[248]

A fifth characteristic of a 'stress peptide' is that stressor-induced peptidergic changes should be attenuated by treatments believed to reduce perceived or actual stressor effects with anxiolytics such as benzodiazepines. There are a number of

experiments which indicate that anxiolytics do indeed attenuate stressor-induced changes in the CRH system(s). Specifically, as previously mentioned Chappel et al.[66] observed a decrease in ir-CRH at the ME and an increase in ir-CRH at the LC following both acute and chronic stressor exposure. However, following alprazolam (an atypical anxiolytic with properties of typical benzodiazepines) treatment, the changes of CRH concentrations in these two brain regions were opposite to those observed following stressor exposure.[318] In addition, Imaki & Vale (1993)[177] demonstrated that the benzodiazepine, chlordiazepoxide, attenuated the stressor elicited increase of CRH mRNA at the PVN ordinarily observed following chronic footshock exposure. Further evidence in support of interactions between CRH and benzodiazepines is provided by the finding that alprazolam attenuated serotonin-induced CRH release from hypothalamic organ cultures.[189] Contrary to these findings, recent studies from our laboratory indicate that diazepam pretreatment fails to block the stressor (novel environment exposure)-elicited increase in CRH release at the CeA. Interestingly, in this particular study, pretreatment with αh-CRF failed to block the suppression of food intake observed in the novel environment, whereas diazepam pretreatment successfully reversed this behavioral effect. While these findings suggest that certain stressor-elicited behavioral responses may not by mediated by endogenous CRH system(s), further research is needed to explore this contention. Indeed, in the majority of studies, pretreatment with benzodiazepines suppressed CRH-induced neurochemical and/or behavioral effects.[47,49,177,235,318] In terms of behavioral experiments, CRH-induced locomotor activation was found to be reversed by pretreatment with diazepam.[235] In addition, using the Geller-Seifter conflict test (a behavioral test of anxiety), CRH administration produced a dose-dependent attenuation

of both punished and nonpunished responding in the conflict test.[47,49] Pretreatment with chlordiazepoxide completely antagonized the effect of CRH on the punished component and partially reversed the CRH-induced attenuation of nonpunished responding.[47,49]

In summary, as can be appreciated by the preceding review of the literature, there is overwhelming evidence suggesting that CRH does meet all five of the stipulated criteria defining a 'stress peptide'.

Arginine-Vasopressin (AVP)

Research into the role of arginine-vasopressin (AVP) as a mediator of the stress response began almost 50 years ago. In the early 1950s, it was independently suggested by two teams of researchers; McCann and Brobeck (1954)[266] as well as Martini and Morpurgo (1955)[264], that AVP (which had already been structurally characterized at that time and established as a major regulator of fluid and electrolyte balance) was the purported 'corticotropin-releasing factor'. Although this contention turned out to be false and the 'real CRH' was eventually isolated and characterized, over the years an abundance of evidence emerged suggesting that AVP works in concert with CRH to regulate ACTH release and is, in its own right, a major contributor to the regulation of the HPA axis.

Does AVP meet the stipulated characteristics of a stress peptide?

In accordance with the first characteristic of a 'stress peptide', the anatomical distribution of AVP encompasses many brain sites thought to be involved in the stress

response. Specifically, immunohistochemical studies confirmed that the primary source of AVP within the CNS is the hypothalamus, where AVP is synthesized, transported within and released from two distinct classes of neurons; 1) the magnocellular neurons of the PVN and supraoptic nucleus, and 2) the parvocellular neurons of the PVN and suprachiasmatic nucleus.[15,209,377] Magnocellular AVP-containing neurons project to the posterior lobe of the pituitary gland (via the internal lamina of the median eminence), where AVP is released into general circulation to act on the kidneys and blood vessels to regulate fluid and electrolyte balance. Parvocellular AVP-containing neurons project to the external layer of the ME where AVP is released into the portal blood system to regulate anterior pituitary functioning. Parvocellular AVP-containing neurons (originating from the PVN) have also been shown to project to some extrahypothalamic sites within the brainstem (such as the NTS) and spinal cord.[377,414] Radioimmunoassay and immunohistochemical studies have revealed the presence of extrahypothalamic ir-AVP within the medial amygdaloid nucleus, BNST, lateral septum, hippocampus, medial thalamus, LC, NTS, substantia nigra, and the dorsal vagal nucleus.[97,377] These findings are consistent with the *in situ* hybridization studies, which revealed high levels of AVP mRNA within the hypothalamus as well as in extrahypothalamic structures, such as the BNST and the medial amygdala.[432] AVP gene expression as well as AVP immunoreactivity have also been localized in the anterior and posterior lobes of the pituitary gland.[247,301]

Receptor autoradiography studies have revealed a wide distribution of binding sites for AVP throughout the CNS. It should be noted that while three AVP receptor

subtypes (V_{1a}, V_{1b} and V_2) have been identified so far, only one subtype (V_{1a}) has been found in the CNS.[23] In general, there appears to be a high degree of correlation between the distribution of ir-AVP, AVP mRNA and the relative distribution of AVP receptors.[23] In addition, the distribution of AVP receptor mRNA has been shown to be remarkably consistent with that of AVP binding sites.[316] Briefly, within the hypothalamus, AVP receptors and AVP receptor mRNA are located in the suprachiasmatic, arcuate and preoptic nuclei and the lateral hypothalamic area. Extrahypothalamic sites of AVP receptors include the olfactory nucleus, lateral septum, nucleus accumbens, BNST, CeA, dentate gyrus, certain thalamic nuclei, LC, dorsal vagal complex, NTS and inferior olive.[23,316]

In keeping with the second characteristic of a 'stress peptide', there is considerable evidence suggesting that exogenous AVP administration produces endocrine, autonomic and behavioral effects similar to those observed during stressor exposure.[15,91] In addition, several studies have demonstrated that pretreatment with AVP antiserum or AVP receptor antagonists attenuate the neurochemical and behavioral effects induced by exogenous AVP administration or stressor exposure.[15,91] Thus, AVP would appear to meet the third characteristic of a 'stress peptide'. In terms of endocrine effects, both *in vitro* and *in vivo* studies have shown that in rats, AVP is a weak ACTH secretagogue by itself; however, it strongly potentiates CRH-induced ACTH release from the anterior pituitary.[16,136,351,399,404] Interestingly, in some species such as sheep, while synergism between AVP and CRH has also been observed, AVP by itself was found to be more potent than CRH at stimulating ACTH release.[118,246] Further support in favor of a

physiological role for AVP in HPA axis regulation is provided by studies using either AVP antiserum or AVP antagonists to block the effects of exogenous AVP administration or stressor exposure. For example, pretreatment with V_1 receptor antagonists blocked the AVP-induced increase of plasma ACTH, as well as the ability of AVP to potentiate CRH-induced ACTH release.[33,351] Pretreatment with AVP antiserum or an AVP receptor antagonist attenuated the elevation of plasma ACTH normally observed following stressors such as ether exposure or restraint.[245,355] Moreover, pretreatment with a V_1 receptor antagonist significantly attenuated the hypoglycemia- and hemorrhage-induced ACTH secretions.[337]

Morphological studies also supported a role for AVP in HPA axis activity. For example, as previously mentioned, anatomical data confirmed the existence of a vasopressinergic pathway from the parvocellular division of the PVN to the external zone of the ME.[15] High concentrations of ir-AVP in the hypophysial portal blood have also been observed.[436] Many studies have shown that AVP is co-localized with CRH.[15,423] Specifically, it has been demonstrated that most, if not all immunodetectable AVP in the external zone of the ME is located in CRH-containing nerve terminals. In this region, up to 50% of the CRH-containing terminals have been shown to produce and release AVP.[35,422,423]

While there is unequivocal support for a role for AVP in the regulation of the HPA axis, there is some debate regarding the exact source of AVP in stimulating ACTH secretion. Although it is thought that the primary source of AVP for stimulating ACTH

release originates in parvocellular neurons of the PVN (which project to the external zone of the ME), there is also evidence suggesting that AVP originating from magnocllular neurons may also play a role in ACTH secretion.[15] Although there are no direct projections from magnocellular AVP neurons to the portal blood system, it has been suggested that AVP may diffuse from magnocellular axons located in the internal zone of the ME to the external zone of the ME or from short portal vessels connecting the posterior to the anterior pituitary.[20,164] Indeed, c-fos expression studies have shown activation of both parvocellular and magnocellular AVP neurons, depending on the type of stressor exposure.[11,24,25,162,243] Not only do these findings support a role for both parvo- and magnocellular AVP in HPA activity, but they also suggest that these systems may be regulated independently of one another.

In addition to its influence on HPA axis activity, AVP also appears to have a stimulatory effect on autonomic functioning.[198,263] Indeed, central AVP administration has been reported to elicit an increase in plasma catecholamine levels in several species.[198,263] For example, King et al. (1985)[198] found that fourth ventricular AVP administration significantly increased plasma NE and epinephrine concentrations in rats. Similar effects have also been observed in rabbits following i.c.v. administration of AVP.[263] Furthermore, it appears that AVP plays a significant role in cardiovascular regulation.[30,332,390,434] Several studies have shown that central AVP administration induced a dose-dependent increase of mean arterial pressure and heart rate in several species including rats, dogs and rabbits.[30,332,390,434] For example, Szczepanska-Sadowska et al. (1993)[390] found that central administration of AVP as well as a specific V_1 (but not

V_2) agonist elicited a long lasting increase in mean arterial pressure and heart rate in dogs, suggesting that the cardiovascular effects of AVP are mediated via the V_1 receptor. The neural mechanism(s) mediating AVP-induced cardiovascular effects are currently not known; however, it was suggested that AVP may affect blood pressure regulation through its central action on the baroreceptor reflex. In support of this contention, it has been demonstrated that direct microinjection of AVP into the NTS (which is the sensory nucleus of the barareceptor reflex) dose-dependently increased blood pressure and heart rate.[265] It has also been hypothesized that AVP may exert its cardiovascular effects by stimulating noradrenergic neurons. Indeed, Olpe & Baltzer, (1981)[312] showed that microiontophoretic administration of AVP into the LC increased the firing rate in the noradrenergic neurons at this site. Moreover, microinjection of AVP into the LC (the major source of norepinephrine cell bodies) elicited a dose-dependent increase in mean arterial pressure and heart rate, an effect which was blocked with central administration of an AVP receptor antagonist into the LC or peripheral administration of α- or β-adrenergic receptors antagonists.[31]

As alluded to earlier, AVP has also been shown to produce a wide range of behavioral effects, many of which could be considered stress-relevant. In this context, similar to moderate stressor exposure, evidence strongly indicates that AVP modulates learning and memory processes.[37,91,407] More than 30 years ago it was found that removal of the posterior pituitary gland impaired maintenance of an avoidance behavior in rats and that this deficit could be reversed with AVP treatment.[90] Subsequent studies revealed that AVP has significant nmenonic effects.[91] For example, central administration of AVP

enhanced retention on passive avoidance tasks and pretreatment with AVP antiserum or AVP antagonists blocked these AVP-induced effects.[38,115,409,411] In addition, Brattleboro rats (which lack the ability to synthesize AVP) show impaired memory function when tested in active and passive avoidance tests, and administration of AVP restored avoidance behavior in these rats.[91] Moreover, AVP also has a facilitory effect on retrieval, as AVP treatment prevents or reverses retrograde amnesia induced by CO_2 inhalation, electroconvulsive shock or circadian disruption.[91] The possible site(s) of action for these AVP-effects appear to be located within various limbic structures. For example, Van Wimersma Greidanus & Baars (1991)[407] showed that microinjections of AVP antiserum into the hippocampus or septum impaired passive avoidance behavior. In contrast, direct microinjection of AVP into the hippocampus facilitated passive avoidance behavior.[207]

While the effects of AVP are not characterized as well as those of CRH, there is some indication that central administration of AVP elicits other stress-relevant behavioral effects, including enhanced locomotor activity[100,393] and an increase in grooming.[95,273,408] Di Michele et al. (1996)[100] reported that central AVP administration dose-dependently increased locomotor activity in a familiar environment. It has also been shown that central AVP administration induced excessive grooming, an effect that was attenuated by pretreatment with naloxone or a dopamine D_1 receptor antagonist, suggesting that the dopaminergic and/or opioid systems may mediate, at least in part, some of the AVP effects on grooming behavior.[408]

It has been suggested that AVP may be physiologically important in fear conditioning, as homozygous Brattleboro rats display deficits in the conditioned freezing paradigm.[379] Moreover, Stoehr et al. (1993)[379] have demonstrated that central administration of a V_1 receptor antagonist attenuated the expression of conditioned fear. Evidence also suggests that AVP may play a role in anxiety-related behaviors. Landgraf et al. (1995)[229] demonstrated that chronic infusion of AVP antisense into the septum reduced anxiety-related behaviors as measured on the elevated-plus maze. Finally, AVP has also been implicated as a modulator of nocioception. Central AVP administration produces antinocioception in rats and this effect was blocked by pretreatment with a V_1 receptor antagonist.[32,34]

The fourth characteristic of a 'stress peptide' is that in response to stressors, peptide release, levels and/or turnover, mRNA expression, and receptor densities should change. There are several studies which document stressor-induced changes in the AVP system(s) providing support for a functional role for AVP in the mediation of the stress response. Given that AVP plays an important role in fluid and electrolyte balance, many studies examined the effects of hypertonic and hypotensive stressors on the vasopressinergic system. Hypertonic saline injection and hemorrhage were associated with increased plasma and CSF concentrations of AVP.[184,199,337] In addition, hypertonic saline injection caused increased expression of AVP mRNA at the PVN and such osmotic stimulation elicited the release of AVP from the PVN and supraoptic nucleus.[59,230] Likewise, alterations of AVP system(s) have been associated with many other stressors. For example, inescapable footshock, or single injections of IL-1 or lipopolysaccaride

(LPS) have been shown to produce long-term increases in ir-AVP concentrations within the ME.[366,406] Acute or chronic exposure to immobilization likewise induced a significant up-regulation of AVP mRNA production within the parvocellular division of the PVN.[24,25,251] In addition, insulin-induced hypoglycemia has been associated with increased plasma concentrations of AVP, as well as increased AVP stores within the external zone of the ME.[92,122] Moreover, push-pull perfusion has revealed that AVP is released from the PVN and ME in response to IL-1ß administration[419], while a recent microdialysis study indicated the *in vivo* release of AVP from the septal area in response to a swim stressor.[111] While the majority of the evidence had pointed to AVP playing an important role in response to physical stressors, there are some findings that suggest the involvement of this peptide in mediating the response to emotional stressors as well. For example, an up-regulation in AVP mRNA expression was observed in the parvocelluar PVN following social isolation[341], and chronic psychosocial stressor exposure induced an increase of AVP content within the external zone of the ME.[93] In addition, using *in vivo* microdialysis, the release of AVP from the PVN was observed following exposure to an emotional stressor (social defeat).[428]

It is of interest to note that certain manipulations, such as adrenalectomy or exposure to various chronic (psychosocial, immobilization, insulin-induced hypoglycemia) or acute (footshock, IL-1 or LPS injection) stressors, cause increased co-expression of AVP and CRH at the external zone of the ME.[92,93,366,396,406] Not only did such manipulations increase production of AVP by neurons co-expressing CRH and AVP, but a phenotypic change was induced such that some of the CRH neurons that were

not producing AVP, began to co-express this peptide. This action, in effect, leads to a shift in the composition of the signal driving ACTH secretion from predominantly CRH-driven to one that is predominantly driven by AVP. While the significance of this phenotypic shift in CRH neurons has yet to be determined, it should be noted that CRH- and AVP-induced ACTH secretion appears to differ in their sensitivity to glucocorticoids. Indeed, evidence suggests that AVP is more sensitive than CRH to glucocorticoid negative feedback and that enhanced AVP production in CRH neurons may involve a reduced glucocorticoid sensitivity of CRH neurons.[208,254] In support of this contention, it has been demonstrated that exposure of rats to repeated immobilization stress elicits a corticosterone surge, which serves to down-regulate glucocorticoid receptors in the hippocampus and PVN.[254,261] This may in turn, temporarily impair glucocorticoid negative feedback, hence, facilitating AVP expression.

In support of the fifth characteristic of a 'stress peptide', there is some evidence, albeit limited, that benzodiazepines can attenuate stressor-induced changes in the AVP-system. For example, Yagi & Onaka (1996)[431] demonstrated that the benzodiazepine, chlordiazepoxide, dose-dependently attenuated the footshock-induced rise of circulating AVP levels.

In summary, although the stress-relevant effects of AVP are not as well characterized as those of CRH, there does appear to be ample evidence suggesting that AVP does meet many, if not all of the stipulated criteria for a 'stress peptide'.

Bombesin

Bombesin (BN) is a tetradecapeptide originally isolated from the skin of the European frog *Bombina bombina*.[6] Since its discovery, many BN-like peptides have been isolated and subsequently divided into three subfamilies based on their carboxy-terminal tripeptide sequences. The three subfamilies include; Bombesin, Ranatensin and Phyllolitorin (see Table 1). Currently, only two mammalian BN homologues have been identified. One is gastrin-releasing peptide (GRP), in both of its molecular forms; GRP_{1-27} (a 27 amino acid peptide often referred to as mammalian BN), and GRP_{18-27} (a carboxy terminal decapeptide of GRP often referred to as Neuromedin C (NMC)) belong to the BN subfamily[268], whereas the second homologue, neuromedin B (NMB) (in both its molecular forms; NMB_{1-32} and NMB_{23-32}) belongs to the ranatensin subfamily.[287]

Table 1 Amino acid sequence comparisons of representative members of each BN-like peptide subfamily

Bombesin subfamily	
BN	pGlu-Gln-Arg-Leu-*Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-*NH_2
GRP_{1-27}	X-Lys-Met-Tyr-Pro-Arg-*Gly-Asn*-His-*Trp-Ala-Val-Gly-His-Leu-Met-*NH_2
GRP_{18-27}	*Gly-Asn*-His-*Trp-Ala-Val-Gly-His-Leu-Met-*NH_2
Alytesin	pGlu-Gly-Arg-Leu-*Gly*-Thr-*Gln-Trp-Ala-Val-Gly-His-Leu-Met-*NH_2
Ranatensin Subfamily	
NMB_{1-32}	X-Val-Hys-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-Glu-Thr-Pro-*Gln-Trp-Ala*-Thr-*Gly-His*-Phe-*Met-*NH_2
Liktorin	pGlu-*Gln-Trp-Ala-Val-Gly-His*-Phe-*Met-*NH_2
Phyllolitorin Subfamily	
Leu^8-Phyllolitorin	pGlu-Leu-*Trp-Ala-Val-Gly*-Ser-*Leu-Met-*NH_2
Phe^8-Phyllolitorin	pGlu-Leu-*Trp-Ala-Val-Gly*-Ser-Phe-*Met-*NH_2
Thr^5-Leu^8-Phyllolitorin	pGlu-Leu-*Trp-Ala*-Thr-*Gly*-Ser-*Leu-Met-*NH_2

Note. Structural homology of BN-like peptides at the decapeptide C-terminal region in italics. "X" represents the amino acid chain preceding the sequence indicated.

Do BN-like peptides meet the stipulated characteristics of a stress peptide?

In keeping with the first characteristic of a 'stress peptide', mammalian BN-like peptides are widely distributed throughout the gastrointestinal tract and the CNS, encompassing many brain sites thought to be involved in the stress response. Within the CNS, relatively high concentrations of BN-like peptide immunoreactivity (ir-BN) have been detected within several hypothalamic nuclei including the PVN, medial preoptic area, suprachiasmatic, arcuate, ventromedial and anterior nuclei. High concentration of ir-BN have also been detected in several extrahypothalamic regions including certain brain stem structures such as the NTS and substantia gelatinosa as well as in several midbrain structures including the interpeduncular nucleus, central gray matter and the medial and central amygdaloid nuclei. Low to moderate concentrations of ir-BN have also been detected in several other regions including the periventricular and dorsomedial hypothalamic nuclei, hippocampus, LC, olfactory bulb and tubercle, caudate putamen and nucleus accumbens.[70,297,322] In the periphery, apart from peptide concentrations seen in the gastrointestinal tract, high concentrations of ir-BN have been observed in the pituitary gland and the adrenal medulla.[103,170,232,238]

More specific measurements of BN-like peptides have come from radioimmunological and immunohistochemical studies which have examined the separate distributions of immunoreactive NMB and GRP within the CNS. Interestingly, the localization of NMB and GRP appears to be relatively distinct.[296,323] Consistent with these findings, *in situ* hybridization studies have revealed a heterogeneous distribution pattern for NMB and GRP mRNA expression.[415] Briefly, a strong signal for NMB

mRNA has been detected in the olfactory bulb and dentate gyrus while a moderate signal has been detected in the CeA, CA2 pyramidal layer of the hippocampus and the ventral tegmental area. Relatively weak NMB mRNA signals were detected in the BNST, nucleus accumbens, arcuate nucleus and the medial preoptic nucleus. In contrast, the distribution of GRP mRNA appeared to be more widespread with the strongest signals being detected in the forebrain (throughout the hippocampus formation) and the cortical amygdaloid nucleus. Moderate GRP mRNA hybridization signals were expressed in cortical layers 11 and 111, the medial, lateral and basomedial amygdaloid nuclei, the suprachiasmatic and medial preoptic nuclei, the NTS and the parabrachial nucleus. A weak signal for GRP mRNA was detected at the PVN, the posterior hypothalamic area, the septum and the LC.[415]

Autoradiographic and membrane binding studies have demonstrated that, similar to its peptidergic distribution, receptors for BN related peptides are also localized in discrete sites throughout the CNS and gastrointestinal tract.[295,325,427,433] The highest density of BN binding sites within the CNS were detected in the olfactory bulb and tubercle, nucleus accumbens, suprachiasmatic and paraventricular hypothalamic nuclei, central and medial paraventricular thalamic nuclei, hippocampus, dentate gyrus, medial amygdaloid nucleus and NTS. Moderate densities of BN binding sites were observed in the parietal and rhinal cortices, caudate putamen, stria terminalis, basomedial and lateral amygdaloid nuclei, LC and parabrachial nucleus, whereas low densities of binding sites were detected in the globus pallidus, lateral thalamus and hypothalamus and midbrain. In

the periphery, BN binding sites have been located in the gastrointestinal tract as well as in the anterior pituitary.[168,298]

Subsequent to the discovery of a distinct distribution of NMB and GRP immunoreactivity as well as mRNA expression, receptor binding studies revealed that the total population of receptors for BN-like peptides within the CNS is composed of NMB- and GRP- preferring subtypes (denoted BB_1 and BB_2, respectively).[26,222] Thus, NMB binds with higher affinity to BB_1 than BB_2 receptors and the opposite holds true for GRP (BN is not selective for either receptor subtype). The distribution of BB_1 and BB_2 binding sites shows a strong correlation with the relative distributions of NMB and GRP immunoreactivity and mRNA expression within the brain.[26,222] There are, however, a few brain regions where poor correlation exists between binding sites and immunoreactivity for the peptide. One striking example is the nucleus accumbens, which contains a very high density of BB_2 binding sites but surprisingly, no GRP or NMB immunoreactivity or mRNA expression.[26,221,297] While the reasons for such discrepancies are currently not known, it should be noted that receptor and peptide distribution mismatches have been observed with other peptides, and may signal the existence of as yet unidentified BN-like peptide(s) that may interact with those receptors.

More recently, three new BN receptor subtypes (BB_3, BB_4 and BB_5) have been identified, however information pertaining to their functions is currently limited as no known natural ligands have been shown to interact with high affinity to these receptors.[9,259] With respect to BB_3 receptors, their distribution appears to be

predominantly in the brain and spinal cord (as compared to peripheral tissues), with prominent expression in hypothalamic regions including the PVN, arcuate and dorsomedial nuclei.[307] Although the function of the BB_3 receptor is currently not known, mice lacking this receptor subtype exhibit obesity and metabolic defects, suggesting the involvement of BB_3 receptor in the regulation of energy balance.[308]

In summary, ir-BN, mRNA expression and receptor density have been localized in many stress-relevant brain regions including several hypothalamic nuclei, the CeA, the hippocampus, LC and NTS as well as the pituitary gland and adrenal medulla. Interestingly, there is considerable overlap in the anatomical distributions of both BN-like peptides and CRH. Taken together, these findings support the notion that BN-like peptides meet the first characteristic of a 'stress peptide' and may be involved in both HPA axis and autonomic activity.

The second characteristic of a 'stress peptide' is to demonstrate that exogenous administration of the peptide mimics the endocrine, autonomic and behavioral components of the stress response. Increasing evidence has accumulated suggesting that BN-like peptides do indeed possess this characteristic.[53,215,256] Recent evidence suggests that administration of BN receptor antagonists can block the neurochemical effects of exogenously administered BN, providing evidence that BN-like peptides meet the third characteristic of a 'stress peptide'.[132,133,313] In terms of endocrine effects, several laboratories, including ours, have shown that BN, along with its mammalian counterparts, GRP and NMB, can potently stimulate the release of ACTH from the anterior pituitary

and/or corticosterone from the adrenal medulla.[19,132,133,150,255,257,313] For example, central administration of GRP dose-dependently increased plasma levels of ACTH and corticosterone in rats.[133,313] Recent findings suggest that the ability of GRP to stimulate ACTH and corticosterone release is mediated via BB_2 receptors as Garrido et al. (1998)[133] demonstrated that central administration of a competitive and specific BB_2 receptor antagonist completely blocked the GRP-induced increases in plasma levels of ACTH and corticosterone. Results have been somewhat less consistent with BN-induced endocrine effects following peripheral administration. In this context, both Olsen et al. (1992)[313] and Garrido et al. (1998)[133] found that intravenous injection of GRP did not affect plasma ACTH or corticosterone levels in rats. In contrast, others have reported that intravenous infusion or intraperitoneal administration of GRP or BN significantly elevated plasma ACTH and corticosterone levels in rats and dogs.[361,362,395] In addition, intravenous infusion of GRP in normal men stimulated the release of ACTH, ß-endorphin and cortisol.[201] It has been suggested that the discrepancies in the endocrine results following peripheral administration of BN-like peptides may result from species-dependent differences in sensitivities to BN-like peptides and/or differences in the dosages of BN-like peptides administered.[313] Indeed, in a majority of the studies where peripheral BN-like peptide administration had a stimulatory effect on ACTH and/or corticosterone release, the doses used were higher (over 2100 pmol) than when peptide administration was without effect (typically under 700 pmol).[133,313,361] Inconsistent results with peripheral BN administration, as well as the need for relatively high doses of the peptide in order to provoke a stimulatory effect on ACTH or corticosterone secretion, has led to the suggestion that BN-like peptides may be acting predominately through

central mechanisms at suprapituitary sites, such as the PVN or ME to stimulate the HPA axis. In support of this contention, Gunion et al. (1989)[150] demonstrated that infusion of BN into the PVN caused a significant increase in blood corticosterone levels. In addition, Garrido et al. (1999)[132] demonstrated that low to moderate concentrations of GRP (1 and 10 nM) stimulated the release of CRH-like material from the hypothalamus, whereas high concentrations of the peptide (100 and 1000 nM) were needed to stimulate the release of ACTH and corticosterone from the anterior pituitary and adrenal medulla, respectively. The effects of GRP at the hypothalamus, anterior pituitary and adrenal medulla appear to be mediated via BB_2 receptors, as the BB_2 receptor antagonist (Leu^{13}-ψ-CH_2-NH-LEU^{14}) BN blocked GRP's effects on hormone secretion from these sites.

While the neuronal mechanisms underlying these BN-induced endocrine effects remain largely unknown, there is evidence that BN-like peptides stimulate the HPA axis by acting through other ACTH secretagogues, in particular CRH and/or AVP. In support of this contention, Olsen et al. (1992)[313] demonstrated that central GRP administration dose-dependently stimulated ACTH release and potentiated the ACTH-stimulating effects of CRH and AVP. In addition, pretreatment with either CRH or AVP antisera blocked GRP-induced ACTH release by 60% whereas, pretreatment with combined antisera completely blocked this effect. It has also been shown that central administration of αh-CRF blocked the GRP-induced increase of ACTH and corticosterone.[133] In addition, there is evidence from *in vitro* studies indicating that BN-like peptides may be acting via CRH neurons. For example, using isolated perfused anterior pituitary cells, either BN or GRP can enhance the CRH-induced ACTH release.[117] Moreover, Au et al.

(1997)[19] showed that administration of GRP or BN could dose-dependently potentiate CRH-induced ACTH release from mixed anterior pituitary cell cultures. This effect was completely blocked by the BB_2 receptor antagonist, D-Tyr^6 BN(6-13) propylamide, suggesting that BN-like peptides exert their effects by binding to BB_2 receptors on anterior pituitary cells.

Aside from its ability to elicit the release of ACTH from the anterior pituitary, BN and related peptides have been shown to alter the secretions of other pituitary hormones. In this context, administration of BN-like peptides inhibited the release of growth hormone from the anterior pituitary.[197] Similar to findings with CRH, the ability of GRP to inhibit growth hormone release appeared to by mediated, at least in part, via somatostatin release, as pretreatment with antiserum against somatostatin prevented GRP's inhibitory actions on growth hormone release.[196]

There is also strong evidence for the involvement of BN-like peptides in activation of the sympathetic nervous system. Central administration of BN dose-dependently increased plasma levels of epinephrine and NE.[52,53,64,311] In addition, central administration BN and NMB elevate plasma glucose levels.[52,171,309,333] The ability of BN to increase levels of plasma glucose is dependent on adrenal epinephrine secretion which diminishes plasma insulin levels and increases plasma glucagon levels.[52] While the underlying mechanisms mediating BN's sympathoadrenomedullary functions are currently not known, direct electrophysiological evidence has shown that centrally applied BN elevates nerve activities of both sympathetic and adrenal branches of

splanchnic nerves, suggesting that BN has a direct effect on sympathetic outflow.[310] In addition, hypophysectomy did not prevent the development of BN-induced hyperglycemia and adrenalectomy did not prevent the BN-induce rise in plasma concentrations of NE.[52,53] These findings suggest that BN's involvement in autonomic activation is centrally mediated and independent of the HPA axis. Moreover, BN-induced elevations in plasma epinephrine levels are not blocked by central administration of dopaminergic, adrenergic and cholinergic antagonists.[53] To determine the relevant central site(s) of action, this peptide was microinjected into various brain sites of unanesthetized rats.[53,64] Injection of BN into the NTS produced a dramatic elevation in circulating plasma levels of catecholamines whereas injection of BN into other sites produced less pronounced effects. Iguchi et al. (1984)[171] also performed a similar microinjection study in an attempted to localize the site(s) of action of BN to elevate plasma glucose levels. Microinjection of BN into the ventromedial and lateral hypothalamic nuclei resulted in a marked increase in plasma glucose levels suggesting that these sites play an important role in mediating BN-induced hyperglycemia.

Further evidence supporting a role for BN-like peptides in autonomic activation is provided by the finding that central BN administration induced a significant increase in mean arterial pressure.[64,123] It has been suggested that this action is secondary to the increase in plasma epinephrine levels. Consistent with this contention, acute adrenalectomy or pretreatment with the α-adrenergic receptor antagonist phentolamine, blocked the increase in mean arterial pressure induced by BN injection.[123]

Behavioral experiments also support the notion that BN and related peptides might qualify as 'stress peptides', in that central or systemic administration of this family of peptides can induce many behavioral effects resembling those elicited by stressor-exposure or central CRH administration.[215] For example, it has consistently been shown that both peripheral and central administration of BN and its mammalian counterparts (GRP and NMB) dose-dependently suppress food intake in a variety of species.[96,134,302,391] Central mechanisms are required to elicit these effects as total neural disconnection of the gut from the brain attenuates satiety induced by systemic BN injection.[385] More specifically, pretreatment with capsaicin (a neurotoxin that selectively destroys small unmyelinated fibers in the vagus and spinal nerves) attenuated suppression of food intake induced by peripheral BN administration, suggesting the involvement of afferent fibers in this BN-induced effect.[225,285] Further support for the involvement of central mechanisms in BN-induced satiety, as well as for a physiological role of BN-like peptides in the mediation of satiety, is provided by studies using BN-like peptide receptor antagonists or BN antiserum. For example, central pretreatment with BN antiserum significantly attenuates satiety induced by systemic BN injection.[279] In addition, blockade of central BB_1/BB_2 receptors enhances food intake in rats and attenuates the satiety effects induced by central or systemic BN administration.[279,280,382] Several studies have attempted to elucidate the central site(s) of action of BN on food intake. Direct microinjection of BN into specific hypothalamic and extrahypothalamic nuclei have been shown to suppress food intake.[186,219,384,425] Hypothalamic structures have further been implicated in BN-induced suppression of food intake by results that show alterations of the levels of BN-like peptides in the paraventricular, arcuate and dorsomedial nuclei, as well as the *in vivo*

release of BN-like peptides from the PVN, in a meal-dependent manner.[334,336] In addition, there is evidence that caudal brainstem structures, such as the NTS, are particularly sensitive to the feeding suppressant effects of BN as injection of BN into the 4th ventricle suppresses food intake at much lower doses than injection into the lateral ventricles.[224] Moreover, microinjection of exquisitely low doses of BN-like peptides into the NTS suppresses food intake[186] and lesions to the area postrema and NTS block the ability of either central or systemic BN to inhibit feeding.[220,226]

Central BN and GRP administration has also been shown to enhance grooming in rats.[73,139,186,215] While central BN administration elicited grooming, peripheral BN injection was without such effect implicating central mechanisms in the mediation of this BN-elicited behavior. Although the central mechanisms are currently not known, there is evidence of involvement of both the dopaminergic and cholinergic systems, as pretreatment with either a selective D_1 receptor antagonist or a muscarinic receptor antagonist can attenuate BN-induced grooming.[276,281] Interestingly, Van Wimersma et al. (1988)[410] examined in detail the elements that comprise BN-induced grooming (of which the main component is scratching) and found that pretreatment with haloperidol induced a general reduction of BN-induced grooming, whereas pretreatment with naloxone suppressed the scratching element of BN-induced grooming behavior. These findings suggest that the opioid system may be involved in the display of the scratching element of BN-induced grooming, whereas the DA system appears to be involved in other elements of BN-induced grooming. In addition, neither adrenalectomy nor

hypophysectomy prevented the excessive grooming induced by BN administration, suggesting that these BN-induced effects are independent of the HPA axis.[139]

Another behavioral effect of BN related peptides which resembles the stress response is the ability of this family of peptides to increase exploratory behavior in a familiar environment. Indeed, several investigators have shown that central administration of BN dose-dependently enhanced locomotor activity in rats in a familiar environment.[275,325,369] There is evidence that the ability of BN to enhance locomotor activity is mediated, at least in part, via dopaminergic neurons as pretreatment with dopamine receptor antagonists attenuated this BN-induced effect.[275,369] While central administration of BN-like peptides elicit an increase of exploratory behavior in a familiar environment, the opposite effect occurs when these peptides are administered in a novel environment. Again, these BN-induced behavioral effects are consistent with those observed in response to stressors. In this context, Itoh et al. (1994)[180] demonstrated that central administration of BN, NMB, NMC and other related peptides induced a marked reduction of locomotion and rearing in rats placed in an open field.

Another stress-relevant behavioral effect of exogenously administered BN is its ability to enhance learning and memory. In this context, Flood and Morley (1988)[125] demonstrated that, in mice, BN or GRP administered immediately after training or 1 week before testing, enhanced performance in a test of retention. While the neural mechanisms are currently not known, it has been suggested that this BN-induced effect is peripherally mediated, as higher doses of BN or GRP were needed to enhance retention

when administered i.c.v. as compared to peripherally. Moreover, vagotomy blocked the BN- and GRP-induced enhanced retention suggesting that BN and GRP may modulate memory through activation of ascending vagal pathways.[125] Additional support for this contention is provided by the demonstration that microinjection of BN into the NTS (a brain structure that receives direct sensory input from the vagal nerve) enhanced retention in an inhibitory avoidance and radial maze task.[424] Moreover, Rashidy-Pour & Razvani (1998)[345] recently demonstrated that unilateral inactivation of the NTS or amygdala (which receives direct projections from the NTS) with lidocaine attenuated the memory enhancing effects of peripheral BN administration.

Finally, exogenous BN administration has also been shown to produce antinocioception in rats. Pert et al. (1980)[325] demonstrated that microinjection of BN into the periaqueductal gray matter produced an antinocioceptive reaction in both the hot plate and tail flick tests. These effects were not blocked by naloxone administration, suggesting that the opioid system is not involved in mediating this effect of BN.

The fourth characteristic of a 'stress peptide' is to show that in response to stressors, the putative stress peptide system is activated (e.g. enhanced release and/or associated receptor-based changes). At present, no studies have explored the effects of stressor exposure on BN-like peptide system(s). The fifth characteristic of a 'stress peptide' is to show that treatments believed to reduce stressor effects, such as benzodiazepines, should effectively reverse stressor-induced changes of the BN-like peptide system(s), as well as other stress relevant neurochemical changes or behavior

effects induced by central BN administration. Although there is little evidence in support of this characteristic for BN-like peptides, one behavioral study by Crawley et al. (1983)[75] showed that pretreatment with a non-sedating dose of diazepam markedly suppressed the ability of BN to induce excessive grooming behavior.

Thesis research objectives

As can be appreciated from the preceding review of the literature, there are many similarities between the anatomical distribution of BN-like peptides and the primary regulators of the stress response, namely CRH and AVP. In addition, there is a growing body of evidence suggesting that BN-like peptides share many similarities with the stress response (as well as CRH or AVP administration) in terms of endocrine, autonomic and behavioral effects. The striking similarities between BN-like peptides, CRH, AVP and stressor exposure in terms of regional distribution, endocrine, autonomic and behavioral effects have led us to hypothesize that i) BN-like peptides may be important in the mediation and/or integration of the stress response and ii) that BN-like peptides may in part, mediate their stress relevant effects by activation of CRH and/or AVP circuits. The overall objective of this research project was to test these working hypotheses using a variety of physiological and/or pharmacological approaches.

In an attempt to attain this overall objective, we have proposed the following set of five specific objectives addressing particular questions from both physiological and pharmacological perspectives:

Physiological Approach

1.1 Is exposure to an acute stressor associated with fluctuations in the endogenous regional levels of ir-BN and/or receptor density (of BN-related peptides)? This particular set of experiments was aimed at addressing the fourth criteria characterizing a 'stress peptide' (stressor exposure should alter peptide release, levels and/or turnover, mRNA expression, and/or receptor function) by assessing whether stressor exposure is associated with endogenous changes in BN-like peptide levels and/or receptor functioning.

1.2 Are the fluctuations in the BN-related peptides (or CRH) dependent on a) the nature of the stressors, and/or b) the 'stress proneness' of the rats? Although results from the preceding experiment (1.1) suggested that BN-like peptides appear to meet the fourth criteria of a 'stress peptide', many questions remained unanswered particularly with respect to the nature of the role of the BN family of peptides in the stress response. Thus, the next study examined the effects of a pure psychogenic stressor (ferret exposure) or a more neurogenic stressor (physical restraint) on changes in the regional levels of ir-CRH and ir-BN as well as on the release of CRH from the CeA, in rat lines with differential proneness to stressors.

1.3 Is exposure to an acute stressor associated with increased availability of BN-like peptides (GRP and NMB), CRH and AVP at the anterior pituitary? And are such stressor-elicited changes of pituitary peptidergic secretagogues influenced by the subject's prior stress-history? Although in the 2 post-mortem studies (experiments

1.1 and 1.2) we showed that stressor exposure is associated with site-specific alterations in endogenous levels of ir-BN, an important question remains unresolved: What do these tissue peptidergic changes mean? Do stressor-induced fluctuations in levels of BN-like peptides represent fluctuations in peptide release, synthesis or metabolism? Thus, in this experiment we decided to use *in vivo* push-pull perfusion to determine the effects of acute stressor (air-puffs) exposure on interstitial levels of GRP and NMB (as well as other stress relevant peptides, namely CRH and AVP) at the anterior pituitary. We also examined whether the subject's prior stress-history (previous exposure to acute or chronic restraint) influenced the stressor-associated elevations in the interstitial levels of GRP, NMB, CRH and AVP at the anterior pituitary.

Pharmacological approach

2.1 Does central administration of BN induce neuroendocrine and/or autonomic activation? Are these effects of BN mediated by the CRH system? Specifically does CRH receptor blockade attenuate these BN-induced changes? In light of the similarities between BN-like peptides and CRH in terms of anatomical distribution, behavioral and neuroendocrine effects, the first part of this experiment characterized the endocrine and autonomic effects of central BN in order to ascertain whether they mimic the effects of CRH. In the second part of this experiment we assessed whether BN mediated its effects through activation of CRH neurons. This was achieved by evaluating the effects of CRH receptor antagonists on BN-induced autonomic and endocrine activation.

2.2 Does central administration of BN elicit changes in regional levels and/or release of CRH and/or AVP? In experiment 2.1 we demonstrated that pretreatment with a CRH antagonist attenuated BN-induced endocrine and autonomic effects, supporting the contention that BN-like peptides may mediate their effects via activation of CRH neurons. In an attempt to determine the potential locus (or loci) of BN/CRH interactions, we examined the effects of central BN administration on regional levels of ir-CRH (and ir-AVP). In order to assess the functional relevance of the observed tissue level alterations, a second more direct push-pull perfusion experiment was performed to assess the dynamics of the *in vivo* release of CRH and AVP from selected brain sites following central BN administration.

These five principal approaches which constitute the chapters of this thesis, are presented in the form of published or submitted scientific papers. These investigations are not necessarily presented in the chronological order by which they were conducted but rather in an order that best represents the logical flow. The general discussion at the end of the thesis will summarize and consolidate the various findings with an attempt to further our understanding of the role of BN-like peptides in the mediation and/or integration of the stress response.

Preface to Chapter I

Prior to the initiation of this thesis research, evidence supporting a role for BN-like peptides in the mediation and/or modulation of the stress response was derived from a limited number of sources. This included studies demonstrating that administration of BN or related peptides (GRP or NMB) elicited endocrine, autonomic and behavioral effects, which resembled those induced by stressor exposure or CRH administration. However, there was no evidence demonstrating a functional link between the BN family of peptides and the stress response. We attempted to address this issue by assessing whether stressor exposure affected the *endogenous system(s)* utilizing BN-like peptides. This study therefore aimed to determine whether acute immobilization stress elicited regional changes in the endogenous levels of BN-like peptides and/or the density of BN binding sites, at various brain sites.

CHAPTER I

Are bombesin-like peptides involved in the mediation of stress response?

Abstract

The neurochemical mechanisms underlying the coincident activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system in response to stress remain unclear. Central injection of the neuropeptide bombesin (BN), potently stimulates the release of epinephrine from the adrenal medulla, adrenocorticotropic hormone (ACTH) from the pituitary gland, and elicits behaviors typically associated with increased emotionality and arousal. The current studies assessed whether stressor exposure is associated with 1) fluctuations in the endogenous regional levels of BN-like peptides and/or 2) changes in the density of BN binding sites. Male Sprague-Dawley rats received either no treatment or were subjected to acute immobilization stress for 10, 30 or 120 min. Plasma ACTH levels increased in response to stressor exposure, peaking at 30 min. BN-like peptide immunoreactivity increased significantly at the hypothalamus and medulla, within 30 min; however with more sustained immobilization (120 min) BN-like immunoreactivity declined to control levels. Levels of BN-like peptides remained unchanged in several other regions, including the hippocampus, striatum, midbrain, pituitary, and pons. Autoradiographic analysis revealed that the density of BN binding sites varied in a regionally specific manner. Significant stressor-related increases in binding were found at the nucleus of the solitary tract (at 30 and 120 min), and at the paraventricular of the hypothalamus (at 120 min). These data indicate the BN-like peptides may play a role in the mediation and/or modulation of response to stress.

Introduction

A characteristic response to stress involves the coincident activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic division of the autonomic nervous system. However the neurochemical mechanism(s) underlying this cascade of responses is (are) less well understood.

There is an abundance of evidence suggesting that the 41-amino acid polypeptide, corticotropin-releasing hormone (CRH) plays a key role in mediating the neuroendocrine, autonomic and behavioral responses to stress.[54,204,350,352,402] Central administration of CRH potently stimulates the release of adrenocorticotropic hormone (ACTH) and β-endorphin from the anterior pituitary in rats.[350,352,402] Studies have also demonstrated that central administration of CRH causes dose-dependent increases in plasma concentrations of norepinephrine, epinephrine and glucose in both rats and dogs.[54,216] In addition, central administration of CRH elicits a number of behavioral responses which resemble those induced by stressors. In particular, central CRH significantly suppresses food intake in food-deprived animals, induces excessive grooming behavior, enhances locomotor activity in a familiar environment, and decreases activity in a novel environment.[45,108,239,274,300,386]

Central administration of Bombesin (BN), a tetradecapeptide of amphibian origin, produces biological and behavioral actions strikingly similar to those elicited by CRH. Central BN injection induces dose-dependent elevations in plasma ACTH, corticosterone, catecholamines and glucose.[52,53,150,282,333] Behaviorally, central BN consistently induces a

satiety-like state in various species ranging from mice to man.[302,391] In addition, central BN elicits grooming behavior as well as locomotor activation in a familiar environment.[186,215,325] A recent study demonstrated that not only could centrally administered gastrin-releasing peptide (GRP), the mammalian analogue of BN, stimulate ACTH secretion, but also that low doses of GRP potentiated the ACTH-releasing effects of both CRH and vasopressin (AVP).[313] Furthermore, pretreatment with either CRH or AVP antiserum attenuated GRP induced ACTH response by about 60% and pretreatment with combined antiserum completely abolished this effect. These findings suggest that central GRP may mediate secretion of ACTH by stimulating the release of central CRH and/or AVP. Indeed, we have recently found that many of the endocrine and behavioral effects of BN can be blocked with CRH antagonists.[282]

In addition to numerous endocrine, autonomic and behavioral similarities between BN and CRH, both peptides also share considerable overlap in terms of their anatomical distribution. For example, high concentrations of both CRH (ir-CRH) and BN-like peptide immunoreactivity (ir-BN) have been found within the hypothalamus (paraventricular nucleus (PVN) and arcuate nucleus (Arc)), the central nucleus of the amygdala (CeA) and the nucleus of the solitary tract (NTS).[70,79,283,297,321,322,388] Furthermore, co-localization of GRP and CRH has been demonstrated in some neurons of the external zone of the median eminence (ME).[138] Some similarities also extend to the distribution of CRH and BN-like peptide receptors. For instance, high densities of binding sites for both neuropeptides are found in the olfactory bulbs, PVN, CeA and NTS.[88,295,433]

The striking similarities between BN-like peptides and CRH in terms of regional distribution, endocrine, autonomic and behavioral effects have led us to hypothesize that the BN family of peptides may play a role in the integration and/or mediation of the stress response; possibly by causing the release of CRH. If this peptidergic system is involved in stress response, one might expect to see stress-related alterations in this system. Thus in the present investigation, we assessed whether acute immobilization stress was associated with time- and region-dependent changes in the endogenous levels of BN-like peptides and/or the density of BN binding sites.

Material and Methods

Animals

Male Sprague Dawley rats (weighing between 300 and 350 g), obtained from Charles River (St-Constant, Quebec) were used in both experiments. The individually housed animals were maintained on a 12-h light/dark cycle (with lights on at 6:00 a.m.), with ambient temperature of 23°C and a relative humidity of about 60%. All animals had free access to food (Purina rat chow) and water. Experiments were run in a separate room between 09:00 and 11:00 a.m.

Experiment 1. Effects of immobilization stress on regional levels of ir-BN

Rats were randomly divided into four groups (n=6-7): non-stressed controls, rats subjected to immobilization stress for 10, 30 or 120 min. Immobilization stress consisted of securing the rat to a Plexiglas platform with adjustable Velcro straps and pieces of tape

holding each limb. On each test day, one rat from each test condition was sacrificed and the brains were processed for radioimmunoassay (RIA).

Tissue Preparation for RIA

Rats were sacrificed by decapitation, and their brains were rapidly removed and dissected on an ice-cold platform. Using blunt dissection, seven brain regions were isolated including; hypothalamus, medulla, pons, hippocampus, striatum, midbrain and pituitary. The brain tissue from each region was then weighed and placed in separate test tubes containing 2.5 ml of 2 M acetic acid heated to 80°C for a period of 1 h. The tissue was then homogenized and sonicated (Kontes, Micro ultrasonic cell disrupter, setting 5; Kontes, Vineland, N.J.) and the homogenate was centrifuged at 10 000 x g for 10 min. Two 1 ml aliquots of supernatant from each area were then frozen at -70°C and lyophilized. The desiccated samples were then stored at -20°C until the RIA was performed. Prior to centrifugation, 100 µl of each homogenate was collected and assayed for protein content using a BCA Protein Analysis kit (Chromatographic Specialties, Canada) and a PC 800 colorimeter (Brinkmann, Canada).

Radioimmunoassay (RIA)

Tyr^4BN was iodinated using a modified version of the technique described by Salacinski et al.[360] and purified using a Sephadex column (Sephadex G-10, 0.6x20 cm). The peak fractions were pooled and heated to 80°C for 1 h in the presence of 1 M 1,4-dithiothreitol (DTT) to reverse the oxidation of amino acids. The fraction was then

aliquoted and stored at -20°C. The specific activity of the peptide was calculated to be approximately 1200 Ci/mmol, using the method of Chiang et al.[68]

On the day of the RIA, the lyophilized aliquots were reconstituted in RIA buffer (0.05 nM phosphate buffered saline, pH 7.4 with 0.25% bovine serum albumin). The RIA was carried out at 0-4°C in duplicate, as previously described by Kateb and Merali.[191] Briefly, to the sample and/or standard tubes, 200 µl of BN antibody (final dilution of 1:300 000) was added followed 1 h later by 100 µl (approx. 5000 cpm) of ^{125}I-labeled BN. The ligand-antibody complex was then isolated 16 h later, using 100 µl of Amerlex-M donkey anti-rabbit antibody coated onto magnetizable beads (Amersham, Canada) and the precipitates were counted in a gamma-counter (Canberra Packard, Cobra II Autogamma, model D5002).

Experiment 2. Effects of immobilization stress on the density of BN binding sites

Rats were randomly divided into three groups (n=7-8): non-stressed controls, and subjects exposed to immobilization stress for 30 or 120 min. On each test day, one rat from each test condition was sacrificed and the brains processed for autoradiography on the same day.

Plasma ACTH

Trunk blood was collected in tubes containing EDTA, centrifuged and the plasma was frozen to -70°C. Plasma ACTH levels were determined using RIA kits (ICN, Canada).

Tissue preparation and sectioning

Rats were sacrificed by decapitation and their brains were rapidly removed and frozen in a rat stainless steel brain matrix (ASI instruments, MI, U.S.A.) resting on dry ice. A razor blade was inserted into the brain matrix to separate the cerebrum from the cerebellum and more caudal brain regions prior to being frozen. For the cerebrum, 20 μm coronal brain slices were obtained using a cryostat (Hacker 5030) set at -17°C, and thaw mounted onto gelatin-chrome alum coated 22 X 22 mm glass coverslips. A total of 15 brain slices were selected from each brain; 5 at the level of the nucleus accumbens (nAcb) (~ +1.70 mm from bregma), 5 at the level of the PVN (~ -1.8 mm from bregma) and 5 at the level of the Arc (~ -3.3 mm from bregma). The remaining five slices were obtained from horizontal sections at the level of the NTS (~ -8.1 mm from bregma). Upon completion of the cryostat sectioning, the slices were air dried at room temperature for 30 min.

In Vitro Receptor Autoradiography

Autoradiography was carried out using a modification of the procedure described by Wolf and colleagues.[427] Briefly, sections were incubated in autoradiography buffer containing 130 mM NaCl, 4.7 mM KCl, 5.0 mM MgCl2*6H20, 1.0 mM EGTA and 10.0 mM HEPES, pH 7.4 with 0.1% BSA and 0.01% Bacitracin. The slices were preincubated for 20 min (to remove endogenous ligand) and then incubated for 1 h 45 min in the presence of I^{125}-Tyr^4-BN (at a final concentration of 3 nM). The slices were then rinsed twice (4 min), quickly dipped in distilled water and then dried by a stream of cold air. The slices were then apposed to Hyperfilm Bmax (Amersham, Canada) along

with [^{125}I] microscales (RPA 523, Amersham, Canada) for 40 h. The autoradiograms were then developed (Kodak D-19 developer (4 min); Kodak rapid fixer (8 min)) and quantified as described below.

Staining

The brain sections used for autoradiographic exposure were subsequently recovered and stained using thionin, for structural/anatomical identification during image analysis.

Image Analysis

The autoradiograms were analyzed using the Microcomputer Imaging Device (MCID; Imaging Research, St. Catherines, Ontario). Histological overlay of the stained section onto the autoradiogram facilitated identification of precise nuclei. The optical densities of seven brain regions were determined and then converted to DPM/mg using the ^{125}I calibration standards. The sampled regions include PVN, Arc, NTS, CeA, hippocampus (Hipp), paraventricular thalamic nucleus (PV), and nucleus accumbens (nAcb).

Statistical Analysis

All results are expressed as means ± S.E.M. In the first experiment, concentrations of ir-BN were analyzed by a two-way analysis of variance (ANOVA) with Brain regions and Stressor duration as factors. Post hoc comparisons were conducted using Tukey post hoc procedure. In the second experiment, plasma ACTH values were

analyzed by a one factor ANOVA (Stress duration) followed by a Tukey post hoc comparison. The density of BN binding sites was analyzed by a one factor (Stressor duration) repeated measures ANOVA for individual brain regions. A repeated measures analysis was employed in order to control for potential variability across autoradiographic films (processed on different days). Post hoc comparisons were again conducted using Tukey post hoc procedure ($\alpha = 0.05$).

Results

Experiment 1. Effects of immobilization stress on regional levels of ir-BN

The ANOVA revealed that ir-BN varied as a function of the interaction between Brain region and Stress duration ($F_{18,165} = 2.15$; $p < 0.0069$). Tukey's post hoc comparisons revealed that the interaction was attributable to a differential regional responsiveness to the stressor application. In accordance with previously published data, individual brain regions differed significantly in their levels of ir-BN, with the highest concentration being detected in the hypothalamus.[191] Tukey post hoc comparisons revealed significant alterations of ir-BN at the hypothalamus and medulla following acute immobilization stress. At the hypothalamus, ir-BN was significantly elevated following 10 and 30 min of stressor application ($p<0.01$) with return to baseline levels by 120 min. A similar trend was noted at the medulla with a significant increase in ir-BN at the 30 min stress interval ($p<0.05$) and a return to baseline levels by 120 min.

Experiment 2. Effects of immobilization stress on the density of BN binding sites

Figure 2 shows plasma ACTH levels in non-stressed control rats, and subjects exposed to 30 or 120 min of immobilization stress. ANOVA revealed a significant effect of Stressor duration on plasma levels of ACTH ($F_{2,20}$= 47.19; $p<0.0001$). Tukey post hoc comparisons indicated a significant increase in plasma ACTH levels following 30 and 120 min of immobilization stress ($p<0.01$).

As illustrated in figure 3, stressor application affected (in a regionally specific manner) the density of BN binding sites. Autoradiographic analysis of the density of BN binding sites for seven brain regions are listed in ascending order; Hipp < NTS < PVN < Arc < PV < CeA < nAcb. ANOVA revealed a significant effect of Stressor duration at the level of the NTS ($F_{2,20}$=6.92; $p<0.01$), which remained significant using the greenhouse-geisser adjustment ($p<0.03$). Tukey post hoc comparisons showed a significant increase in the density of BN binding sites at this site following 30 and 120 min of immobilization stress. ANOVA also revealed a significant effect of Stressor duration at the level of the PVN ($F_{2,20}$ = 5.85; $p<0.017$), which remained significant using the greenhouse-geisser adjustment ($p<0.02$). Tukey post hoc comparisons indicated a significant increase in the density of BN binding sites following 120 min of stressor application at this site.

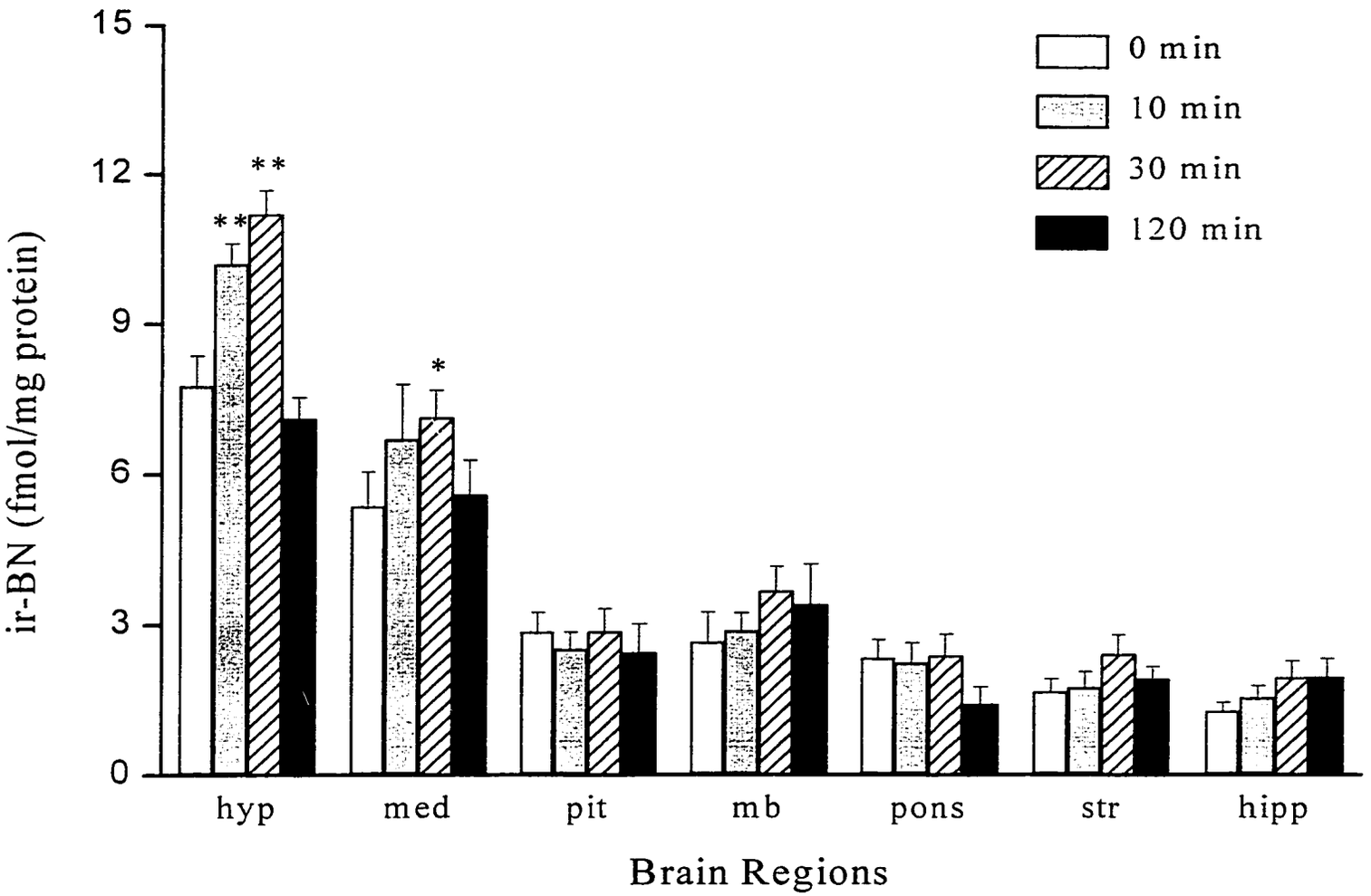

Fig. 1 Content of ir-BN (mean ± S.E.M) in brain regions under non-stressed control condition and following exposure to 10, 30 and 120 min of immobilization. Brain regions include: hypothalamus (hyp), medulla (med), pituitary (pit), midbrain (mb), pons, striatum (str) and hippocampus (hipp). *,** significantly different from non-stressed control values at $p<0.05$ and $p<0.01$, respectively.

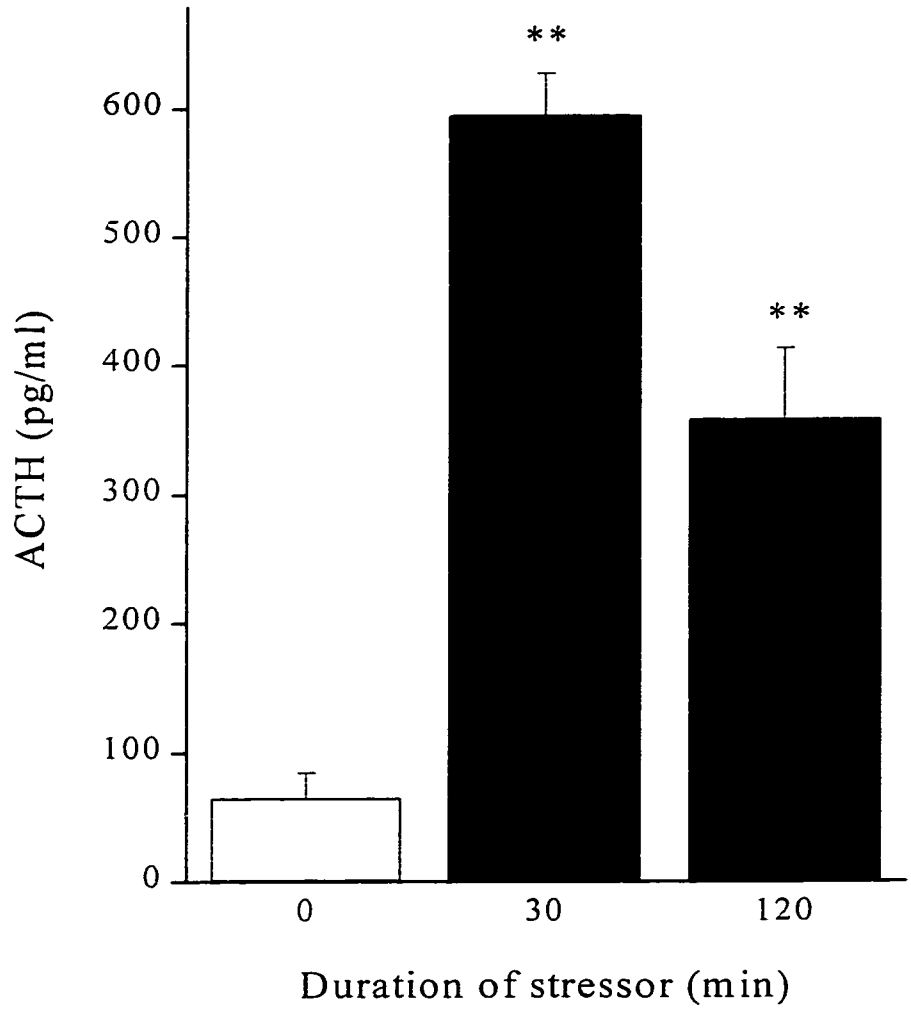

Fig. 2 Plasma levels of ACTH under non-stressed control condition and following exposure to 30 or 120 min of immobilization. Results are expressed in pg/ml as mean ± S.E.M. ** significantly different from non-stressed control values at $p<0.01$.

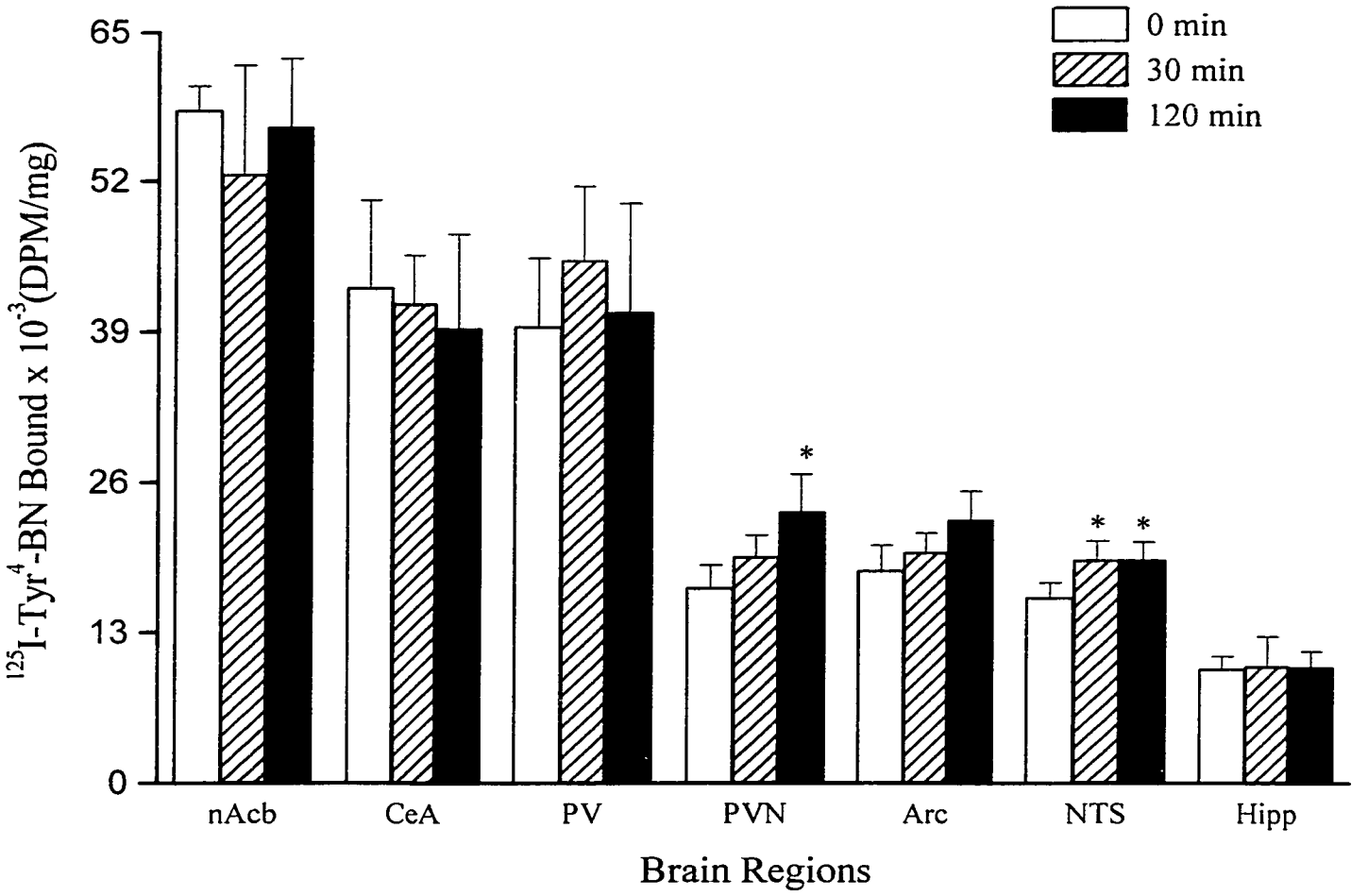

Fig. 3 Density of BN binding sites in various brain regions under non-stressed control condition and following exposure to 30 and 120 min of immobilization. Brain regions assessed included the following: nucleus accumbens (nAcb), central amygdaloid nucleus (CeA), paraventricular thalamic nucleus (PV), paraventricular hypothalamic nucleus (PVN), arcuate nucleus (Arc), nucleus of the solitary tract (NTS) and hippocampus (Hipp). Results are expressed in DPM/mg as mean ± S.E.M. *, ** significantly different from non-stressed control values at $p<0.05$ and $p<0.01$, respectively.

Discussion

The objective of the present study was to determine whether the endogenous system(s) utilizing BN-like peptides was involved in the mediation of some of the effects of acute stressor exposure. These results demonstrated that acute immobilization stress induced site-specific changes in the endogenous levels of BN-like peptide(s) as well as in the density of BN binding sites. To our knowledge, this is the first account of stressor-induced alterations in the BN-like peptide system in the brain, and supports our notion that this system may be involved in the mediation and/or integration of the stress response.

Stressor application was associated with a significant elevation in ir-BN at the hypothalamus following 10 and 30 min of immobilization and at the medulla following 30 min of stressor exposure. While these findings support a physiological role for BN-like peptides in stress response, they do not reveal their mechanism(s) of action, as alterations in tissue peptide concentrations can be interpreted in many ways. For instance, increased tissue levels of BN could indicate either 1) an increased synthesis of the peptide and/or 2) a decreased release of the peptide, or 3) an increased release of the peptide with an even greater increase in its synthesis. Using the *in vivo* microdialysis approach, our laboratory has recently shown that increased tissue levels of BN-like peptides at the CeA translates to increased release of that peptide.[271] If a similar mechanism of action applies to other brain sites, it could be postulated that the increased ir-BN noted at the hypothalamus and medulla during immobilization stress may be indicative of increased release of the peptide accompanied by an even greater rate of its

synthesis. Conversely, the lower peptide content (e.g. at 120 min of stressor application) could reflect lower rates of synthesis and/or release of BN-like peptides. However, this interpretation is only speculative at this time and needs to be tested empirically using dynamic *in vivo* sampling techniques such as push-pull perfusion or microdialysis.

The autoradiographic analysis of changes at the receptor level also support a role for BN-like peptides in the mediation of stress response. A significant increase in the density of BN binding sites was noted at the NTS (30 and 120 min of immobilization) and at the PVN (120 min of stressor application). At present, an interpretation of the observed up-regulation of BN binding sites remains equivocal. Recent *in vitro* experiments have found that in Swiss 3T3 cells, BN-like peptide receptors respond to agonist exposure by desensitization, internalization and down-regulation.[29] If we contend that stressor exposure is associated with an increased synaptic availability of BN-like peptides, then we might expect to see a down-regulation of BN binding sites. However, since the converse was apparent, we think that the *in vitro* responses of Swiss 3T3 cells may not reflect the processes occurring *in vivo* within the brain. Another major factor explaining this discrepancy may have to do with the physiological vs. pharmacological doses of the peptide used; the Swiss 3T3 cells were exposed to relatively high doses of BN and the tissue response(s) may not be relevant to the current physiological conditions. It is of interest to note that like our observations with BN binding sites, 120 min of immobilization stress up-regulated CRH_1 receptor mRNA at the PVN.[173] It was suggested from these results that an ultra-short positive feedback loop may exist for the production of CRH which would have the net effect of increasing CRH expression and

thus amplifying the stress response. It is possible that the observed up-regulation of BN binding sites in the present study may likewise be a response designed to amplify the response to stress. At present it is not clear whether this response is due to *de novo* receptor synthesis or the activation of latent (or dormant) receptors. In yet another experiment focusing on the cholinergic system, an increase in muscarinic acetylcholine receptor activity was noted following 2 h of immobilization stress. The authors of that study suggested that the increase in receptor density might reflect a compensatory response to depleted transmitter stores.[141] In our experiment, immobilization stress had a transient effect on ir-BN at the hypothalamus and medulla; peptide levels increasing during the initial 30 min but declining to control levels with 120 min of protracted exposure, potentially reflecting increased synthesis/release followed by depletion/exhaustion. Thus, the up-regulation of BN binding sites noted at the PVN and NTS at the 2 h time point, could potentially reflect a compensatory response to the depletion and/or exhaustion of the BN-like peptide stores. Although there is an anomaly with this interpretation at the NTS at the 30 min time point (when an up-regulation of BN binding sites was observed along with high peptide levels within the medulla), it remains possible that the tissue levels may not necessarily reflect the synaptic availability of the peptide. In keeping with this observation, one must be cautious whilst making any direct comparisons between the results obtained for ir-BN to those obtained for the density of BN binding sites. When measuring ir-BN in relatively large brain structures, the effects of immobilization stress within discrete nuclei could be obfuscated; if two nuclei within the same region had peptidergic changes in the opposite direction, the effect would be "washed out" in the analysis of the whole region. Thus techniques with higher

anatomical resolution (such as brain micropunch or *in situ* hybridization) would need to be employed in order to get a more accurate and nucleus specific measure of ir-BN.

Although BN-like peptides appear to be involved in the mediation and/or integration of the stress response, it is not clear which particular peptide (and respective receptor subtype) of this family is more stress relevant. The mammalian brain has been shown to express many structurally related BN-like peptides, including GRP_{1-27}, GRP_{18-27} (neuromedin C (NMC)) and the more distinct neuromedin B (NMB; NMB-10 or NMB-32).[268,287] In the present experiment, the antibody used for the RIAs recognized the C-terminal of BN and has been shown to strongly cross react with GRP_{1-27} (110%), BN (100%) and GRP_{18-27} (82%), but not with NMB-10 (<0.1%). Therefore, the BN-like peptides being measured in the present study are most likely to be GRP and/or NMC, but not NMB. It is of interest to note that the PVN and the NTS both appear to express GRP but not NMB.[26,415] In the case of receptor binding studies, the ligand we used (^{125}I-[Try^4]BN), has a high affinity for the GRP (BB_2), as well as for the NMB (BB_1) receptor subtype.[222] In the case of the receptor subtypes, when one compares the receptor binding profile with the receptor mRNA content of the same regions, the results are concordant at some regions but discordant at others. For instance, the PVN has a high preponderance of BB_2 receptor mRNA but little if any BB_1 receptor mRNA. These findings are similar to results obtained with receptor binding studies which have shown hypothalamic structures to possess high densities of BB_2 binding sites.[222] However, at the NTS, messages for both the receptor subtypes are present, but receptor binding studies seem to indicate that this nucleus contains more BB_1 binding sites than BB_2 sites.[26,222] Of course,

the development of more specific agonists and antagonists will ultimately permit characterization of the roles of specific members of the BN family of peptides.

The regional specificity of the observed changes in BN-like peptide levels to the hypothalamus and medulla suggests that BN-like peptides at these regions may be important in mediating the stressor effects. The regional dissections, however, precluded identification of the precise nuclei within these regions that might be stress-relevant. Indeed, the receptor autoradiography experiment, that had a higher anatomical resolution, suggests that distinct sites located within the hypothalamus and medulla may be involved in the mediation of the stress response by BN-like peptides. Various other studies have identified the hypothalamic and brain stem structures as being stress-relevant sites.[131,160,166,181,203,233] *In situ* hybridization studies examining the pattern and time-course of immediate early gene expression following acute restraint stress have consistently found a dramatic induction of c-fos messenger RNA expression at the PVN as well as other hypothalamic nuclei.[77,176,373] A moderate induction of c-fos was also noted at the NTS.[77] Interestingly, the time-course of this stressor-induced c-fos expression paralleled those of ir-BN noted in the present study; peaking at 30 min and returning to baseline with more protracted stressor exposure (120 min).

Several hypothalamic nuclei contain high concentrations of ir-BN, including the PVN, Arc, anterior and ventromedial nuclei.[70,297,322] A high density of BN binding sites is also apparent at specific hypothalamic nuclei, particularly at the PVN.[295,433] Microinfusions of BN directly into the PVN causes dose-related increases in blood

corticosterone, whereas no increases occurred after infusions into other regions including the lateral hypothalamus and caudate-putamen.[150] Recent immunohistochemical studies have revealed strong fos-like immunoreactivity at the PVN following central administration of either BN or CRH, suggesting that both of these peptides stimulate neuronal activity in this area.[176,240] Taken together, these studies provide support for the hypothalamus, particularly the PVN, as a likely site of action for BN in the mediation and/or modulation of the stress response. CRH-containing neurons in the PVN project to the ME where CRH is released into the portal blood system. It is possible that BN-like peptides interact with receptors located on the cell bodies and/or dendrites of CRH expressing neurons of the PVN, causing the release of CRH at the terminal regions of these neurons at the ME. Alternatively, receptors for BN-related peptides at the external zone of the ME may cause the release of CRH directly from this region. In support of this contention, co-localization of ir-BN and ir-CRH has been observed in the external zone of the ME.[138]

Among the CNS structures that play a role in the mediation of stress response, there is clear evidence that catecholaminergic nuclei of the lower medulla have a leading position in HPA axis regulation.[77,131,233] Within the medulla, the NTS is known to receive direct sensory input from the vagal and glossopharyngeal nerves and gives rise to noradrenergic projections that extend to the parvocellular division of the PVN and other basal forebrain nuclei.[347] It is of interest to note that in the current study, stressor-induced increases in the density of BN binding sites were observed at the NTS. Immunohistochemical studies have demonstrated that centrally administered CRH or BN

stimulate neuronal activity at the same nucleus reflected by an increased c-fos expression.[176,240] Other studies have found the NTS to be a site of action for BN to increase plasma epinephrine and norepinephrine levels.[53] The NTS contains high levels of both ir-BN and ir-CRH and contains high densities of BN-like peptide and CRH receptors making this region another likely site for BN-like peptide-CRH interactions.[88,295,297,321,322]

In conclusion, results from the present study provide the first account of stressor-induced alterations of endogenous system(s) utilizing BN-like peptides and thus support the role of this family of peptides in the mediation and/or modulation of the stress response. While the exact role of this system in the regulation of the stress response is not understood, various findings suggest that some effects may be mediated via the activation of CRH and/or AVP neurons. It has recently been shown that pretreatment with CRH and/or AVP antiserum can attenuate GRP-induced increases in plasma ACTH.[313] Moreover, we have found that pretreatment with the CRH antagonist, αh-CRF, significantly attenuates the BN-induced endocrine (increases is plasma ACTH, norepinephrine, epinephrine and glucose) and behavioral effects (suppression of food intake and increased grooming behavior).[282] Thus it appears that BN-like peptides in conjunction with CRH may play an important role in activation of both the HPA as well as the sympatho-adrenal arms of the stress circuits. These results suggest that investigation of the temporal sequence of the localized release of BN-like peptides, CRH and AVP may shed more light into the mechanisms responsible for the activation of sympatho-adrenal medullary and adrenal cortical outflow associated with stressor

exposure.

Preface to Chapter II

Although the first set of experiments provided functional evidence suggesting a linkage between BN-like peptides and the mediation and/or modulation of the stress response, the mechanisms for such an interaction remain undetermined. For example, it is not known whether this peptidergic activation is stressor specific or generalizable across a wide array of stressors. It is known that the pattern and degree of activation of the HPA axis is dependent on the type of stressor and also on the animal's susceptibility to stress. Thus, the objective of the present study was to assess the effects of qualitatively different stressors (predator exposure and physical restraint) on regional levels of ir-CRH and ir-BN as well as on the release CRH from the central amygdaloid nucleus in two rat lines with differential proneness to stressors.

CHAPTER II

Differential effects of psychogenic and neurogenic stressors on central systems utilizing corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rat

Abstract

In as much as marked interindividual differences exist in response to stressors, we assessed the effects of exposure (15 min) to a pure psychogenic stressor (predator (ferret) exposure) or a more neurogenic stressor (restraint) on regional levels and release of corticotropin-releasing hormone (CRH) and bombesin (BN)-like peptides as well as on endocrine stress markers (plasma ACTH and corticosterone) in 'Slow' and 'Fast' seizure susceptible rats. Although these lines of rats were selectively bred for proneness to kindling effects, we have noted behavioral differences implicating differential responsiveness to stressors. Results showed that the rat lines differ in their endocrine responses to the stressors; ferret exposure elicited a greater increase in plasma ACTH and corticosterone concentrations in the Slow rats than in the Fast rats. In contrast, exposure to restraint stress elicited a more pronounced rise in plasma ACTH levels in Fast rats as compared to Slow rats. In terms of basal levels of immunoreactive-CRH (ir-CRH) and BN-like peptides (ir-BN), Slow rats had higher levels of ir-CRH at the hippocampus, lateral hypothalamus and motor cortex and lower levels of ir-BN at the cingulate cortex, anterior hypothalamus (AH) and nucleus of the solitary tract (NTS) as compared to Fast rats. In response to stressor exposure, Slow animals showed increased levels of ir-BN at the AH and increased levels of ir-CRH at the median eminence/arcuate nucleus (Me/Arc), paraventricular hypothalamic nucleus (PVN) and pituitary (Pit) in response to both ferret and restraint, whereas decreased levels of ir-BN were found at the NTS in response to both stressors. Fast rats showed decreased levels of ir-BN at the NTS and decreased levels of ir-CRH at the Me/Arc, PVN and Pit in response to both ferret and restraint exposure, whereas increased levels of ir-CRH were found at the central

amygdaloid nucleus (CeA) in response to restraint stress. A subsequent *in vivo* microdialysis experiment revealed that restraint elicited a more pronounced release of CRH at the CeA in Fast rats as compared to Slow rats, whereas in response to ferret exposure, the Slow rats showed a greater CRH release as compared to Fast rats. Taken together the current results demonstrate that these two lines of rats show differential endocrinological and neurochemical response patterns to psychogenic and neurogenic stressors

Introduction

Exposure to stressors activates various structures within the brain, which converge upon the hypothalamic-pituitary-adrenal (HPA) axis.[77,78,160,319] One of the most prominent effects of stressors is the release of the 41 amino acid peptide, corticotropin-releasing hormone (CRH). It has been observed, for instance, that a variety of different stressors provoke alterations in CRH immunoreactivity as well as the induction of CRH mRNA in various brain regions.[25,66,172,210,400] Moreover, *in vivo* microdialysis studies indicated release of CRH from the CeA in response various stressful conditions.[278,328] Supporting the contention that this peptide may subserve many of the responses to stressors, it has been shown that central administration of CRH produces endocrine, autonomic and behavioral changes which resemble those elicited by stressor exposure[107,317] and these effects are blocked by pretreatment with CRH antiserum or antagonists.[48,54,167,350,356]

In addition to CRH, it appears likely that bombesin (BN)-like peptides are also involved in the mediation and/or modulation of the response to stressors. In this context, acute immobilization stress induced site-specific alterations of endogenous levels of BN-like peptide as well as in the density of BN binding sites.[195] As well, like stressor exposure, central administration of BN and related peptides (gastrin-releasing peptide and neuromedin B) potently activate both the HPA axis and sympathetic division of the autonomic nervous system.[52,64,133,150,257,311,313] Moreover, application of BN-like peptides directly into the brain elicits a variety of behavioral responses which resemble those induced by stressor exposure, including suppression of food intake, enhanced grooming

behavior, decreased locomotor activity in a novel environment and increased exploratory behavior in a familiar environment.[134,180,215,325]

It has been argued that HPA activation evoked by different types of stressors may involve distinct neuroanatomical pathways. Herman and Cullinan (1997)[160] suggested that an immediate physiological threat to survival (systemic stressor) would necessarily require little sensory processing and therefore activate brain stem nuclei that project directly to the PVN where activation of the HPA axis would be initiated. In contrast, stressors requiring emotional appraisal through higher order sensory processing (processive stressors) via limbic brain structures, such as the hippocampus, amygdala, and prefrontal cortex, leads to indirect or rather 'filtered' HPA activation. It is important to note that the responses to various so-called processive or psychogenic stressors may also vary in a stressor-specific manner. Indeed, the pattern of central c-fos mRNA induction is different for the two psychogenic stressors, physical restraint and swim stress.[77]

The physiological changes evoked by stressor exposure depend not only upon the nature of the stimuli, but also on the inherent individual differences of the organisms being assessed.[185] Predictably, the sensitivity to the effects of stressors also varies across different strains of mice and rats.[12,98,99,269,378] Moreover, it seems likely that the central neurochemical changes elicited by stressors may also be influenced by the behavioral styles animals adopt to deal with the stressor.[272] Interestingly, the strain differences can be used to identify the mechanism(s) contributing to specific stressor-elicited

neurochemical alterations and behavioral changes engendered by the treatment. In this context, we have assessed stressor responses in two rat lines that had been selectively bred for differences of amygdala excitability, as realized by either fast or slow seizure development following mild electrical amygdala stimulation.[106] These rat lines, dubbed Fast and Slow seizing, also displayed marked behavioral differences indicative of greater fear/anxiety in the Slow rats as compared to the Fast rats.[292] In addition, we characterized behavioral, endocrine and immunological differences between the Slow and Fast rat lines in terms of their response to two different stressful stimuli; a pure psychogenic stressor (predator exposure) and another pyschogenic stressor with a "neurogenic" or physical component (restraint stress).[13] The Fast rats exhibited a protracted struggle and a more pronounced endocrine response during restraint stress as compared to the Slow rats which remained relatively immobile during exposure to this stressor. In contrast, the Slow rats showed less immobility and a greater endocrine response to ferret exposure as compared to the Fast rats.

In the current study, it was our intention to further characterize endocrine and neurochemical changes in the Fast and Slow rat lines in response to either predator exposure or to restraint. In particular, these studies assessed the effects of the stressors on levels of circulating ACTH and corticosterone, and immunoreactive CRH (ir-CRH) and BN-like peptides (ir-BN) across a number of discrete brain regions (using brain micropunch technique). One of the pitfalls with experiments of this nature is, of course, that hormonal or post-mortem tissue peptide alterations represent only a "snapshot assessment " and do not reflect the dynamic changes associated with stressor application.

Accordingly, a second more direct experiment was carried out to assess the dynamics of *in vivo* release of ACTH, corticosterone and amygdala CRH (through *in vivo* microdialysis) in response to the physical restraint and predator exposure stressors. The central nucleus of the amygdala (CeA) was selected for detailed examination as it plays an important role in the animals' response to stressors[28,143,144,165], and is a distinguishing nucleus between the Fast and Slow rat lines in terms of kindling susceptibility.[106,292]

Materials and Methods

Animals

Male Fast and Slow rats (weighing between 300 and 375 g) were used in all experiments. These rat lines, currently established at Carleton University (Institute of Neuroscience) were selectively bred from an original parent population comprising Wistar and Long-Evans Hooded rats. Animals were individually housed in wire mesh cages and maintained on a 12-h light/dark cycle, in a temperature (23°C) and humidity (60%) controlled room. Animals were permitted free access to food and water. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the local Research Ethics Committee of Carleton University.

Experiment 1. Effects of predator exposure and physical restraint on plasma ACTH and corticosterone levels and on ir-CRH and ir-BN levels at discrete brain regions

Fast and Slow rats were randomly assigned to 3 conditions (n=10 rats per condition) and were subjected to either 1) no stress (control), 2) 15 min of non-contact ferret exposure, or 3) 15 min of restraint stress. The procedures for restraint and predator stress have been described elsewhere.[13] Briefly, restraint stress involved placing a rat

into a cone shaped plastic bag with an opening at the narrow end to permit the rat's snout to protrude. Once completely inside, the bag was taped shut around the rat's tail and placed on a tabletop for 15 min. The bag provided a snug fit, which restrained both lateral as well as forward movements, while permitting limited paw and head movement. The procedure for predator exposure comprised placing a rat in a typical shoebox clear polycarbonate cage (26 x 48 x 20 cm) with a stainless steel lid. The cage containing the rat was then place inside a larger ferret cage (60 x 60 x 75 cm) containing a single ferret. The 15 min of ferret exposure permitted an exchange of olfactory and visual cues, but the ferret could not make physical contact with the rat. However, the ferret could sit atop the rat cage, and attempt to push the cage about (albeit to a limited extent). Typically, upon placing the rat cage into the ferret cage, the ferret would explore the top and sides of the rat cage, and make obvious attempts to gain access to the rat. In order to maintain a ferret's interest in the inaccessible rat, exposure of rats was alternated between 4 ferrets, and ferrets were only exposed to 2 rats on any given day. Since the ferrets differed in their effort to gain access to the rats, the ferrets were counterbalanced between the 2 rat lines. Immediately following stressor exposure rats were transported, while in their cages, to a necropsy area where they were sacrificed immediately by decapitation. Trunk blood was collected for ACTH and corticosterone analysis by radioimmunoassay (RIA), while the brains were rapidly extracted, frozen on dry ice, and stored at -80°C for subsequent neuropeptide determinations.

Plasma ACTH and Corticosterone

Trunk blood was collected in tubes containing EDTA (0.1 M/ 5 µl), centrifuged and the plasma was frozen and stored at -80°C. Plasma ACTH and corticosterone levels were determined in duplicate using commercial RIA kits (ICN Pharmaceuticals (ACTH); R&D Biochemicals (corticosterone)). These assays yielded intra- and interassay variations of less than 10%.

Tissue Preparation for RIA

Using a cryostat, the brains were sectioned coronally (600 µm thick) and the following 15 discrete nuclei were dissected from the sections, using a modification of the micropunch method described by Palkovits and Brownstein[320]: the median eminence/arcuate nucleus (Me/Arc), paraventricular hypothalamic nucleus (PVN), lateral (LH), anterior (AH), ventromedial (VMH) and dorsomedial (DM) hypothalamic nuclei, central amygdaloid nucleus (CeA), hippocampus (Hipp), locus coeruleus (LC), olfactory tubercle (OT), nucleus accumbens (nAcb), caudate (Cau), cingulate cortex (Cg Cx), somatosensory cortex (S Cx) and motor cortex (M Cx). A 16th region, the nucleus of the solitary tract (NTS), was micropunched from a horizontal section (600 µm thick) of the brain stem, while the pituitary was taken as a whole at the time of brain extraction. All nuclei were derived bilaterally (Micro Punch MP-600, ASI instruments, U.S.A.) using a 1mm diameter punch, except for the Me/Arc, which was obtained using a single medial 1 mm diameter punch.

The tissue punches were placed in microcentrifuge tubes containing 200 µl of 0.2 M acetic acid and heated to 80°C for 1 h. The tissue was then sonicated (Kontes Micro-ultrasonic Cell Disruptor, Kontes, NJ, U.S.A.) and 40 µl of the homogenate was removed for protein content analysis (BCA Protein Analysis kit; Chromatographic Specialties, Canada) using a PC colorimeter (Brinkmann, Canada). The remainder of the homogenate was centrifuged at 10 000 x g for 4 min and the supernatant of each region was then divided into two 75 µl aliquots, lyophilized and stored at -20 °C.

Experiment 2. Effects of predator exposure and physical restraint on the time-course of plasma ACTH and corticosterone changes in Fast and Slow rats

Rats were anesthetized with halothane and implanted with indwelling arterial catheters (PE 50 tubing) via the pulmonary artery. The catheter, which was filled with saline containing heparin, was passed subcutaneously to the nape region and exteriorized. Rats were then fitted with harnesses around their front paws, which attached to a tether (to enclose the exteriorized catheter) at the base of the neck. Each rat was then placed individually into a cage and the catheters connected to a peristaltic pump (to permit continuous infusion of saline containing heparin through the catheter) via polyethylene tubing and a swivel. A 3-way valve permitted blood sampling on demand.

Following a five-day recovery period, testing began with collection of a baseline blood sample (control condition). Rats in their cages were then taken to another room where they were exposed to the predator or were restrained for 15 min. Restraint stress involved manual immobilization of the rat for 15 min. Predator exposure involved

placing the rat (in its cage) into a 10 x 12 foot room containing a single ferret. As in the first experiment, the ferret could move freely around the rat's cage, but could not make direct contact with the rat. Blood samples were taken at the 5, 15, 30 and 60 min time intervals.

Experiment 3. In vivo release of CRH at the CeA following predator exposure and physical restraint in Fast and Slow rats

Surgical Procedure

Groups of male Fast and Slow rats (n =13-14/group) were anesthetized (60 mg/kg pentobarbital, i.p.) and stereotaxically implanted with unilateral 20 gauge guide cannulae containing removable 24 gauge obturators aimed at the CeA. The placement coordinates[324] with level skull were anteroposterior, -2.1 mm; dorsoventral, -7.0 mm; and lateral, ± 4.2 mm. The guide cannulae, protruding from a custom-manufactured Derlin® pedestal, were secured to the skull with four stainless steel screws and dental cement. After a minimum 7 day surgical recovery, animals were transferred to individual testing chambers and allowed to acclimate for 2 days before testing. The testing chambers comprised of Plexiglas cages (25 x 35 x 34 cm) with stainless steel grid floors. Food (Purina Lab Chow) and tap water were available *ad libitum*.

Microdialysis

Approximately 90 min before testing, rats were briefly anesthetized with halothane, and the obturator within the guide cannula was replaced with a microdialysis probe. The concentric microdialysis probe had 2.5 mm of active membrane (250 μm

outer diameter) of regenerated cellulose (6000 KD cut-off; Spectrum Medical Industries) that protruded into the CeA. Each probe was secured with a retaining screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel and a 2.5 ml gas-tight infusion syringe (Hamilton) attached to a pump (model 22, Harvard). Microdialysis probes were perfused at 3 μl/min with filtered Kreb's-Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K^+, 145 Na^+, 1.35 Ca^{2+}, 1.0 Mg^{2+}, 150 Cl^-, 0.05% ascorbate, pH 7.4[291], and BSA (0.1%). On collection, each sample (90 μl) was immediately frozen on dry ice and stored at -80°C until radioimmunoassay (RIA) analyses. The efficiency of the microdialysis probes was assessed *in vitro* (details previously described by Merali et al.[278]) with the average peptide recovery for CRH being 3.3 ± 0.6%.

Histology

At the end of the experiment, the brains were extracted, rapidly frozen on dry ice, sectioned, and stained for histological examination of the microdialysis probe placements. Only those probes confirmed to be in the CeA were used in the data analysis.

Radioimmunoassay (RIA)

A solid-phase RIA procedure which has previously been described[253,278] was employed for the detection and quantification of CRH and BN-like peptides. Briefly, 96-well plates (Immunilon-4, Dynatec Laboratories Inc., Chantilly, VA) were coated with protein A/G (Calbiochem Corp; La Jolla) at least 24 h before use and stored at 0-4°C. On

the day of the RIA, the plates were rinsed with buffer (15 M PBS) and then coated with either CRH or BN antibody (1:100,000 final dilution). Following a 2 h incubation period, the plates were rinsed with buffer and the standards (reconstituted in KRB solution; ranging from 0.05 to 250 fmol/well for CRH and 0.25 to 512 fmol/well for BN), blanks and samples were then pipetted into the designated wells and incubated for 24 h at 0-4°C. Next, ^{125}I-labelled CRH or BN (Amersham, Canada; 5000 cpm) was added. Following a 24 h incubation period (at 0-4°C), the plates were rinsed with wash buffer and the individual wells counted for residual radioactivity using a gamma-counter (Cobra II Autogamma, model D5002, Canberra Packard,). Sensitivity of the assay was typically ~0.1 fmol/well for CRH and BN. Inter and intra-assay variability was less than 3 %.

The specific anti-CRH serum (rC70, kindly provided by W. Vale) recognizes CRF_{1-41} and has been shown to cross-react negligibly with other related peptides including urotesin 1 and urocortin.[403] The BN antibody (α-BN2 was kindly provided by Dr. T. Moody) used in this study recognizes the C-terminal of BN and has been shown to cross react strongly with GRP_{1-27} (110%), BN (100%) and GRP_{18-27} (82%) and negligibly with neuromedin B (<0.1%) and other related peptides.

Statistical Analysis

All results are expressed as means ± S.E.M. In the first experiment, the effects of the stressor treatments on plasma ACTH or corticosterone, as well as ir-CRH and ir-BN at the various brain regions, were analyzed by two factor (Rat line x Treatment) ANOVA's followed by Newman-Keuls multiple comparisons of the means or, in the case

of significant interactions, comparisons between the means comprising the simple effects. In the second experiment, the effects of ferret exposure on plasma ACTH and corticosterone values were analyzed separately by two factor (Rat line x Time) repeated measures ANOVAs. Post hoc comparisons were conducted using Newman-Keuls multiple comparisons wherein post-stressor hormone levels were compared between the rat lines, as well as to the basal hormone levels. Microdialysis data from the ferret exposure and restraint stressor experiments were analyzed by first averaging the baselines (denoted as 100%) with subsequent samples expressed as a percentage of that baseline. The changes in ir-CRH levels were analyzed using two factor repeated measures ANOVAs with Rat lines as the between-groups factor (Fast vs. Slow) and Treatment conditions (baseline, early post-stress, late post-stress) with nested sample bins being the within-subjects factor. Post hoc analyses were conducted using Newman-Keuls multiple comparisons. For all experiments, a value of $p<0.05$ was considered statistically significant.

Results

Experiment 1. Effects of predator exposure and physical restraint on plasma ACTH and corticosterone levels

Plasma ACTH and corticosterone levels in Fast and Slow rats exposed either to 1) no stress (control), 2) ferret exposure, or 3) restraint stress are shown in Figure 1. The concentrations of ACTH varied as a function of the Stressor treatment x Rat line interaction, $F_{2,42} = 8.00$; $p<0.01$. Newman-Keuls comparisons of the simple effects revealed that levels of ACTH were comparable in the two rat lines. Relative to non-

stressed rats, both ferret exposure and restraint significantly increased ACTH in the two lines. However, in response to ferret exposure, the increase was greater in the Slow rats, such that the levels exceeded those evident in the Fast line. Following restraint, the ACTH increase among the Slow rats was comparable to that elicited by ferret exposure, whereas in Fast rats the increase of ACTH was considerably greater, such that the hormone levels significantly exceeded that of Slow rats.

Like ACTH, plasma corticosterone levels were altered by the stressor manipulation, $F_{2,52} = 25.89$; $p<0.01$, but the interaction between the Stressor and the Rat lines was not statistically significant. The multiple comparisons indicated that ferret exposure and restraint increased corticosterone levels to a comparable extent in both rat lines.

Effects of predator exposure and physical restraint on regional ir-CRH levels

Figure 2 shows ir-CRH concentrations at the PVN, Me/Arc and Pit in Fast and Slow rats exposed to either 1) no stress (control), 2) ferret exposure, or 3) restraint stress. In each of these brain regions the concentrations of ir-CRH varied as a function of the Rat line x Stressor treatment interactions, $F_{2,54} = 6.22$, 6.12 and 4.4; p's<0.01, respectively. Subsequent multiple comparisons of the simple effects confirmed that in the absence of any stressor treatment the concentrations of ir-CRH were comparable in the two rat lines, in each of these three regions. Following stressor exposure, the peptide concentrations within the PVN, Me/Arc and Pit were reduced in the Fast rats, but were increased in the Slow line. As a result, the post-stressor levels of ir-CRH were significantly higher in

Slow rats as compared to Fast rats. The effects observed in other brain regions, including several hypothalamic nuclei, were less dramatic. Within the LH, the Slow rats displayed a small but statistically significant elevation of ir-CRH, $F_{1,54}$=6.89; $p<0.01$ (the mean peptide level of Slow rats exceeded that of Fast rats (0.156 ± 0.013 and 0.124 ± 0.012 pmol/mg protein, respectively). However, neither the stressor main effect nor the Rat line x Stressor interaction reached significance. Likewise, within the VMH and AH, the ir-CRH levels were not altered as a function of any of the variables. The ir-CRH concentrations at the Hipp among Slow rats (mean = 0.075 ± 0.008 pmol/mg protein) exceeded that of Fast rats (mean = 0.048 ± 0.004 pmol/mg protein), $F_{1,48} = 14.08$; $p<0.01$, but the Stressor treatment was without effect. In the CeA, the levels of ir-CRH did not vary as a function of either the Rat lines main effect or the Rat line x Stressor interaction, although the latter approached significance, $F_{2,51} = 2.27$; $p = 0.11$. However, as the two rat lines were selected based on amygdala excitability, it was expected that they would be differentially affected at this site. Given this a priori hypothesis, Newman-Keuls multiple comparisons were conducted for the simple effects of this interaction. These comparisons confirmed that the stressors did not affect ir-CRH within the CeA of Slow rats. However, a significant rise of ir-CRH was induced by restraint (0.289 ± 0.021 pmol/mg protein) in the Fast rat line as compared to controls (mean = 0.209 ± 0.008 pmol/mg protein). The effect of the ferret exposure was not significantly different (mean = 0.227 ± 0.022 pmol/mg protein) from that of control rats. The levels of ir-CRH within the Cg Cx and S Cx were unaffected by the Stressor treatment or the line of rats. Likewise, in the M Cx, the peptide levels were unaffected by the stressor, whereas concentrations of ir-CRH in Fast rats (mean = 0.107 ± 0.012 pmol/mg protein) were

lower than those seen in Slow rats (mean = 0.137 ± 0.012 pmol/mg protein), $F_{1,50} = 4.25$; $p<0.05$. Finally, striatal sites (NAc, Caudate and OT) were unaffected by the treatments and similarly brainstem sites such as the LC and NTS were comparable between the two rat lines and were unaffected by the stressor treatments.

Effects on predator exposure and physical restraint on regional ir-BN levels

In contrast to the well-defined CRH variations observed in response to the stressor treatments, few changes of ir-BN were observed between the Fast and Slow rats or as a function of the stressor treatments. In neither the PVN nor the Pit were appreciable differences observed as a function of the treatments, whereas at the Me/Arc, the levels of ir-BN of Fast rats exceeded those of the Slow line following stressor treatments although control levels were comparable between the two rat lines, $F_{1,42} = 5.59$; $p<0.05$. Within the AH, the ir-BN levels in Fast rats appreciably exceeded that of Slow rats (mean = 0.196 ± 0.017 and 0.145 ± 0.016 pmol/mg protein, respectively). Relative to non-stressed rats, the concentrations of the peptide were significantly elevated by ferret exposure (0.211 ± 0.016 pmol/mg protein) and restraint (0.211 ± 0.017 pmol/mg protein) to a comparable extent in Slow rats, $F_{1,54} = 4.40$; $p<0.05$, but remained relatively unchanged in Fast rats. In the remaining hypothalamic nuclei (VMH, LH and DM) treatment effects were not evident. Likewise, unlike the CRH variations, in neither the Hipp nor the CeA were there differences as a function of the stressor treatment or the line of rats, a pattern also evident in both the M Cx and S Cx. In the Cg Cx, however, ir-BN levels of Fast rats were higher than those of Slow rats, $F_{1,49} = 20.01$; $p<0.01$ (mean = 0.184 ± 0.01 and 0.130 ± 0.013 pmol/mg protein, respectively). Differences in ir-BN

were not apparent as a function of the treatments within striatal regions (caudate, NA and OT) or within the LC. Within the NTS, however, the ir-BN levels in Fast rats appreciably exceeded that of Slow rats, $F_{1,51} = 6.56$; $p<0.05$ (mean = 0.355 ± 0.031 and 0.274 ± 0.02 pmol/mg protein, respectively), and relative to non-stressed rats, the concentrations of the peptide were significantly reduced by ferret exposure in both Fast (mean = 0.212 ± 0.026 pmol/mg protein) and Slow rats (mean = 0.176 ± 0.021 pmol/mg protein) and by restraint in Fast rats (mean = 0.256 ± 0.028 pmol/mg protein), $F_{2,51} = 12.36$; $p<0.01$. Similarly, a slight but statistically non-significant decrease in post-restraint levels of ir-BN was also observed in Slow rats (mean =0.215 ± 0.019 pmol/mg protein).

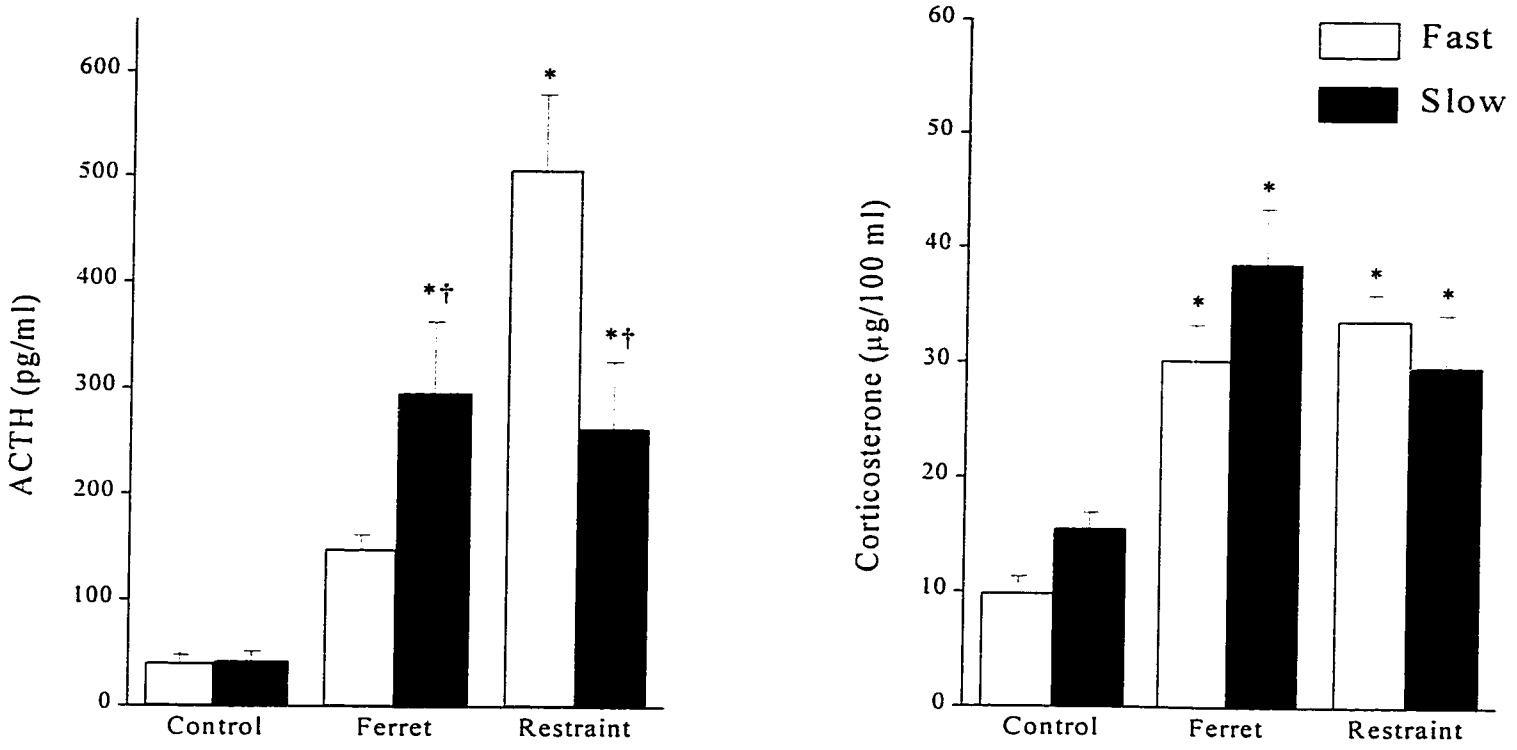

Fig. 1 Plasma levels of ACTH and corticosterone (mean ± S.E.M.) in Fast and Slow rats under non-stressed control condition and following 15 min of ferret exposure or physical restraint. * significantly different from non-stressed (within-rat line) control values at $p<0.05$. † significantly different from Fast (between-rat line) treatment-matched values at $p<0.05$.

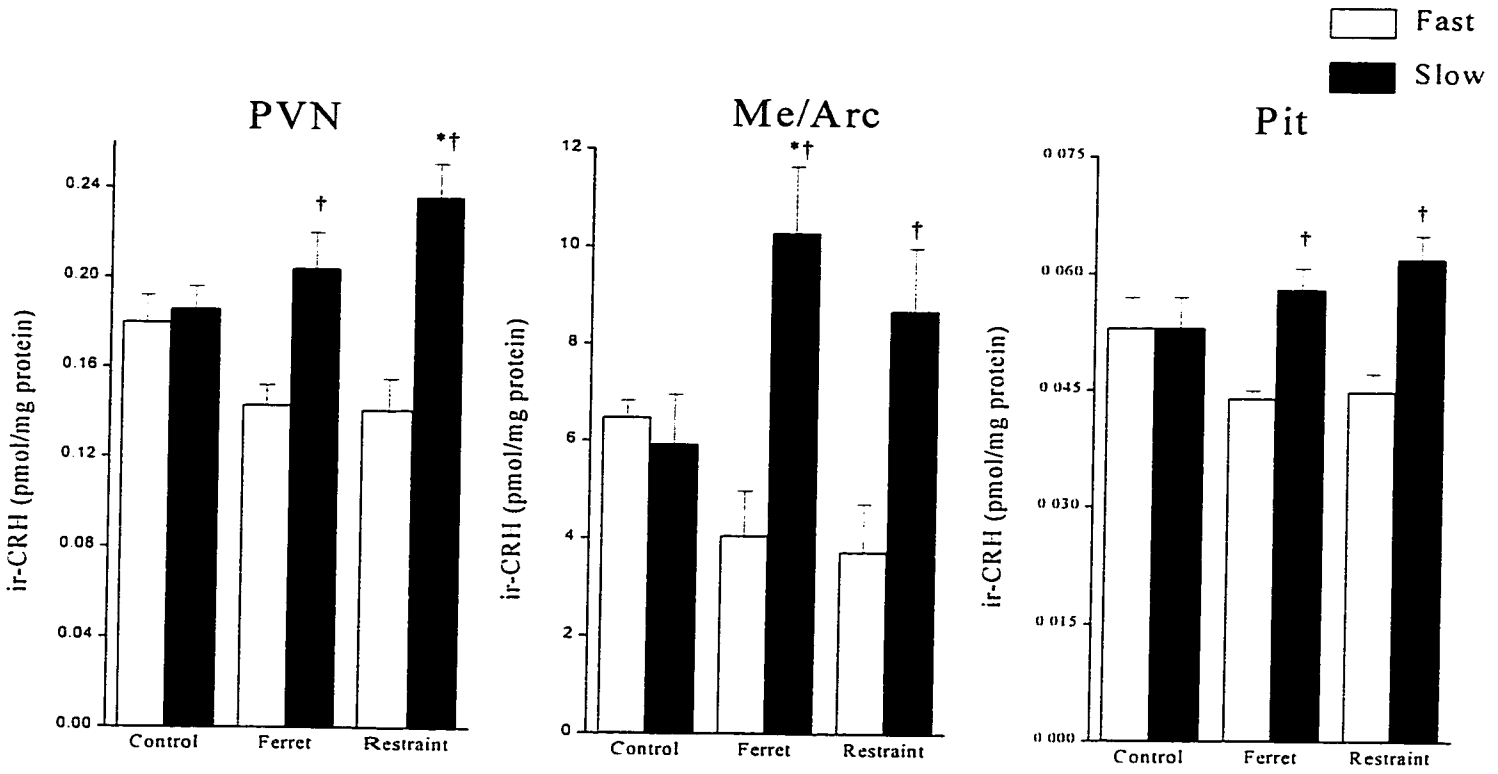

Fig. 2 Content of ir-CRH (mean ± S.E.M.) at the paraventricular hypothalamic nucleus (PVN), median eminence/arcuate nucleus (Me/Arc) and pituitary (Pit) in Fast and Slow rats under non-stressed control condition and following 15 min of ferret exposure or restraint stress. * significantly different from non-stressed (within-rat line) control values at $p<0.05$. † significantly different from Fast (between-rat line) treatment-matched values at $p<0.05$.

Table 1. Content of ir-CRH at various brain regions in Fast and Slow rats

	CONTROL		FERRET		RESTRAINT	
Regions	Fast	Slow	Fast	Slow	Fast	Slow
Me/Arc	6.478 ± 0.349	5.928 ± 1.030	4.052 ± 0.925†	10.27 ± 1.366*	3.729 ± 0.976†	8.691 ± 1.293
LC	0.418 ± 0.050	0.389 ± 0.021	0.305 ± 0.038	0.432 ± 0.091	0.323 ± 0.056	0.361 ± 0.038
DM	0.363 ± 0.022	0.406 ± 0.028	0.385 ± 0.034	0.472 ± 0.055	0.477 ± 0.057	0.473 ± 0.071
VMH	0.206 ± 0.022	0.236 ± 0.017	0.208 ± 0.021	0.222 ± 0.017	0.214 ± 0.028	0.206 ± 0.019
CeA	0.209 ± 0.008	0.219 ± 0.011	0.227 ± 0.022	0.230 ± 0.019	0.289 ± 0.021*	0.223 ± 0.016
PVN	0.180 ± 0.012	0.186 ± 0.010	0.143 ± 0.009*	0.204 ± 0.016*†	0.141 ± 0.014	0.236 ± 0.015†*
Olf Tub	0.185 ± 0.026	0.167 ± 0.019	0.178 ± 0.023	0.131 ± 0.020	0.144 ± 0.011	0.170 ± 0.009
LH	0.124 ± 0.012	0.156 ± 0.013†	0.125 ± 0.009	0.140 ± 0.008	0.119 ± 0.006	0.138 ± 0.010
M Cx	0.107 ± 0.012	0.137 ± 0.012†	0.123 ± 0.010	0.134 ± 0.012	0.109 ± 0.008	0.122 ± 0.010
NTS	0.111 ± 0.008	0.127 ± 0.012	0.104 ± 0.008	0.106 ± 0.011	0.109 ± 0.011	0.130 ± 0.012
AH	0.116 ± 0.010	0.126 ± 0.012	0.116 ± 0.008	0.137 ± 0.012	0.120 ± 0.008	0.115 ± 0.007
NAcb	0.097 ± 0.006	0.112 ± 0.009	0.112 ± 0.093	0.116 ± 0.011	0.113 ± 0.007	0.100 ± 0.006
S Cx	0.097 ± 0.008	0.106 ± 0.014	0.128 ± 0.007	0.120 ± 0.009	0.114 ± 0.013	0.117 ± 0.009
Cg Cx	0.094 ± 0.009	0.083 ± 0.008	0.101 ± 0.099	0.086 ± 0.011	0.093 ± 0.010	0.094 ± 0.010
Cau	0.083 ± 0.008	0.071 ± 0.006	0.082 ± 0.010	0.063 ± 0.006	0.074 ± 0.010	0.082 ± 0.010
Hipp	0.048 ± 0.004	0.075 ± 0.008†	0.048 ± 0.068	0.059 ± 0.006	0.034 ± 0.006	0.057 ± 0.007
Pit	0.053 ± 0.004	0.053 ± 0.004	0.044 ± 0.001	0.058 ± 0.003†	0.045 ± 0.002	0.062 ± 0.003†

Note. Each cell represent the mean ± S.E.M. (pmol/mg protein) under non-stressed control condition and following 15 min of ferret exposure or restraint stress.
* significantly different from non-stressed (within-rat line) control values at $p<0.05$.
† significantly different from Fast (between-rat line) treatment-matched values at $p<0.05$.

Table 2. Content of ir-BN at various brain regions in Fast and Slow rats

	CONTROL		FERRET		RESTRAINT	
Regions	Fast	Slow	Fast	Slow	Fast	Slow
DM	1.125 ± 1.844	0.879 ± 0.095	0.815 ± 0.099	0.793 ± 0.095	0.936 ± 0.138	0.781 ± 0.133
LC	0.366 ± 0.056	0.356 ± 0.042	0.370 ± 0.040	0.397 ± 0.068	0.319 ± 0.025	0.465 ± 0.032
NTS	0.355 ± 0.031	0.274 ± 0.020†	0.212 ± 0.026*	0.176 ± 0.021*	0.256 ± 0.028*	0.215 ± 0.019
VMH	0.189 ± 0.031	0.308 ± 0.061	0.231 ± 0.025	0.228 ± 0.034	0.234 ± 0.036	0.207 ± 0.025
NAcb	0.220 ± 0.025	0.259 ± 0.039	0.289 ± 0.044	0.238 ± 0.040	0.280 ± 0.032	0.199 ± 0.024
Olf Tub	0.203 ± 0.027	0.186 ± 0.018	0.175 ± 0.028	0.214 ± 0.036	0.198 ± 0.023	0.270 ± 0.041
AH	0.196 ± 0.017	0.145 ± 0.016†	0.199 ± 0.018	0.211 ± 0.016*	0.228 ± 0.018	0.211 ± 0.017*
Me/Arc	0.110 ± 0.035	0.105 ± 0.029	0.180 ± 0.064	0.068 ± 0.020	0.111 ± 0.035	0.017 ± 0.010
CeA	0.178 ± 0.011	0.160 ± 0.015	0.226 ± 0.040	0.179 ± 0.019	0.190 ± 0.014	0.190 ± 0.025
Cg Cx	0.184 ± 0.010	0.130 ± 0.013†	0.193 ± 0.020	0.130 ± 0.015†	0.195 ± 0.014	0.142 ± 0.021†
M Cx	0.155 ± 0.023	0.132 ± 0.011	0.126 ± 0.019	0.156 ± 0.025	0.175 ± 0.014	0.148 ± 0.013
PVN	0.121 ± 0.011	0.146 ± 0.015	0.139 ± 0.019	0.135 ± 0.012	0.131 ± 0.016	0.152 ± 0.013
LH	0.113 ± 0.020	0.131 ± 0.017	0.123 ± 0.014	0.149 ± 0.024	0.092 ± 0.011	0.110 ± 0.016
S Cx	0.140 ± 0.026	0.140 ± 0.019	0.123 ± 0.019	0.103 ± 0.010	0.129 ± 0.014	0.101 ± 0.011
Cau	0.099 ± 0.017	0.101 ± 0.017	0.111 ± 0.012	0.102 ± 0.009	0.095 ± 0.013	0.102 ± 0.017
Hipp	0.028 ± 0.003	0.033 ± 0.005	0.049 ± 0.011	0.062 ± 0.011	0.063 ± 0.017	0.052 ± 0.012
Pit	0.024 ± 0.002	0.023 ± 0.001	0.023 ± 0.001	0.023 ± 0.001	0.025 ± 0.001	0.022 ± 0.001

Note. Each cell represent the mean ± S.E.M. (pmol/mg protein) under non-stressed control condition and following 15 min of ferret exposure or restraint stress.
* significantly different from non-stressed (within-rat line) control values at $p<0.05$.
† significantly different from Fast (between-rat line) treatment-matched values at $p<0.05$.

Experiment 2. Effects of predator exposure and physical restraint on the time-course of plasma ACTH and corticosterone changes in Fast and Slow rats

The time-course of predator-elicited changes in the release of ACTH and corticosterone in Fast and Slow rats are depicted in Figure 3. The ANOVA revealed a significant Rat line x Time interaction for the release of ACTH, $F_{4,44} = 5.24$; $p<0.01$. Newman-Keuls comparisons of the simple effects comprising this interaction revealed that in Fast rats, ferret exposure elicited a relatively modest, but significant rise of ACTH at the 5 min interval, which persisted to the 60 min post-stress period. The extent of the increase was much greater in Slow rats, even at the 5 min interval, which peaked at 15 min and was still greatly elevated above baseline at the 30 min post-stressor period. Although baseline ACTH levels were comparable for both rat lines, in response to ferret exposure the Slow rats had significantly higher ACTH levels than the Fast rats at the 15 and 30 min intervals.

The analysis of plasma corticosterone levels yielded significant main effects of the Rat line ($F_{1,11} = 6.98$; $p<0.05$), indicating that corticosterone levels were higher in the Slow than in the Fast line. As well, a significant effect was found for the Time after treatment ($F_{4,44} = 13.35$; $p<0.001$), and the multiple comparisons indicated that corticosterone levels were elevated at each of the post-stressor periods relative to baseline values. Although the interaction between the Stressor Treatment and Rat line was not significant, Newman-Keuls multiple comparisons of the simple effects were conducted since a priori predictions had been made concerning this interaction. Newman-Keuls comparisons of the simple effects revealed that at baseline the rat lines displayed

comparable levels of plasma corticosterone. In response to ferret exposure, a rise of plasma corticosterone levels was evident in both rat lines. Among the Slow rats this increase was apparent as early as the 5 min time point. In both lines, the corticosterone levels continued to rise, peaking at the 30 min time point (i.e. 15 min after stressor termination), after which the levels began to decline. The levels of corticosterone among Slow rats at 15 min significantly exceeded that of Fast rats.

Figure 4 illustrates the time-course of restraint-induced release of plasma ACTH and corticosterone in Fast and Slow rats. ANOVA revealed that plasma ACTH levels varied as a function of the Rat line x Time relative to treatment interaction, $F_{4,44} = 4.986$; $p<0.01$. Newman-Keuls comparisons of the simple effects revealed that restraint produced an increase of plasma ACTH in both rat lines, which was significantly elevated above baseline levels at the 5, 15, 30 and 60 min time points for Fast rats and at the 5 and 15 min time points for Slow rats. While baseline plasma ACTH levels were comparable for both rat lines, Fast rats appeared more responsive to the restraint stressor as indicated by significantly higher plasma levels of ACTH at the 5, 15, 30 and 60 min time points. Relative to ferret exposure, restraint induced far greater elevations of ACTH in both rat lines. For instance, while peak ACTH levels in response to the ferret were less than 180 pg/ml (in the more reactive rat line), in response to restraint the peak ACTH levels exceeded 750 pg/ml in the more reactive line and 350 pg/ml in the less reactive line.

The analysis also revealed that restraint-induced release of corticosterone varied as a function of the post-treatment time ($F_{4,44} = 13.5$; $p<0.0001$). In both rat lines,

restraint stress produced an increase of plasma corticosterone, which was significantly elevated from baseline levels at the 5, 15, 30 and 60 min intervals. In contrast to the ACTH levels, the restraint-induced rise of corticosterone was comparable in the two rat lines.

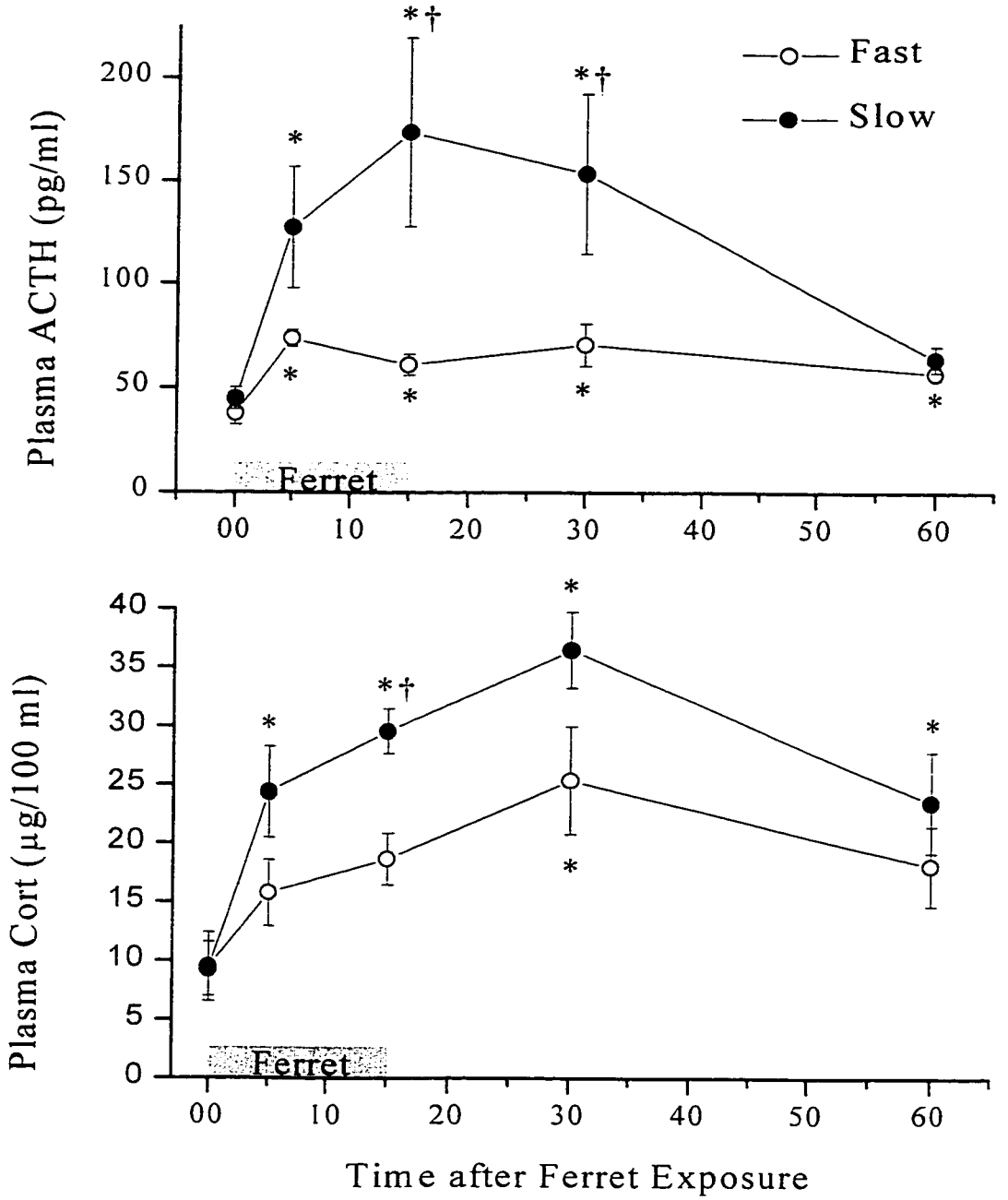

Fig. 3 The *in vivo* release of plasma ACTH and corticosterone (Cort) (mean ± S.E.M.) over 60 min in Fast and Slow rats under basal (non-stressed) conditions and following 15 min of ferret exposure. * significantly different from (within-rat line) baseline values (0 min time point) at $p<0.05$. † significantly different Fast (between-rat line) time-matched values at $p<0.05$.

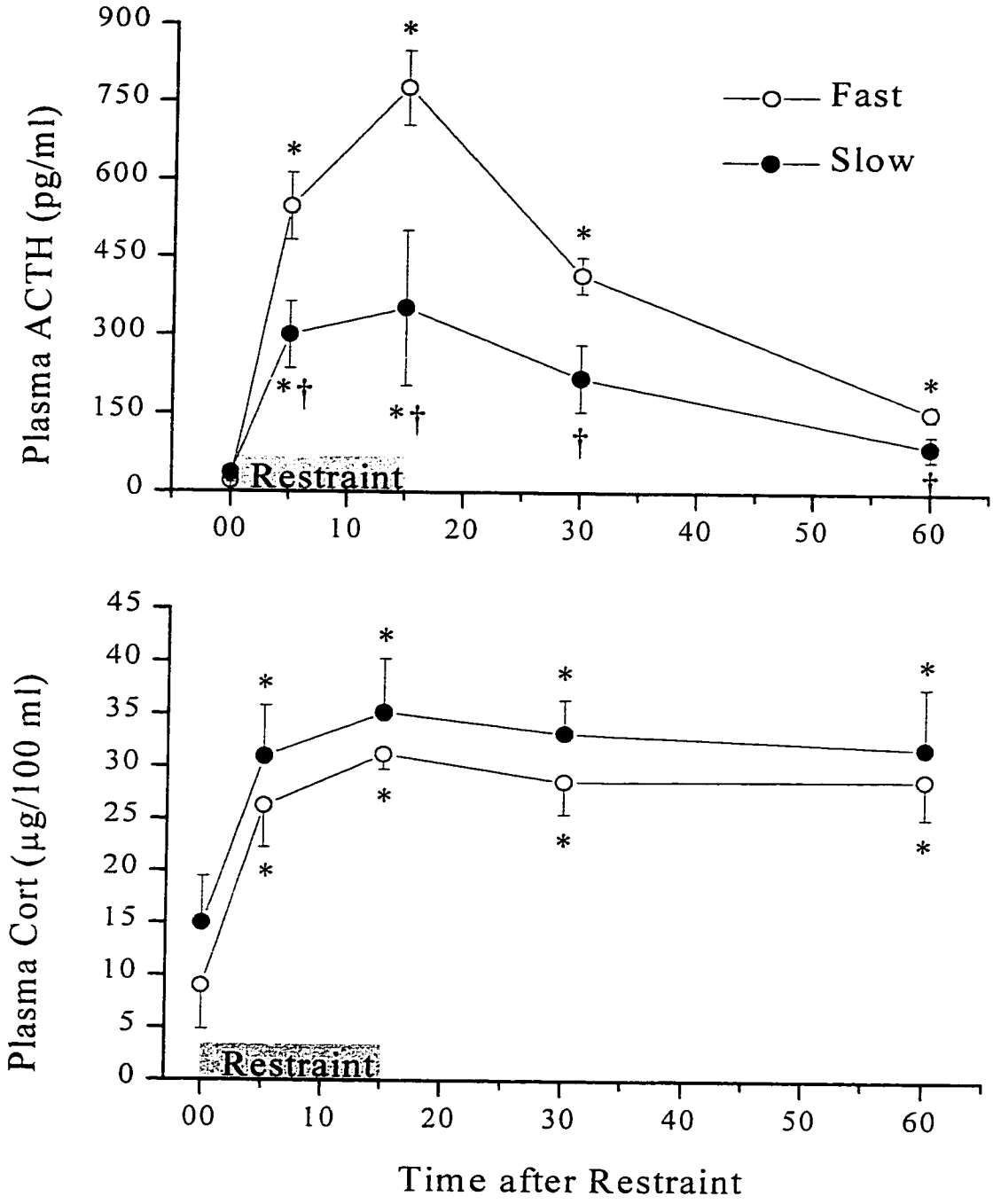

Fig. 4 The *in vivo* release of plasma ACTH and corticosterone (mean ± S.E.M.) over 60 min in Fast and Slow rats under basal (non-stressed) conditions and following 15 min of ferret exposure. * significantly different from (within-rat line) baseline values (0 min time point) at $p<0.05$. † significantly different Fast (between-rat line) time-matched values at $p<0.05$.

Experiment 3. In vivo release of CRH at the CeA following predator exposure and physical restraint in Fast and Slow rats

Figure 5 shows interstitial CRH levels at the CeA as a percent of baseline during baseline conditions and following 15 min of ferret exposure (shown as early and late post-stress time periods). A two factor repeated measures ANOVA was conducted to determine the effects of ferret exposure on CRH release in Fast and Slow rats. The between-groups factor was Rat line (Fast vs Slow) and the within-subjects factor was Treatment condition (Baseline, early and late post-stress time periods) with sample bins (n = 2 samples/bin) nested within each treatment condition. Among animals with correctly positioned probes (Fast n = 10; Slow n = 9), ANOVA revealed a significant effect of sample bin on CRH release at the CeA, $F_{2, 34} = 8.10$; $p<0.001$. Newman Keul's comparisons of the simple effects revealed the predator exposure produced a pronounced but delayed increase in CRH release in the late post-stress time interval in Slow rats that significantly exceeded CRH release measured in the baseline time interval. In contrast, ferret exposure produced only a slight if any (statistically non-significant) increase in CRH release at the CeA in Slow rats. In animals with off-target CeA placements (Fast n=4; Slow n=4), ferret exposure fail to alter CRH levels in both Fast and Slow rats (data not shown).

Figure 6 shows interstitial ir-CRH levels at the CeA as a percent of baseline during baseline conditions and following 15 min of restraint (shown as early and late post-stress time periods). A two factor repeated measures ANOVA was conducted to determine the effects of restraint stress on CRH release in Fast and Slow rats. The between-groups factor was Rat line (Fast vs Slow) and the within-subjects factor was

Treatment condition (Baseline, early and late post-stress time periods) with sample bins (n = 3 samples/bin) nested within each treatment condition. Among animals with correctly positioned probes (Fast n = 8; Slow n = 7), ANOVA revealed a significant effect of sample bin on the *in vivo* CRH release at the CeA, $F_{2, 48} = 8.32$; $p<0.001$. Newman-Keuls comparisons of the simple effects further revealed that the main effect was attributable to a pronounced, but delayed, increase in CRH release in the Fast rat line such that the interstitial levels of CRH as measured in the late post-stress time interval were significantly elevated above levels measured in both the baseline and the early post-stress time intervals. In Slow rats, restraint was associated with only a slight if any (statistically non-significant) increase in CRH release. In animals with off-target CeA placements (Fast n=6; Slow n=7), restraint fail to alter CRH levels in both Fast and Slow rats (data not shown).

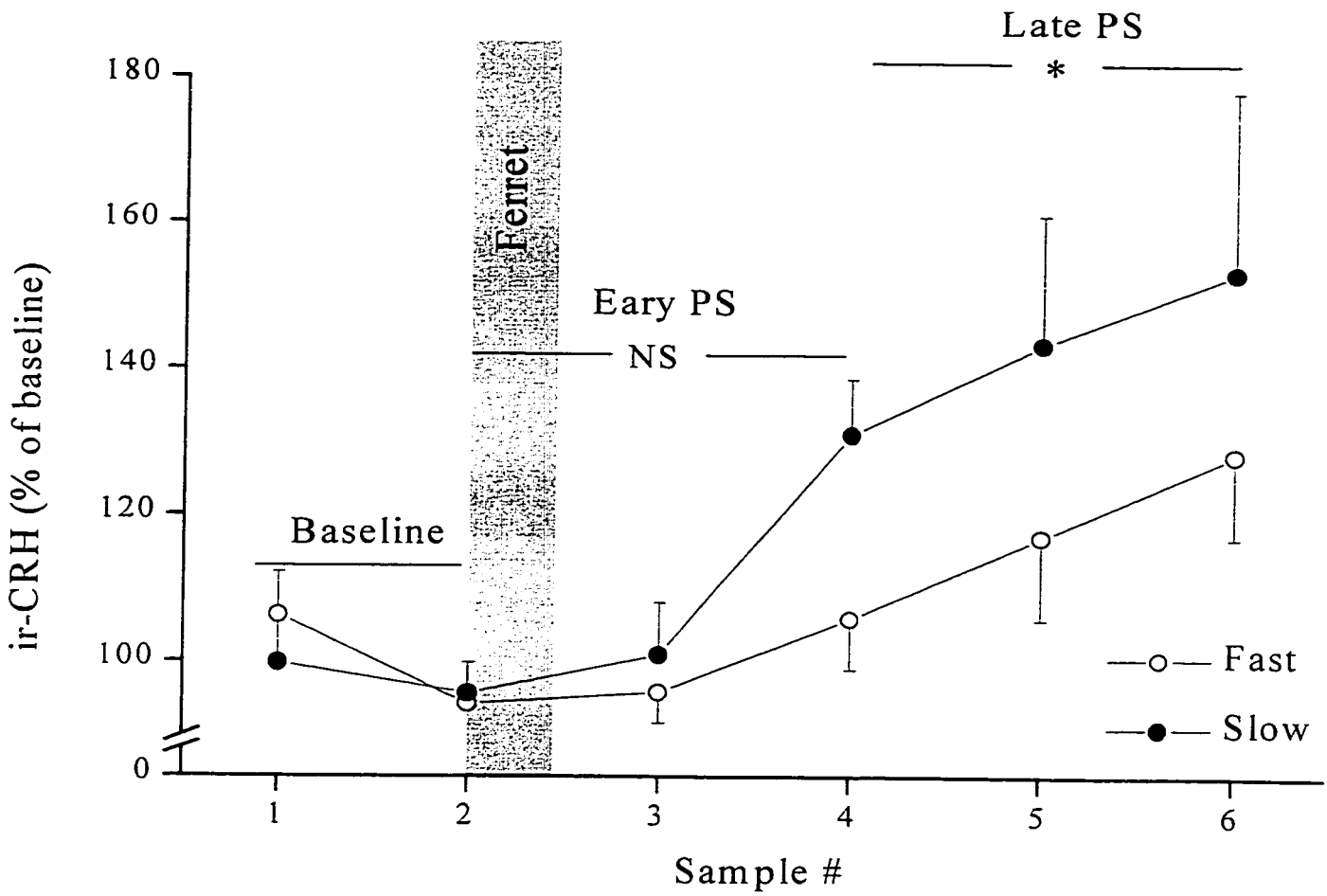

Fig. 5 Levels of ir-CRH at the central amygdaloid nucleus as measured by *in vivo* microdialysis under baseline condition and following 15 min of ferret exposure in Fast and Slow rats. "Early PS" represents the early post-stress time interval and "Late PS" represents the late post-stress time interval. The baseline samples from each subject were averaged and defined as 100%. All values were then expressed as a percent of that baseline. * significantly different from (within-rat line) baseline condition at $p<0.05$.

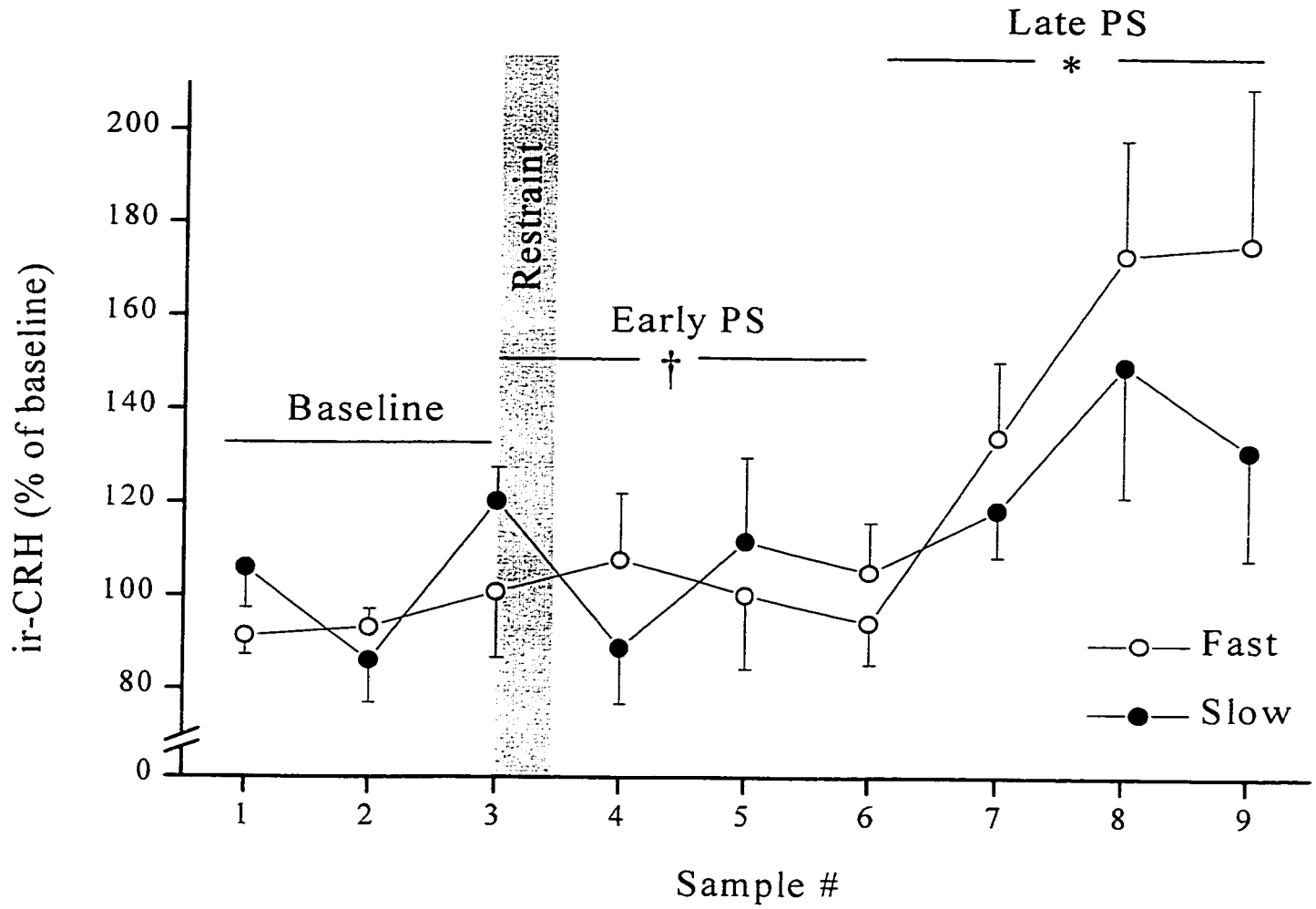

Fig. 6 Levels of ir-CRH at the central amygdaloid nucleus as measured by *in vivo* microdialysis under baseline condition and following 15 min of restraint in Fast and Slow rats. "Early PS" represents the early post-stress time and "Late PS" represents the late post-stress time interval. The baseline samples from each subject were averaged and defined as 100%. All values were then expressed as a percent of that baseline.
* significantly different from (within-rat line) baseline condition at $p<0.05$.
† significantly different from (within-rat line) Late PS time interval at $p<0.05$.

Discussion

The major aim of the present investigation was to further characterize endocrine and neurochemical differences between the Fast and Slow rats in response to a purely psychogenic stressor (predator exposure) or to a stressor with mixed neurogenic and psychogenic properties (restraint). It will be recalled that although the rat lines were originally selected on the basis of amygdala excitability, culminating in seizure development in response to electrical stimulation (kindling), the rat lines also display marked behavioral differences. In particular, relative to the Fast line, the Slow line of rats was previously shown to display behavioral responses associated with high levels of anxiety.[292] As described earlier, in response to a ferret exposure, the Fast rats froze (became immobile) far more than did the Slow rats; however, upon being restrained the Fast rats display vigorous and protracted struggling, whereas the Slow rats tended to adopt an immobile response style. Thus, one would expect to find that the two stressors would differentially influence neuroendocrine or neuropeptide functioning.[13,272]

Paralleling the behavioral effects of the two stressors, the neuroendocrine profile in response to restraint in the rat lines was distinguishable from that associated with predator exposure. Consistent with our previous findings[13,272], ferret exposure elicited a more pronounced increase of plasma ACTH concentrations in the Slow than in the Fast rat line. This effect was evident both when blood was collected immediately after stressor exposure (at a single time point) as well as when blood samples were collected *in vivo* (at several time points) through indwelling cannulae within the pulmonary artery. Similarly, ferret exposure elicited a more pronounced rise of plasma corticosterone levels

in the Slow rats than in the Fast rats, such that the corticosterone levels rose more quickly and were more pronounced in the Slow rats as compared to the Fast rats.

Unlike the effects of ferret exposure, restraint elicited a more pronounced rise in plasma ACTH levels among Fast rats as compared to the Slow rats (both in blood taken 15 min post-mortem and during serially collected blood at several time intervals). However, in contrast to the between-rat line difference in ACTH response, the restraint-elicited corticosterone rise was comparable in the two rat lines. Clearly, the changes of ACTH are not necessarily proportional to the variations of corticosterone. Such discrepancies between ACTH and corticosterone levels have been noted by other investigators[156,406], and may be attributable to altered adrenal sensitivity and/or the potential influence of other secretagogues on adrenal responses.[8,147,163,330,389] Globally, it appears that whereas the Slow rat is generally more stressor-reactive across a range of behavioral tests, the Fast rat appears differentially sensitive to the nature of the stressor.

As previously observed, stressor exposure elicited marked brain region-specific variations of both ir-CRH and ir-BN. In the main, such effects were elicited by both the pure psychogenic stressor (predator exposure) and by restraint. However, to a considerable degree these effects were dependent upon the rat line being examined. Specifically, while the stressors provoked elevations of ir-CRH at the key elements of the HPA axis (PVN, Me/Arc and to a moderate extent within the Pit) among the more emotional Slow rats, among the Fast rats ir-CRH levels were moderately reduced at these sites. The rat line-specific alterations in ir-CRH in the elements of the HPA axis are

consistent with the behavioral and endocrine findings suggesting that the Fast and Slow rat lines may show differential sensitivities to stressor exposure. The elevated levels of ir-CRH at the PVN, Me/Arc and Pit in Fast rats could be interpreted as either increased peptide synthesis and release, or alternatively decreased peptide release, whereas the decreased levels of ir-CRH in Slow rats could be attributed to decreased synthesis or increased peptide release. Given that the release of CRH from the median eminence in response to stressor exposure is a well documented event[22,181,417-419], it is likely that the alterations in ir-CRH content (at least at the Me/Arc) in both Fast and Slow rats reflects enhanced peptide release, accompanied by enhanced peptide synthesis in Fast rats.

In contrast, at the CeA, restraint stress increased ir-CRH levels among Fast rats, but had no effect in the Slow rat line. There is considerable evidence for the involvement of the CeA in HPA activation.[27,63,109,119,145,405] Moreover, the CeA appears to be a distinguishing nucleus between Fast and Slow rats in terms of kindling susceptibility.[106] It was therefore somewhat surprising that alterations in ir-CRH content at the CeA were only observed in the Fast rats following exposure to restraint. As indicated earlier, analyses of neurotransmitters in post-mortem tissue collected at a single time point may not provide an adequate reflection of the dynamic changes that may be occurring, particularly in response to stressors. This is certainly the case with respect to CRH variations where synthesis of the peptide occurs readily, and hence changes of level may not reflect ongoing changes of activity. To be sure, post-mortem analyses are useful in that it permits the determination of proteins in a wide sampling of brain regions; however, the absence of an effect does not necessarily imply that variations of the

neurotransmitter are not occurring as a result of the treatment. Indeed, CRH variations within the CeA were detected in response to stressors using *in vivo* microdialysis. These effects, like the ACTH alterations, were dependent on the line of rat and on the specific stressor to which the animals had been exposed. Specifically, in response to restraint stress the Fast rats showed a more pronounced release of CRH at the CeA as compared to Slow rats, whereas ferret exposure elicited a greater release of CRH in the Slow rats as compared to the Fast rats. The significant restraint-elicited release of CRH in Fast rats is interesting in that the post-restraint intracellular concentration of CRH in the CeA were greater in the Fast rats than the Slow rats (who did not demonstrate a significant release of CRH in response to restraint stress). This supports the idea that restraint stress may result in an enhanced expression of CRH mRNA in the Fast rat line more so than in the Slow rat line. Moreover, it is noteworthy that the most pronounced stressor-induced endocrine changes were observed in the Fast rats following exposure to restraint leading to the possibility that the perceived intensity of the stressor may be an important factor mediating activation of this particular nucleus. In contrast, ferret exposure evoked an increase in the release of CRH in only the Slow rats, in the face of unaltered intracellular content, suggesting that the expression of CRH mRNA may be up-regulated to compensate for the enhanced release in this rat line. Interestingly, for both Fast and Slow rats, the stressor-elicited release of CRH at the CeA was somewhat delayed (observable by the 3rd or 4th sample post-stressor exposure). Although these findings are not concordant with our past results showing a more pronounced and rapid restraint-elicited increase in CRH release at the CeA (observable by the 2nd sample post-stressor exposure)[278], there are several possible explanations for this discrepancy. For example, it

is possible that the Fast and Slow rats lines employed in the present experiment differ in levels of ir-CRH and/or mRNA expression, or in the rate of synthesis, turnover and/or release of this peptide as compared to other rat strains (including Sprague Dawley rats which were employed in the previous experiment). Indeed, inter-strain differences in many of these parameters have previously been observed by other investigators.[151,227] Inter-strain differences as well as regional differences may also be found in the levels of CRH binding protein within the brain. Since CRH binding protein binds this peptide with high affinity, and the protein-bound CRH may not cross the membrane of the dialysate probe, the portion of the released peptide sequestered by this binding protein may be masked. Indeed, high levels of CRH binding protein are present in limbic structures such as the CeA[237], and could affect the dynamics of CRH release and its availability. At present, these explanations are speculative and efforts will be needed to elucidate the genomic changes that occur in these rat lines when they are exposed to either ferret or restraint.

In terms of alterations in ir-BN, stressor treatments provoked elevations in ir-BN at the AH in Slow rats, whereas a decrease in ir-BN at the NTS in both rat lines. There is considerable evidence suggesting that certain brain stem nuclei, in particular the NTS, play a pivotal role in HPA activation.[77,131,233,241,373] The NTS serves as a primary relay station for information traveling via the glossopharyngeal and vagus nerves.[347] Moreover, this nucleus gives rise to noradrenergic projections that extend to the parvocellular division of the PVN as well as the other brain stem nuclei, which, in turn innervate the PVN.[80] BN-like peptides are thought to mediate sympathetic activation via

the NTS as central administration of BN into this nucleus elevates the circulating levels of epinephrine and norepinephrine.[64] Moreover, ir-BN fibers extend from the parvocellular PVN to the NTS/dorsal vagal complex leading to the possibility the BN-like peptides released from the NTS may modulate HPA activity (either directly or through CRH neurons).[72,249] In terms of peptidergic changes at the AH, it is interesting that alterations in levels of BN-like peptide following exposure to ferret and restraint were only observed in Slow rats. Consistent with the behavioral evidence suggestive of greater fear and anxiety responses in the Slow rat line, the AH appears to be an important region involved in mediating defensive behavioral responses such as 'freezing' behavior (tonic immobility) to stressor exposure.[86]

Aside from site-specific alterations in ir-BN and ir-CRH following stressor exposure, these two rat lines also showed brain-region specific basal differences in peptidergic concentrations. In particular, Slow rats had higher basal levels of ir-CRH at the Hipp and LH and lower basal levels of ir-BN at the Cg Cx, AH and NTS as compared to Fast rats. It is of interest to note that all of these regions have been implicated in HPA activation, leading to the possibility that pre-existing differences in the basal concentrations of stress relevant peptides such as CRH and/or BN-like peptides at these sites may contribute to the endocrine and behavioral differences observed between Fast and Slow rats in response to stressor exposure.[77,102,319,426]

In summary, it is well established that the physiological changes evoked by stressor exposure are influenced by a multitude of variables including the severity,

duration, quality (physical or psychological) of the stressor as well as individual (or organismic) factors (e.g. species, genetics, age or gender).[185,381] Given that a response to stress will vary depending on the complex interplay of these various factors underscores the need for more elaborate experiments that take into consideration the interactions between at least some of these different variables. In the present investigations we have shown that in addition to previously reported behavioral, endocrine, and immunological differences[13,292], the Fast and Slow rat lines can also be distinguished from each other in terms of their neurochemical changes (alterations in ir-CRH and ir-BN) in response to two different psychogenic stressors; restraint and ferret exposure. Taken together, these findings suggest that the Fast and Slow rat lines may provide a valuable tool in future stress-related studies to assess the mechanisms underlying the individual differences in endocrine, neurochemical and/or behavioral responses to stressors.

Preface to Chapter III

In the previous post-mortem studies we demonstrated that stressor exposure was associated with site-specific alterations in endogenous levels of ir-BN. One of the caveats of analyzing neurotransmitters/neuropeptides changes in post-mortem tissue is that the ensuing results may not necessarily provide an accurate reflection of the *dynamic* changes in peptide utilization, particularly in response to stressors. For instance, elevated tissue levels of a peptide may be related to a) increased synthesis, b) decreased release, or c) increased synthesis as well as increased release. Similarily, the lack of change in tissue levels may indicate a) no change in utilization of this peptide, b) increased release matched by increased peptide synthesis, or c) decreased release accompanied by decreased synthesis of this peptide. Thus, in the next experiment we assessed (using push-pull perfusion) the dynamics of the *in vivo* interstitial levels of BN-like peptides (as well as other stress relevant peptides, namely CRH and AVP) at the anterior pituitary following exposure to an acute stressor (air-puff).

Several groups, including Tilders and his collegues, have demonstrated protracted changes (including pheotypic changes) in peptide expression following acute and/or chronic stressor exposure. There is no evidence about the potential role of BN-related peptides in this phenomenon. Thus, we also examined whether the subject's prior stress-history (previous exposure to acute or chronic restraint) influenced the subsequent stressor-induced responses of the BN-like peptides, CRH and AVP systems.

CHAPTER III

Impact of differential stressor-exposure history on the release of CRH, AVP and BN-related peptides at the anterior pituitary

Abstract

Corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) play a critical role in the mediation of ACTH secretion from the anterior pituitary. However, the expression of these ACTH secretagogues and their subsequent release may be dictated by the history of stressor exposure. Indeed, exposure to repeated stressors or to an acute stressor episode, over time leads to a long-lasting increase of AVP stores in CRH containing terminals within the external zone of the median eminence (ZEME). Thus, over time, the role of AVP appears to become more dominant in promoting ACTH secretion. Like AVP, bombesin (BN)-like peptides are co-localized with CRH in the ZEME. Moreover, increasing evidence suggests that the BN family of peptides (including its mammalian analogs gastrin-releasing peptide (GRP) and neuromedin B (NMB)) play a role in the mediation and/or modulation of the stress response. In the current study, using push-pull perfusion, we monitored the *in vivo* interstitial levels of CRH, AVP, GRP and NMB at the anterior pituitary in response to an acute stressor (air-puff) exposure. We also examined whether the subject's prior stress-history (previous exposure to acute or chronic restraint) influenced the stressor-induced release of GRP, NMB, CRH and AVP from the anterior pituitary. Results showed that basal interstitial levels of CRH, AVP, GRP and NMB at the anterior pituitary were significantly elevated in animals previously exposed to either repeated episodes (14 sessions) or a single episode of restraint stress followed by the passage of time (14 days) as compared to non-stressed controls. However, basal corticosterone levels remained comparable for all three groups. In response to an acute novel stressor (air-puffs), the naïve controls showed a more rapid and in general, a more pronounced enhancement of peptide availability at the

anterior pituitary as compared to rats previously subjected to restraint stress (either acute or chronic). Taken together, these findings suggest that the role of the BN family of peptides in HPA activation is indeed influenced by the prior stress history of an organism. Like in the case of AVP, GRP and NMB may take on a more dominant role in the stress response when the system has been primed by chronic stressor exposure or a single stressor exposure followed by adequate passage of time.

Introduction

One of the key regulators of the hypothalamic-pituitary adrenal axis is corticotropin-releasing hormone (CRH).[107,317] During stressor exposure, CRH, synthesized in the parvocellular neurons of the paraventricular hypothalamic nucleus (PVN), is secreted into the hypophysial portal blood from axon terminals in the external zone of the median eminence (ZEME).[160,185,286] Subsequently, CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) from the corticotroph cells in the anterior pituitary, which in turn stimulates the secretion of glucocorticoids from the adrenal cortex.[160,185,286] In addition to CRH, other peptides including arginine-vasopressin (AVP) can also influence ACTH secretion. AVP, produced in a subset of parvocellular CRH neurons in the PVN[422,423], acts as a weak ACTH secretagogue on its own, but shows strong synergistic actions in combination with CRH.[15,136,351,404]

It has repeatedly been shown that exposure to an acute stressor causes a predictable rapid and transient increase in pituitary ACTH and adrenal glucocorticoid secretion.[83,84,286] The endocrine response to chronic stress is more complex and less well characterized.[286] It appears that several factors including; the nature, intensity, duration and frequency of the stressful event will determine whether the HPA axis shows habituation (blunted ACTH secretion), sensitization (enhanced ACTH secretion) or whether both of these processes are affected in an interactive manner.[146,331] It is believed that the mechanisms mediating these different types of adaptive responses involve changes at the hypothalamic level.[250] In this context, activation of the HPA axis during an acute stressor exposure is associated marked up-regulation of pavocellular CRH

mRNA at the PVN as well as a less marked increase in AVP mRNA levels.[24,152,178,210,251] Although chronic stressor exposure also affects HPA responsiveness as reflected by changes in hypothalamic CRH mRNA expression, such changes appear to be dependent upon the nature of stressors deployed. Depending on the stimulus employed, levels of CRH mRNA in the PVN have been shown to increase, decrease or remain unchanged.[3,159,244,254] In contrast to the unpredictable changes in CRH mRNA, chronic stressor exposure consistently increased parvocellular AVP mRNA at the PVN as well as its expression (increased immunoreactive AVP content) at CRH containing terminals within the ZEME.[25,85,93,94,250] Although only limited data is available, it has been observed that exposure to certain chronic stressors such as intermittent insulin injection, are not only associated with increased stores of AVP in CRH containing terminals but also with enhanced secretion of AVP from this region following exposure to an acute stressor.[92] Based on these observations, it has been suggested that whereas AVP plays a relatively minor role in the mediation of the acute stress response, following chronic stressor exposure, AVP becomes a more dominant signal driving ACTH secretion. Furthermore, increased AVP stores in CRH containing terminals have been observed 2 weeks after exposure to certain acute stressors as well[366,367,406], suggesting that the passage of time since the initial stressor encounter (independent from the number of stressor repetitions) may also be an important variable influencing the relative role of AVP in ACTH secretion.

In addition to AVP, several other neuromodulators are co-produced in parvocellular CRH neurons in the PVN and some are even co-localized within CRH in

nerve terminals at the ZEME.[396] However, with the exception of AVP, little is known about the physiological significance of the other substances co-localized with CRH. Gastrin-releasing peptide (GRP), a mammalian analogue of bombesin (BN), is one such peptide co-localized with CRH within the external zone of the ovine ME.[138] It is possible that like AVP, BN-like peptides may interact with CRH to influence HPA activation. Indeed, findings from our laboratory and others have shown that central administration of BN or GRP dose-dependently increase plasma levels of ACTH and corticosterone and this effect is attenuated or blocked not only by pretreatment with GRP receptor antagonists but also by the CRH receptor antagonist, αh-CRF.[133,282,313] Moreover, like AVP, central administration of GRP *in vivo* and application of GRP or BN in mixed pituitary cells *in vitro* potentiates CRH-induced ACTH release.[19,313] These finding suggest that the ability of the BN/GRP family of peptides to activate the HPA axis is mediated, at least in part, via release of CRH.

In addition to GRP, neuromedin B (NMB) (another mammalian analog of BN) also appears to be involved in HPA activation. Although, at present, there is no data on the effects of central NMB administration on adrenocortical functioning, subcutaneous administration of NMB significantly elevated plasma ACTH and corticosterone levels in rats.[257] Moreover, NMB (like GRP) is present in all major components of the HPA axis including the hypothalamus, pituitary and adrenal gland.[104,187,288,296] Perhaps more interestingly, very high levels of NMB mRNA are found in the anterior pituitary suggesting that this peptide may serve an endocrine or paracrine function.[169] In context

of the potential paracrine function, it is likely that NMB may act directly and/or in conjunction with CRH and/or AVP.

Given the similarities between AVP and BN-like peptides in terms of HPA activation as well as the functional and anatomical relationship between these peptides and CRH, the BN-related peptides may modulate the endocrine response(s) to stressors. In the current study we assessed the effects of chronic stressor exposure as well as the protracted effects of an acute stressor episode on the interstitial levels of CRH, AVP, GRP and NMB at the anterior pituitary. Specifically, we exposed animals to either no stress (control), acute or chronic restraint stress and assessed the *in vivo* peptide release (using *in vivo* push-pull perfusion) to determine how the prior stress history of these animals affected the availability of CRH, AVP, GRP and NMB at the anterior pituitary under basal conditions and following exposure to another acute stressor challenge.

Materials and Methods

Animals

Individually housed male Sprague-Dawley rats (weighing between 325 and 400 g), obtained from Charles River (St. Constant, Quebec) were used in this experiment. Animals were maintained on a 12-h light/dark cycle (with lights on at 6:00 a.m.), in a temperature (23°C) and humidity (60%) controlled room and had free access to food and water.

Experimental Protocol

Rats were randomly divided into three groups and exposed to 1) no stress (control), 2) chronic restraint stress (20 min daily for 14 days) or 3) acute restraint stress (a single 20-min session followed by 14 days of no stressor exposure). Following the chronic restraint group's last stressor exposure, rats from all three groups were anesthetized (60 mg/kg pentobarbital; i.p.) and placed in a stereotaxic apparatus to implant 20 gauge guide cannulae (plugged with removable stainless steel stylets) at the anterior pituitary. The placement coordinates for the anterior pituitary were 5.0 mm posterior to bregma, 0.9 mm lateral to the midline and 0.5 mm dorsal to the sphenoid bone. The guide cannulae for push-pull perfusion (custom made Derelin™ pedestals) was anchored to the skull surface using 4 stainless steel screws and acrylic dental cement. After a 5 day recovery period, animals were transferred to individual test cages 48 h prior to the experiment, to acclimate subjects to the test environment.

Pull-Pull Perfusion

On the test day, the stylet within the guide cannula was replaced with a push-pull probe aimed at the anterior pituitary. The push-pull probe consisted of an outer (or pull) stainless steel cannula (protruding 0.4 mm beyond the end of the permanent guide cannula) and an inner (or push) cannula (glass silica) protruding 0.2 mm beyond the end of the pull cannula and into the anterior pituitary. Probes were connected by polyethylene tubing (PE-20 and PE-10) to two independent, pre-equilibrated peristaltic pumps (Minipuls 3, Gilson, Middleton, WI), via a dual channel swivel assembly (Instech Laboratories, Horsham, PA). Kreb's ringer phosphate (KRB) solution consisting of (in

mM): 145 Na^+, 2.7 K^+, 1.35 Ca^+, 1.0 Mg^{2+}, Cl^- and 0.1% BSA, was perfused through the push cannula at the rate of 15 µl/min. Samples were collected every 10 min between 9:00 and 15:00 h. Three baseline samples were collected followed by exposure to air-puff stress (5 min; 1 puff/min) and the subsequent collection of 6 more samples. Each sample (150 µl) was immediately frozen on dry ice and stored at –80°C until radioimmunoassay (RIA) analysis.

Histology

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

Radioimmunoassay (RIA)

A solid-phase RIA procedure was employed for the detection and quantification of CRH, AVP, BN and NMB.[253,278] Corticosterone was also measured in the perfusates using a micro-adaptation[39] of the commercial RIA kit (ICN pharmaceuticals, Canada). Briefly (for CRH, AVP, BN and NMB detection), protein A/G (Calbiochem Corp; La Jolla) coated Immunilon-4 well plates (Dynatec Laboratories Inc., Chantilly, VA) were coated with CRH, AVP, BN or NMB antibody (1:100 000 final dilution) for 2 h at room temperature. The plates were then rinsed 3 times with wash buffer. The standards (reconstituted in KRB solution; ranging from 0.05 to 250 fmol for CRH; 0.0025 to 125

fmol for AVP; 0.25 to 512 fmol for GRP and 3.37 to 690.5 fmol for NMB), blanks and samples were then pipetted into the designated wells incubated for 24 h at 0-4°C. Next, 25 μl of KRB solution containing 5000-6000 CPM of [^{125}I-Tyr^{0}]rCRF, [^{125}I-Tyr^{0}]AVP (Amersham, Canada), [^{125}I –Tyr^{0}] NMB (NEN Life Science Products, Boston, MA) or [^{125}I-Tyr^{4}]BN (iodinated in our laboratory according to Salacinski et al.[359]), was added to each well and incubated for 24 h at 0-4°C. Plates were then rinsed and the individual wells counted for residual bound radioactivity using a gamma-counter (Canberra Packard, Cobra II Autogamma, model D5002).

The specific anti-CRH serum (rC70, kindly provided by W. Vale, Salk Institute, LaJolla, CA) recognizes CRF_{1-41} and cross-reacts poorly with other related peptides including urotesin 1 and urocortin.[403] The anti-AVP serum (Pheonix Pharmaceuticals, U.S.A) recognizes [Arg^{8}]-Vasopressin and cross-reacts 100% with [Arg^{8}]-vasotocin, 38% with [Lys^{8}]-vasopressin, and 0% with other peptides. The BN antibody (α-BN_2 kindly provided by T. Moody, NCI, Rockville, MD) recognizes the C-terminal of BN and cross reacts strongly with GRP_{1-27} (110%), BN (100%) and GRP_{18-27} (82%), but only weakly with NMB-10, NMB-32 or substance P (<0.1%). The NMB antibody (α-NMB, kindly provided by T. Moody, NCI, Rockville) recognizes the C-terminal region of NMB (NMB-10) with which it cross-reacts 100%. It also cross-reacts with NMB-30 (104%) and NMB-32 (105), but shows negligible cross reactivity with GRP, BN, or substance P.

Statistical Analysis

All results are expressed as means ± S.E.M. Basal differences in GRP, NMB, CRH, AVP and corticosterone concentrations were assessed by averaging the actual peptides levels (pmol/10 μl) for the three baseline samples (individually for each peptide/hormone) and then performing one factor ANOVAs followed by Newman-Keuls multiple comparisons. The independent factor was the Treatment condition (no stress (control), acute restraint and chronic restraint), whereas the dependent factor was the peptide concentration. To determine the effects of the subsequent acute stressor challenge (air-puffs) on peptide and corticosterone levels, basal GRP, NMB, CRH, AVP and corticosterone values for each subject were averaged individually, over the 3 baseline samples and defined as 100%. Values were then expressed as a percentage of that baseline. GRP, NMB, CRH, AVP and corticosterone values were analyzed independently using two factor repeated measures ANOVAs. The between-groups factor was Treatment condition (no stress (control), acute restraint and chronic restraint) and the within-subjects factor was the Time bins (beginning with the final baseline value until the 6^{th} post-stress sample). Post hoc comparisons were conducted using Newman-Keuls multiple comparisons. A value of $p<0.05$ was considered statistically significant.

Results

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

Interstitial levels of GRP, NMB, CRH, AVP and corticosterone (expressed as pmol/10 μl) for the non-stressed control group and rats exposed to 14 sessions of restraint

(chronic) or 1 session of restraint (acute) followed by the passage of time (14 days), under basal condition and following exposure to a subsequent acute stressor (air-puffs) are depicted in Figures 1*a*, 2*a*, 3*a*, 4*a* and 5*a*. Individual one factor (Treatment condition) ANOVAs were conducted to evaluate the effects of prior exposure to chronic or acute restraint on basal levels of GRP, NMB, CRH, AVP and corticosterone at the anterior pituitary. The 3 baseline values from each treatment condition (control, acute and chronic restraint) were averaged and the ANOVAs were conducted using these means. ANOVAs revealed significant effects of Treatment condition on interstitial levels of GRP ($F_{2,24} = 4.106$; $p<0.05$), NMB ($F_{2,28} = 10.147$; $p<0.001$), CRH ($F_{2,25} = 70.269$; $p<0.0001$) and AVP ($F_{2,29} = 9.05$; $p<0.001$). Newman-Keuls multiple comparisons of the simple effects indicated that relative to naïve controls, basal levels of GRP, NMB, CRH and AVP at the anterior pituitary were markedly elevated in rats previously exposed to restraint stress. Although both stressor conditions (acute and chronic restraint) provoked comparable increases in basal GRP levels relative to naïve controls, the difference only reached statistical significance for the acute restraint condition. For NMB, AVP and CRH, rats previously exposed to either chronic or acute restraint had significantly higher basal levels at the anterior pituitary relative to naïve controls. For NMB, both prior stress conditions provoked comparable increases in interstitial peptide levels at the anterior pituitary. For AVP, although the increase in basal peptide levels was substantially higher in rats previously exposed to chronic restraint (as compared to one session of acute restraint), the difference between the two groups was not statistically significant ($P<0.052$). For CRH, increased basal levels were significantly higher in rats previously exposed to one session of restraint stress as compared to those exposed to the chronic

restraint condition. In contrast to the marked impact of prior stress history on basal levels of GRP, NMB, CRH and AVP at the anterior pituitary, little if any effect on basal corticosterone levels was evident.

Effects of an acute stress challenge on interstitial levels of GRP, NMB, CRH, AVP and corticosterone at the anterior pituitary

Interstitial levels of GRP, NMB, CRH, AVP and corticosterone (expressed as a percentage of the baseline) for the previously non-stressed control rats and those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days), under basal conditions and following exposure to an acute stressor (air-puffs) are depicted in Figures 1*b*, 2*b*, 3*b*, 4*b* and 5*b*. To determine the effects of a novel acute stressor (air-puffs) on interstitial levels of CRH, AVP, NMB, GRP and corticosterone in animals with differential stressor-exposure histories, the data for each peptide or hormone were analyzed individually using a two factor repeated measures ANOVA. The between-groups factor was Treatment condition (no stress, acute and chronic restraint) and the within-subjects factor was Sample (or time bin) (starting with the final baseline sample until the 6th post-stress sample). The ANOVAs indicated that interstitial concentrations of GRP and CRH varied as a function of the interaction between Treatment condition x Sample, $F_{12,156} = 2.043$; $p<0.05$ and $F_{12,150} = 3.17$; $p<0.001$, respectively. Newman-Keuls multiple comparisons of the simple main effects revealed that in naïve rats, exposure to air-puff stress elicited a more rapid and pronounced increase in the interstitial levels of GRP and CRH relative to the increase observed in rats previously exposed to either chronic or acute restraint stress. In the

control rats, interstitial levels of CRH and GRP were significantly elevated above baseline by the 2nd and 3rd post-stress samples, respectively. A slight delay in the post stress-induced increase in the availability of CRH and GRP was observed in rats previously exposed to chronic restraint stress as levels of CRH and GRP were not significantly elevated above baseline until the 3rd and 4th post-stress samples, respectively. An even longer delay in the post stress-induced increase in GRP and CRH was observed in the rats that had previously been exposed to one session of restraint as levels of GRP and CRH were not significantly elevated above baseline until the 6th post-stress sample. Newman-Keuls multiple comparisons further revealed that at the 2nd and 3rd post-stress samples, interstitial levels of GRP were significantly lower in rats previously exposed to acute restraint as compared to rats previously exposed to either no stress (controls) or chronic restraint stress. Similarily, interstitial levels of CRH were significantly lower in rats previously exposed the either acute (at the 1st, 2nd and 3rd post-stress samples) or chronic restraint (at the 1st and 2nd post-stress samples) as compared to naïve rats.

ANOVA indicated significant Sample effects for interstitial concentrations of NMB ($F_{6,162} = 5.092$; $p<0.001$), AVP ($F_{6,162} = 4.907$; $p<0.0001$) and corticosterone ($F_{6,162} = 12.377$; $p<0.001$). Newman-Keuls comparisons of the simple effects revealed that in response to the acute stressor challenge, interstitial levels of NMB and AVP were significantly elevated in naïve rats and in rats previously exposed to chronic restraint stress, but not in rats previously exposed to only a single episode of restraint. Similar to the findings with GRP and CRH, exposure to air-puffs elicited a more rapid increase in

the availability of NMB and AVP (relative to baseline levels) at the anterior pituitary in naïve controls as compared to the increase observed in rats previously exposed to chronic restraint stress. In the naïve rats, levels of NMB and AVP peptides were significantly elevated above baseline by the 1st and 2nd post-stress samples, respectively, whereas in rats previously exposed to chronic restraint, interstitial levels of NMB and AVP were significantly elevated above baseline by the 3rd and 4th post-stress samples, respectively. Newman-Keuls multiple comparisons of the simple effects further revealed that in response to the acute stressor challenge (air-puffs), the availability of corticosterone was significantly increased (at the 3rd post-stress sample) in naïve rats and in rats previously exposed to either chronic or acute restraint stress.

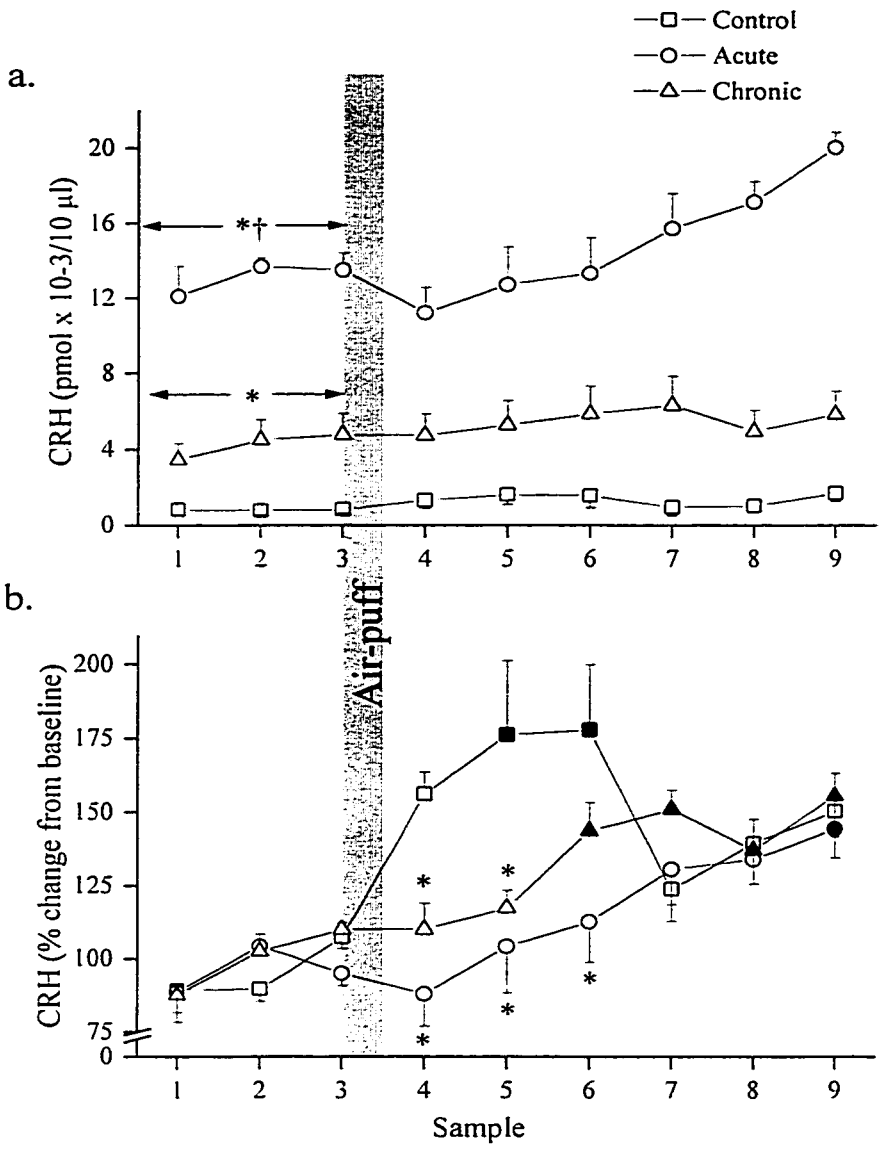

Fig. 1*a* Interstitial levels of CRH (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). * significantly different from baseline values of control condition at $p<0.05$. † significantly different from baseline values of chronic condition at $p<0.05$.

Fig. 1*b* Interstitial levels of CRH (expressed as a percentage of the baseline) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions. * significantly different from (sample-matched) non-stressed control condition at $p<0.05$.

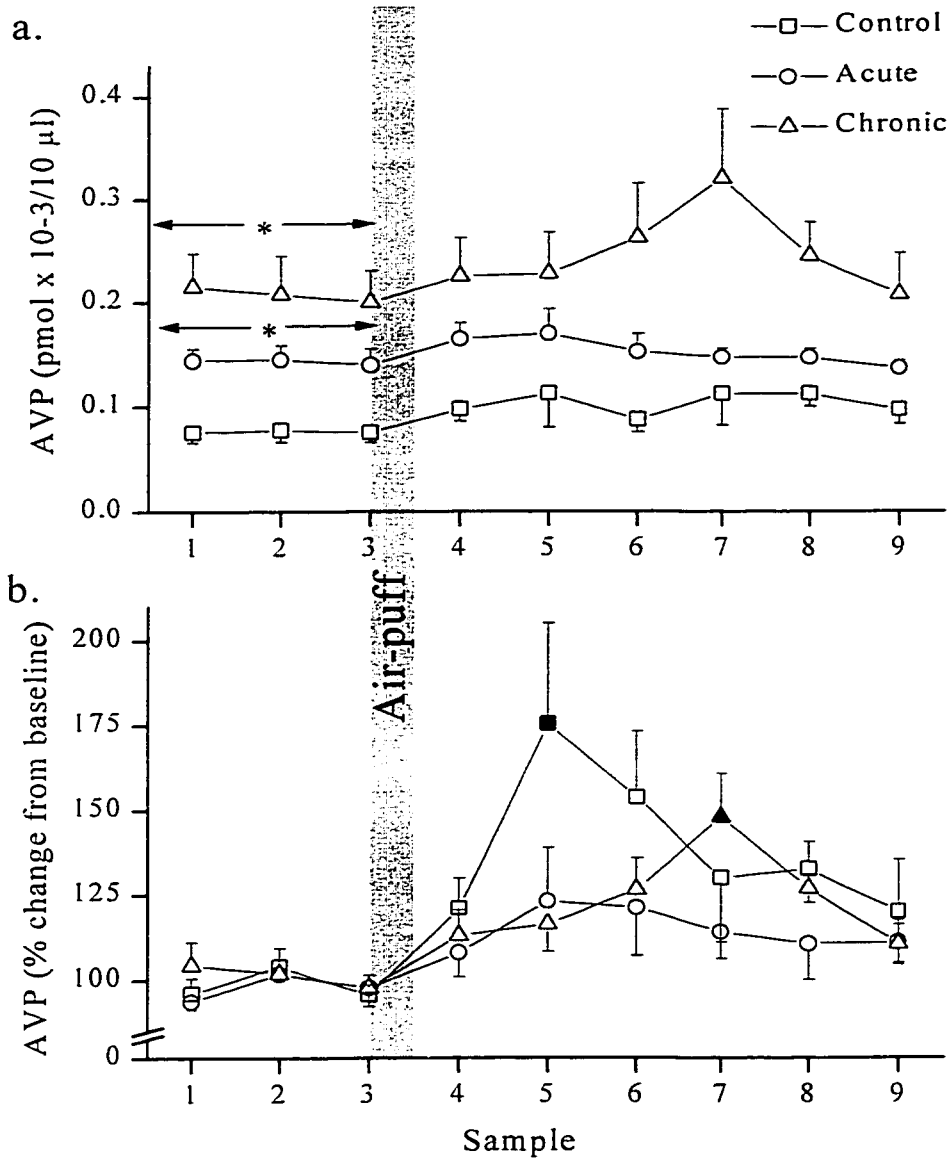

Fig. 2*a* Interstitial levels of AVP (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). * significantly different from baseline values of control condition at $p<0.05$.

Fig. 2*b* Interstitial levels of AVP (expressed as a percentage of the baseline) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). Solid symbols represent points that are significantly different the last baseline sample of respective treatment conditions.

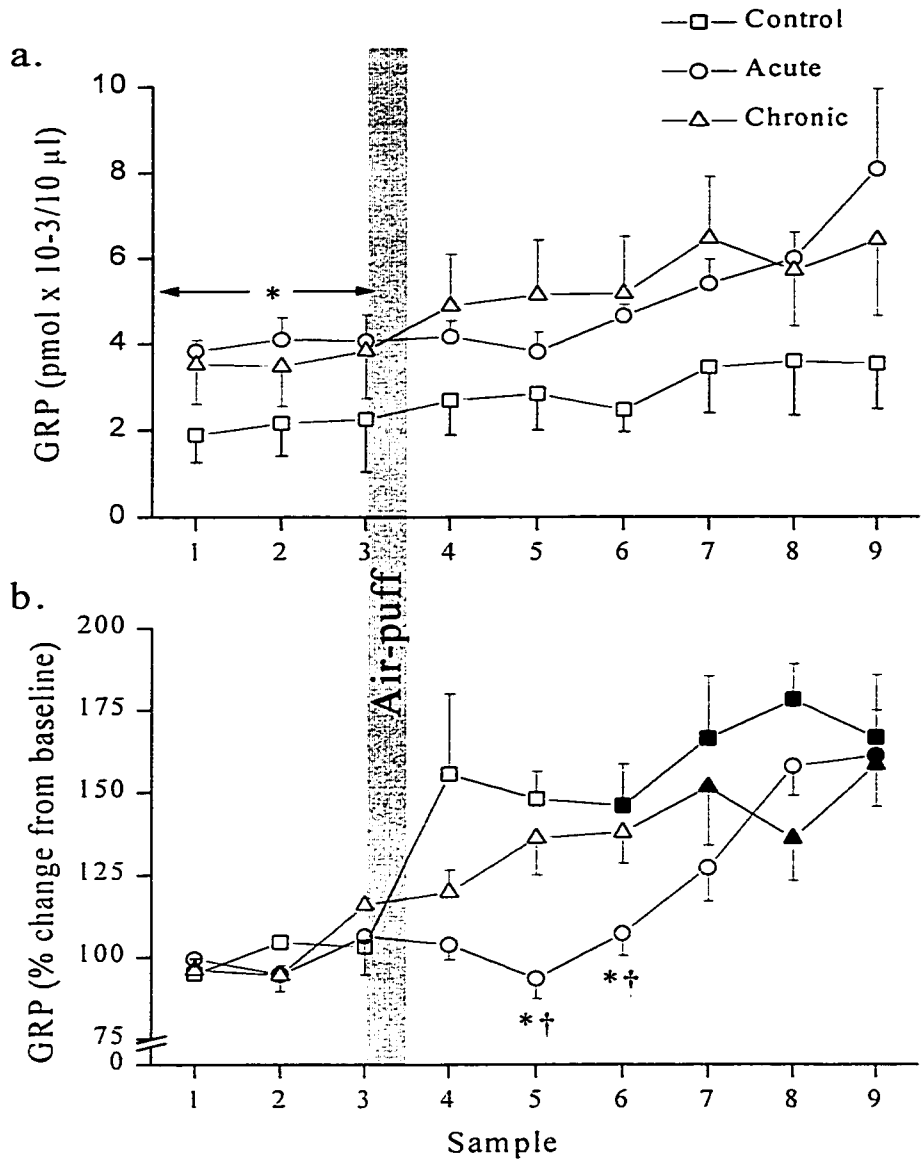

Fig. 3*a* Interstitial levels of BN/GRP (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). * significantly different from baseline values of control condition at $p<0.05$.

Fig. 3*b* Interstitial levels of CRH (expressed as a percentage of the baseline) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions. * significantly different from (sample-matched) non-stressed control condition at $p<0.05$. † significantly different from (sample-matched) chronic condition at $p<0.05$.

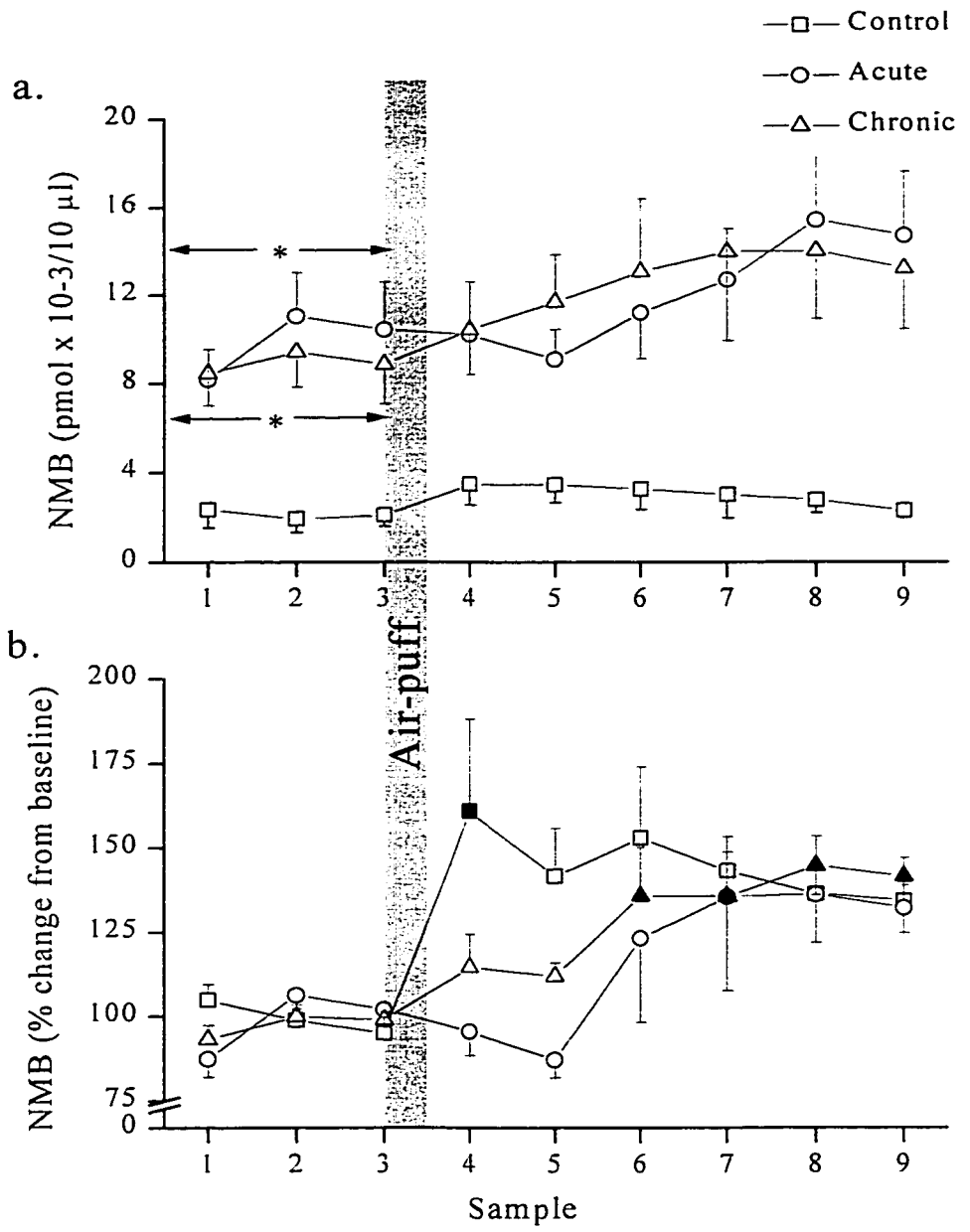

Fig. 4*a* Interstitial levels of NMB (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). * significantly different from baseline values of control condition at $p<0.05$.

Fig. 4*b* Interstitial levels of NMB (expressed as a percentage of the baseline) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in rats exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions.

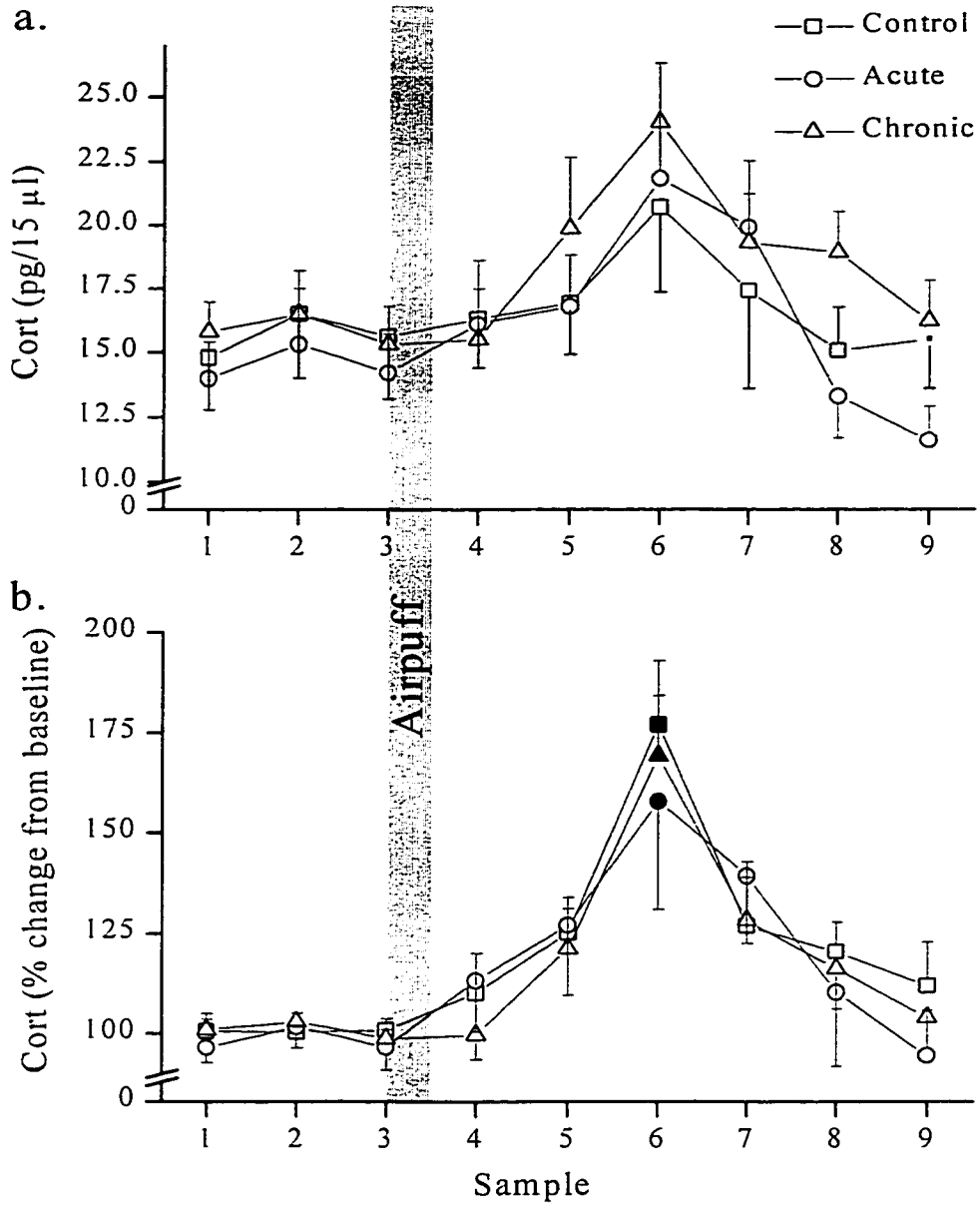

Fig 5*a* Interstitial levels of corticosterone (Cort) (expressed as pg/ 15 µl) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days).

Fig. 5*b* Interstitial levels of corticosterone (Cort) (expressed as a percentage of the baseline) at the anterior pituitary under basal conditions and following air-puff stress (5 min) in the previously non-stressed control rats and in those exposed to 14 sessions of restraint (chronic) or 1 session of restraint (acute) followed by the passage of time (14 days). Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions.

Discussion

In addition to the classical well characterized ACTH secretagogues, namely CRH and AVP, it appears that the BN family of peptides may be involved in regulation of the HPA axis.[132,133,195,257,313] Using molecular techniques, such as peptide mRNA expression, c-fos activation and post-mortem peptide level analyses, several previous studies have *indirectly* implicated increased turnover of CRH and/or AVP following stressor application.[3,24,66,85,93,94,178,250,251,254] Furthermore, such studies also reveal enduring phenotypic changes in the co-expression of these typical secretagogues based on the previous history of stressor exposure.[85,93,94] The current study aimed to 1) assess direct *in vivo* changes in interstitial levels of CRH and AVP at the anterior pituitary following acute and chronic stressor exposure, and 2) to evaluate the potential contribution of the BN family of peptides to HPA activation in response to acute stressor application, in animals with differential stressor histories. Consistent with past findings, our results demonstrated that acute or chronic stressor exposure was associated with significant elevations in basal levels of interstitial AVP and CRH at the anterior pituitary. Similarly, basal levels of interstitial GRP and NMB at the anterior pituitary were significantly elevated in animals previously exposed to either chronic restraint stress or a single session of restraint followed by the passage of time. These findings suggest that the role of the BN family of peptides in HPA activation is indeed influenced by the prior stress history of an organism. It appears that like AVP, GRP and NMB may take on a more dominant role in the stress response when the system has been primed by chronic stressor exposure or a single stressor exposure followed by adequate passage of time.

As mentioned earlier, basal levels of interstitial AVP were significantly elevated in animals previously exposed to restraint stress, as compared to naïve controls. While the most notable enhancement in interstitial ir-AVP levels was observed in rats previously exposed to chronic restraint, exposure to a single session of restraint also produced a significant elevation in basal ir-AVP levels at the anterior pituitary. These findings provide further support for the contention that both acute and chronic stressor exposures can induce long-lasting effects on AVP release that may result in altered composition on the signal driving ACTH secretion in favor of AVP.[396]

As compared to naïve rats, basal levels of interstitial CRH at the anterior pituitary were significantly elevated in animals previously exposed to restraint stress. In particular, exposure to a single session of restraint was associated with a dramatic rise in interstitial levels of CRH at the anterior pituitary as compared to a more modest rise in rats exposed to chronic restraint stress. These finding where somewhat unexpected given that past research has shown that exposure to a variety of acute stressors followed by an adequate passage of time (2 to 3 weeks) is associated with increased AVP stores in the ZEME, but no changes in CRH stores.[368,406] It should be recalled however, that stressor-induced changes in the CRH system are often unpredictable and are largely dependent upon the nature of stressor applied. Indeed, Schmidt et al. (1996)[366] have shown that exposure to a single 5 min session of electric footshock is associated with a significant increase in both AVP and CRH stores in the ZEME 11 days later. A modest enhancement in basal CRH levels was also observed in animals previously exposed to chronic restraint stress. Consistent with this finding, repeated stressor exposure

(including restraint), has been reported to increase both CRH and AVP mRNA at the PVN.[25,254] Indirect evidence suggests that there is a correlation between CRH mRNA levels, peptide synthesis and release into the hypophyseal portal circulation.[112] Moreover, repeated restraint stress or hypertonic saline injections are accompanied by a marked down regulation of pituitary CRH receptors, a finding consistent with enhanced secretion of CRH from the ME.[155,199]

Although basal levels of GRP, NMB, CRH and AVP were lowest in the naïve control group, these rats appeared most responsive to exposure to an acute novel stressor (air-puffs) as reflected by a more rapid and in general, a more pronounced enhancement of peptide levels at the anterior pituitary compared to those noted in the rats previously subjected to acute or chronic restraint stress. It should be noted however, that in the rats with previous stressor exposure, interstitial levels of CRH, AVP, GRP and NMB were significantly elevated above baseline by approximately 30 min post-stressor exposure. Thus, it appears that the peptidergic release in response to an acute stress challenge was delayed in animals with previous stress experience. It is possible that the chronically elevated peptide levels observed in the rats with previous stressor exposure created a 'ceiling effect' in that peptide synthesis, utilization and/or release were functioning at a near maximum capacity.

Although data is limited, it has previously been shown that manipulations that increase AVP and/or CRH content in the ZEME (e.g. repeated stressor exposure, adrenalecotomy or early postnatal maternal separation) are often associated with

enhanced secretion of AVP and/or CRH in response to a subsequent acute stressor challenge.[92,340,396] Although these findings are not concordant with the present data, measurement of peptidergic release in these previous examples was estimated using *indirect* techniques, which assessed peptide depletion by way of blockade of fast axonal transport with colchicine. It is therefore difficult to compare these previous findings with ours, which were obtained using push-pull perfusion, a technique that provides a direct measurement of *in vivo* peptide release over time. Some inconsistencies might also be attributable to the fact that previous studies examined AVP and/or CRH secretion from the ME, whereas we chose to measure interstitial peptide availability at the target site, namely the anterior pituitary, which is located downstream of the ME. Although we have previously shown a tight temporal association between peptide release at the ME and availability of those peptides at the anterior pituitary (unpublished findings), we can not say for certain whether our "pick up" at the anterior pituitary reflects peptide release upstream (at the ME) or peptide released locally at the anterior pituitary or a combination of both. Indeed, evidence suggests that BN-like peptides (including GRP and NMB), AVP and CRH are produced locally at the anterior pituitary and may be involved in the paracrine control of anterior pituitary functions[137,169,346,394]

The corticosterone responses (as measured in the perfusates) to the acute stressor challenge were virtually identical for all three groups. Moreover, there were no basal differences in corticosterone levels between the naïve control group and rats previously exposed to restraint stress. These findings were somewhat unexpected given the large basal difference in interstitial levels of peptidergic ACTH secretagogues between the

naïve rats and those with previous restraint stress exposure. However, it is worth noting that such discrepancies between CRH and AVP systems and ACTH and corticosterone levels have also been noted under other conditions, suggesting that increased CRH or AVP availability does not always produce a corresponding elevation in the end hormones.[5,140,156,250,406] Interpretation of our corticosterone findings is further complicated by the fact that concomitant ACTH levels could not be assessed in the present investigation (due to limited sample size). It is noteworthy, however, that consistent with our findings, there are several reports indicating that repeated stressor exposure, including restraint, had little or no effect on basal corticosterone or ACTH levels, despite significant changes in CRH and/or AVP mRNA expression.[99,251,262] Previous stressor exposure might alter pituitary and/or adrenal responsiveness leading to a normalization of the ACTH and/or corticosterone response in the face of hypersecretion of ACTH secretagogues from the hypothalamus. Indeed, it is well documented that chronic stress exposure is associated with a down regulation of CRH receptors at the pituitary, which is thought to be a consequence of CRH hypersecretion into the portal system.[4,7,154,155,190] Alternatively, other mechanisms, apart from peptide-mediated ACTH release may be involved in the control of glucocorticoid secretion. Such mechanisms may include splanchnic innervation of the adrenals involving the local release of CRH or the stressor-induced release of other peptides into the blood which are able to stimulate corticosterone release.[8,113,147,163,389]

Although our results demonstrate enhanced interstitial levels of CRH, AVP, GRP and NMB at the anterior pituitary in rats with previous stress experience, the neural

mechanism(s) underlying such actions as well as their significance remains speculative at the present time, particularly with respect to GRP and NMB. There is some indication that AVP is more sensitive than CRH to glucocorticoid negative feedback and that enhanced AVP production in CRH neurons may involve a reduced glucocorticoid sensitivity of CRH neurons.[208,254] In support of this hypothesis, it has been demonstrated that exposure of rats to repeated immobilization stress elicits a corticosterone surge, which serves to down-regulate glucocorticoid receptors in the hippocampus and PVN.[254,261] This may in turn serve to temporarily impair glucocorticoid negative feedback and thus facilitate AVP expression. In addition, stressor-related differences between CRH and AVP are also apparent at the level of the pituitary receptors. In this context, repeated stressor exposure is associated with a marked down-regulation and desensitization of pituitary CRH receptors (even in the presence of sustained ACTH responses).[155,199] On the other hand, repeated immobilization stress increased AVP-binding affinity and AVP V1b receptor mRNA expression at the pituitary, suggesting that previous stress experience may result in a more dominant role for the AVP receptor in determining corticotroph responsiveness.[344] With respect to GRP and NMB, little is known about their relative sensitivities to glucocorticoid negative feedback or whether previous stressor exposure up- or down-regulates GRP and/or NMB receptors at the pituitary. It is of interest to note that *in vitro,* BN-like peptide receptors in Swiss 3T3 cells respond to agonist exposure by desensitization, internalization, and down-regulation.[29] In contrast, we have shown that immobilization stress (2 hours) is associated with an up-regulation of BN binding sites at the nucleus of the solitary tract, PVN and arcuate nucleus.[195] Further investigation is needed to determine what effects

stressor exposure (both acute and chronic) has on GRP and NMB receptors at the anterior pituitary.

In conclusion, there is mounting evidence suggesting that hypersecretion of hypothalamic CRH and/or AVP may be involved in the pathogenesis of certain stress-related psychiatric disorders such as major depression and posttraumatic stress disorder.[156,342] In the present study, our results showed that rats chronically exposed to restraint or even a single stressor episode followed by the passage of time, showed enhanced interstitial basal levels of GRP, NMB, CRH and AVP at the anterior pituitary. Although changes in the CRH and AVP systems following previous stress experience have been postulated using indirect markers, our results are the first to *directly* show that the availability of these secretagogues is altered by the prior stress history of an organism, and that the stress history also differentially impacts on the GRP and NMB systems as well. Moreover, in response to a subsequent acute stressor challenge (air-puffs), rats with previous stress experience (and high basal peptide levels) showed a somewhat delayed and less pronounced increase in interstitial levels of GRP, NMB, AVP and CRH as compared to the naïve controls. Although further research is needed to determine the significance of these findings, it seems clear that the BN family of peptides function in a similar manner to that of other stress relevant peptides in the mediation/and or modulation of the response to stress and may be in involved in the pathogenesis of stress-related disorders. In keeping with this contention, we have recently noted that the levels of GRP or BN-related peptides are altered in the brains of suicide victims as compared to brains of subjects who died of other causes (manuscript in preparation).

Preface to Chapter IV

In light of similarities between BN-like peptides and CRH in terms of their anatomical distributions, behavioral and endocrine effects, we wanted to clarify whether BN-like peptides act directly or indirectly via activation of CRH neurons. Thus, in the first part of this experiment we characterized the endocrine and autonomic effects of central BN administration in order to ascertain whether they mimic the effects of CRH. In the second part of this experiment we addressed the question of whether BN mediates its effects through activation of CRH neurons. This was accomplished by assessing whether blockade of CRH receptors attenuated the BN-elicited endocrine and autonomic responses.

CHAPTER IV

Blockade of central corticotropin-releasing hormone receptors attenuates bombesin-elicited hypothalamic-pituitary-adrenal axis and sympathetic activation

Abstract

Bombesin (BN)-like peptides activate the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. There is evidence to suggest that BN-like peptides may mediate their effects via corticotropin-releasing hormone (CRH) neurons. The initial study revealed that central BN increased plasma ACTH, corticosterone, epinephrine, norepinephrine and glucose levels. Blockade of CRH receptors with αh-CRF (10 μg) attenuated the BN (0.25 μg)-induced rise in plasma ACTH, epinephrine, norepinephrine and blood glucose levels, but had no effect on plasma corticosterone levels, seen 30 min after BN administration. The subsequent time-course study revealed that central BN (0.2 μg) increased corticosterone and glucose concentrations above baseline levels at 15, 30, 60 and 120 min relative to vehicle administration. Pretreatment with αh-CRF (10 μg) attenuated the BN-induced rise in corticosterone and glucose levels at all time points. These findings support the notion that BN-induced HPA axis and sympathetic activation is mediated, at least in part, via CRH neurons.

Introduction

While originally discovered in the skin of the European frog *bombina bombina*, bombesin (BN)-like peptides have been shown to be widely distributed throughout the mammalian central nervous system and gastrointestinal tract.[295,297,298,322] When introduced into animals, the BN-related peptides produce a wide spectrum of biological effects including thermoregulation, satiety, smooth muscle contraction and chemotaxis.[116,127,260,267,357] More recent evidence suggests that BN-like peptides may also influence the activity of the hypothalamic-pituitary-adrenal (HPA) axis as well as the sympathetic division of the autonomic nervous system. In this context, it has been reported that exogenous administration of BN, or its mammalian counterparts, gastrin-releasing peptide (GRP) and neuromedin B (NMB), can potently stimulate the release of ACTH from the anterior pituitary and/or corticosterone from the adrenal medulla.[133,150,257,313] Moreover, BN-related peptides appear to activate the autonomic nervous system, as i.c.v. injection of BN dose-dependently increases plasma epinephrine, norepinephrine and glucose levels.[52,53,311,333]

While the neuronal mechanisms underlying these BN-elicited effects remain unclear, it appears that BN-like peptides may mediate some of these effects by acting through ACTH secretagogues, in particular corticotropin-releasing hormone (CRH). Indeed, BN-like peptides share many similarities with CRH in terms of anatomical distribution, endocrine, autonomic and behavioral effects.[195] Moreover, our laboratory has recently shown that pretreatment with the CRH antagonist, αh-CRF, blocked BN-induced behavioral effects (suppression of food intake and enhanced grooming behavior).[335] While not extensively investigated, there is also some indication that BN-

like peptides may act via CRH neurons to produce some of their endocrine effects, as BN and GRP potentiate CRH-induced ACTH release.[19,117,313] In addition, intravenous infusion of CRH antiserum or central administration of αh-CRH attenuates GRP-stimulated plasma ACTH and corticosterone release.[133,313]

The aim of the present study was to characterize the role of CRH in the mediation of BN-induced activation of both the HPA axis and sympathetic nervous system. Thus, in an initial study, we assessed whether 1) central administration of BN would affect plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels and 2) whether blockade of CRH receptors (with αh-CRF) could attenuate these BN-induced effects. While pretreatment with αh-CRF blocked or attenuated the BN-induced increases in plasma ACTH, norepinephrine, epinephrine and blood glucose levels, it failed to alter the rise in plasma corticosterone levels at the 30 min interval. It is conceivable that the failure of αh-CRF to attenuate the BN-induced increases in plasma corticosterone levels may be due to 1) direct effects of exogenous BN administration on the adrenal cortex or 2) an inability to detect the attenuation that may have preceded or followed the 30 min time point selected. In order to test the latter possibility, a second more dynamic experiment was performed where blood samples were collected at different time points to assess the effects of drug administration on blood corticosterone and glucose levels.

Materials and Methods

Animals

Male Sprague-Dawley rats (weighing between 325 and 400 g), obtained from Charles River (St. Constant, Quebec) were used in both experiments. All animals were individually housed and maintained on a 12-h light/dark cycle (with lights on at 6:00 a.m.), in a temperature (23°C) and humidity (60%) controlled room. Animals had free access to food and water.

Surgical procedure

All rats were anesthetized with pentobarbital (60 mg/kg; i.p.) and stereotaxically implanted with permanent guide cannulae (22 gauge) aimed at the 3rd ventricle. The placement coordinates (obtained from Paxinos and Watson[324]) were 4.4 mm posterior to bregma, 0 mm lateral, and 4.4 mm ventral to the skull surface. The cannulae were anchored to the skull surface using 4 stainless steel screws and acrylic dental cement and plugged with removable stainless steel stylettes. During the recovery period, animals were acclimated to handling as well as mock central injections.

Experimental Protocol

Experiment 1a. Effects of central BN administration on plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels

All experiments were performed between 9:00 and 12:00 A.M. On test day, rats were randomly divided into 3 groups (n = 7-10/group) and were microinjected with either vehicle (saline), or one of two doses of BN (0.25 μg or 0.5 μg; i.c.v.). Bombesin

(Bachem, California) was freshly dissolved in 0.9% saline prior to administration. The microinjections were delivered in a 3 μl volume infused over 60 s via an injection cannula (0.5mm longer than the guide cannula) connected by polyethylene tubing to an infusion pump (Harvard Apparatus, Canada). Rats were sacrificed 30 min post injection and trunk blood was collected in tubes containing EDTA, centrifuged and the plasma was frozen at –70°C. Blood glucose levels were determined (from the whole trunk blood) using a portable glucometer Elite™ (Ames, Miles Canada) as previously described by Messier and Kent.[284] Plasma ACTH and corticosterone levels were measured using commercial radioimmunassay (RIA) kits (ICN Pharmaceuticals, Canada). Plasma epinephrine and norepinephrine levels were determined by HPLC using a modification of the method of Seegal et al.[370]

Experiment 1b. Effects of CRH receptor blockade on BN-induced increases in plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels

The design of this experiment was identical to that of experiment 1*a* except that rats were randomly divided into 4 groups (n = 7-10/group) and were microinjected with either vehicle (saline), αh-CRF (10 μg/kg), saline followed 20 min later with BN (0.25 μg; i.c.v.) (saline + BN) or αh-CRF (10 μg /kg) followed 20 min later with BN (0.25 μg/kg) (αh-CRF + BN).

Experiment 2. Effects of CRH receptor blockade on BN-induced increases in corticosterone and glucose as a function of time

The design of this experiment was identical to that of Experiment 1 except that the dose of BN used was 0.2 μg (i.c.v.) and blood samples were collected at 0, 15, 30, 60 and 120 min (following drug or saline injection). Blood samples were collected by lancing the rat's tail close to the tip with a razor blade and then wiping the first droplet of blood away. Blood droplets were then blotted onto preprinted circles on S&S filter paper (Schleicher & Schuell, Mandel Scientific), allowed to dry at room temperature and stored at –20° C. On the day prior to RIA procedure, blood was eluted from the filter paper by placing one 2.5 mm punch (per time interval) of filter paper in a 12 x 75 culture tube containing 100 μl of dulbecco's phosphate buffered saline (Sigma, U.S.A.), covered with parafilm and left to rotate on an automatic shaker (50 rpm) for 1 hr at room temperature. Following an overnight incubation (at 4°C) tubes were again rotated on an automatic shaker for 1 hr at room temperature. Corticosterone levels were then determined from the eluted blood sample using a commercial RIA kit (ICN Pharmaceuticals, Canada).

Statistical Analysis

All results are expressed as means ± S.E.M. In experiments 1*a* and 1*b*, blood glucose, plasma ACTH, corticosterone, epinephrine and norepinephrine values were analyzed separately by one factor (Treatment) analyses of variance (ANOVA) followed by Newman-Keuls multiple comparisons. In experiment 2, blood corticosterone and glucose levels were analyzed separately using two factor repeated measures ANOVAs. The between-groups factor was Treatment condition (saline, αh-CRH, saline + BN and

αh-CRH + BN) and the within-subjects factor was Time (0, 15, 30, 60 and 120 min). Post hoc comparisons were conducted using Newman-Keuls multiple comparisons. A value of $p<0.05$ was considered statistically significant.

Results

Experiment 1a. Effects of central BN administration on plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels

Figure 1 shows plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels in rats centrally injected with saline, or one of two doses of BN (0.25 or 0.5 μg). ANOVA revealed significant effects of BN treatment on plasma levels of ACTH ($F_{2,21}=20.30$; $p<0.0001$), corticosterone ($F_{2,22}=27.29$; $p<0.0001$), norepinephrine ($F_{2,19}= 6.23$; $p<0.01$), epinephrine ($F_{2,19} = 7.66$; $p<0.005$) and glucose ($F_{2,17} = 17.44$; $p<0.0002$). Newman-Keuls multiple comparisons of the simple effects revealed significant elevations in plasma concentrations of ACTH following administration of the higher dose of BN (0.5 μg) and significant increases in plasma corticosterone, norepinephrine, epinephrine and blood glucose levels following administration of both doses of BN (0.25 and 0.5 μg).

Experiment 1b. Effects of CRH receptor blockade on BN-induced increases in plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels

Plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels in rats centrally injected with either vehicle (saline), αh-CRF (10 μg/kg), saline + BN (0.25 μg; i.c.v.) or αh-CRF (10 μg/kg) + BN (0.25 μg; i.c.v.) are depicted in Figure 2.

Individual ANOVAs revealed significant Treatment effects on plasma levels of ACTH ($F_{3,28}$=3.66; $p<0.025$), corticosterone ($F_{3,27}$ = 6.067; $p<0.003$), norepinephrine ($F_{3,23}$ = 4.65; $p<0.012$), epinephrine ($F_{3,25}$ = 14.50; $p<0.0001$) and blood glucose levels ($F_{3,23}$ = 17.07; $p<0.0001$). Newman-Keuls comparisons of the simple effects revealed that saline + BN administration significantly increased plasma levels of ACTH, corticosterone, norepinephrine, epinephrine and blood glucose levels. Whereas, αh-CRF was without effect on its own, when administered prior to BN injection, it significantly blocked the BN-induced increase in plasma epinephrine and ACTH levels and attenuated the BN-induced increases in plasma norepinephrine levels and blood glucose levels. Pretreatment with αh-CRF had no effect on plasma corticosterone levels.

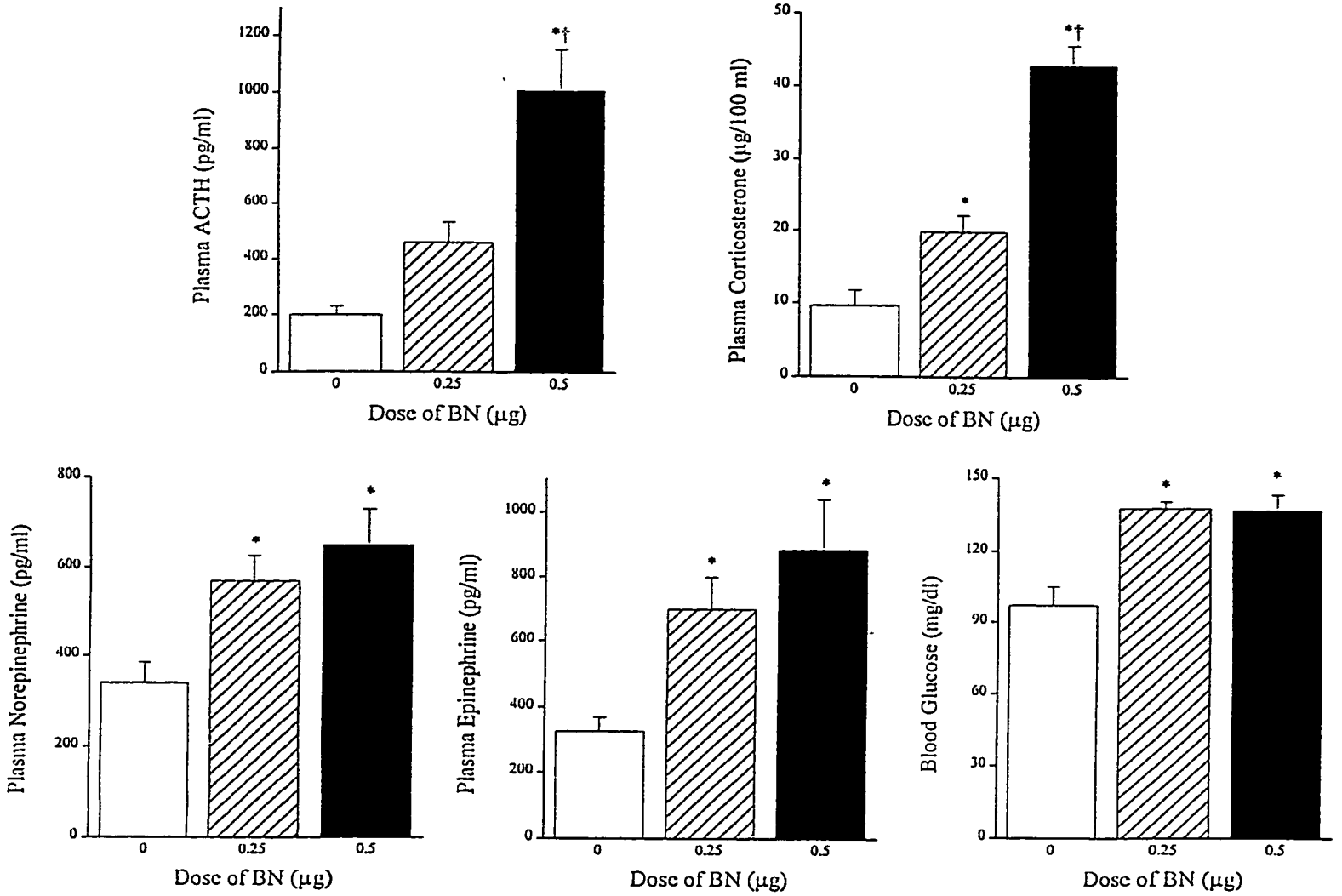

Fig. 1 Plasma levels of ACTH, corticosterone, norepinephrine, epinephrine and glucose (mean ± S.E.M.) following central BN administration. The open columns represent control animals injected with vehicle (saline, 3 μl; i.c.v.) whereas the hatched and solid columns represent animals injects with BN at 0.25 μg and 0.5 μg (i.c.v.), respectively. * significantly different from saline values at $p<0.05$. † significantly different from low dose BN (0.25 μg) values at $p<0.05$.

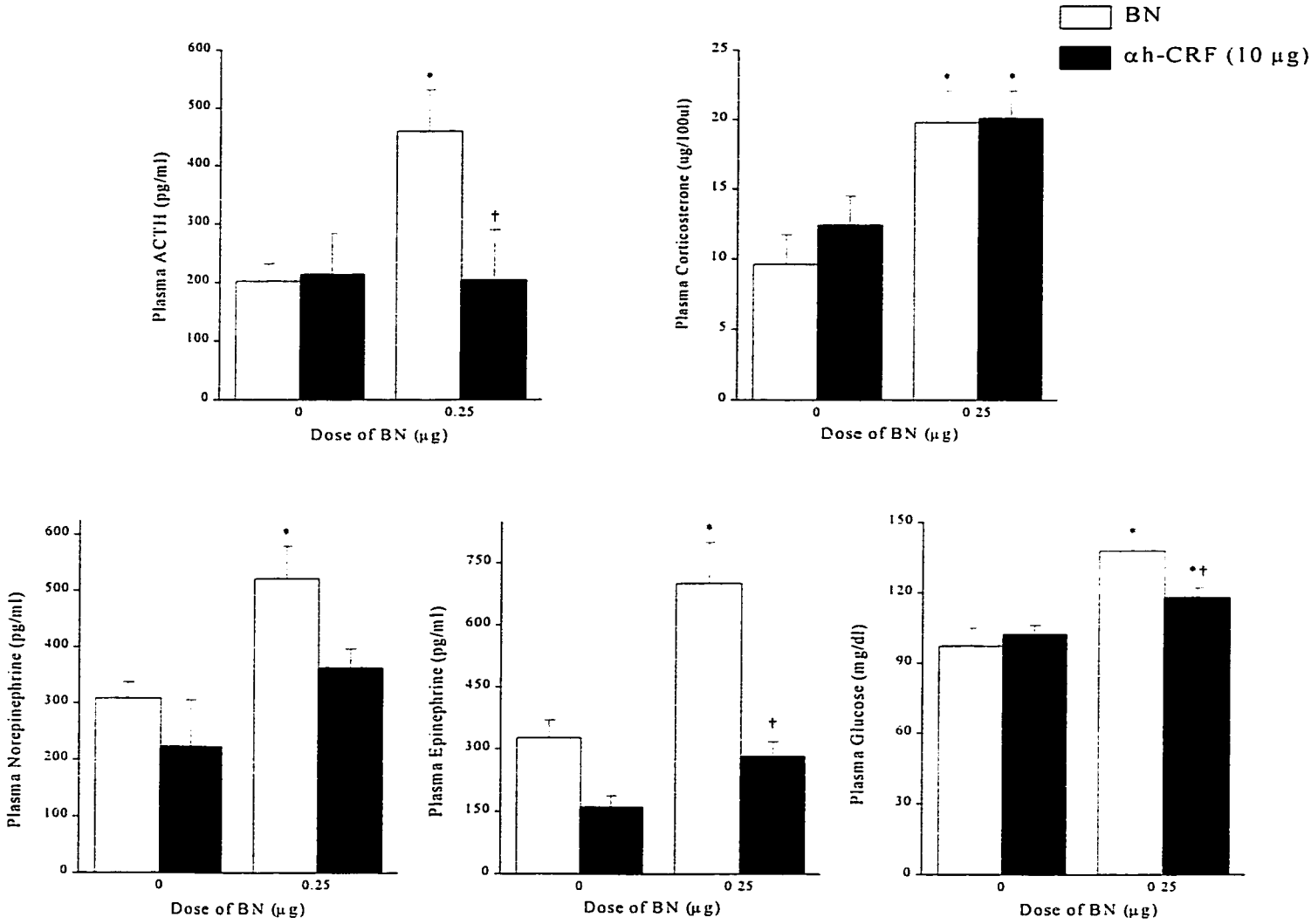

Fig. 2 Plasma levels of ACTH, corticosterone, norepinephrine, epinephrine and glucose (mean ± S.E.M.) in rats that received central injections of either saline, αh-CRF (10 μg/kg), saline + BN (0.25 μg; i.c.v.) or αh-CRF (10 μg/kg) + BN (0.25 μg; i.c.v.). * significantly different from saline values at $p<0.05$. † significantly different from saline + BN values at $p<0.05$.

Experiment 2. Effects of CRH receptor blockade on BN-induced increases in corticosterone and glucose as a function of time

Figure 3 and 4 illustrates blood corticosterone and glucose levels respectively, at 0, 15, 30, 60 and 120 min relative to drug administration in rats that received central injections of either saline, αh-CRF (10 μg/kg), saline + BN (0.2 μg; i.c.v.) or αh-CRF (10 μg/kg) + BN (0.2 μg; i.c.v.). The overall two factor repeated measures ANOVA revealed that blood corticosterone levels varied as a function of Treatment X Time interaction ($F_{12,124} = 9.66$; $p<0.0001$). Newman-Keuls multiple comparisons of the simple effects for this interaction revealed that blood corticosterone levels were significantly elevated above baseline following all treatment conditions, however the rise in corticosterone levels was more pronounced and protracted following administration of saline + BN or αh-CRF + BN as compared to the rise observed following administration of either saline or αh-CRF alone. For the saline + BN and αh-CRF + BN treatment conditions, levels of corticosterone were significantly elevated above baseline values by 15 min post drug injection and remained elevated throughout the 120 min testing period. In contrast, administration of saline or αh-CRF was associated with a rise in corticosterone levels above baseline values by 15 min post drug administration which only remained significantly elevated until 30 min post injection for αh-CRF and 60 min post injection for saline. Newman-Keuls comparisons further revealed that in the absence of drug administration, levels of corticosterone were comparable for all treatment conditions. At the 15 min interval, levels of corticosterone were significantly elevated above the saline condition following saline + BN administration, and αh-CRF attenuated the BN-induced rise in blood corticosterone levels as indicated by the significant

difference in blood corticosterone concentrations between the saline + BN and αh-CRF + BN conditions. At the 30, 60 and 120 min intervals, plasma corticosterone levels were significantly elevated above the saline condition following both saline + BN and αh-CRF + BN administration, and αh-CRF attenuated the BN-induced rise in blood corticosterone levels as indicated by the significant difference in blood corticosterone concentrations between the saline + BN and αh-CRF + BN conditions.

The overall two factor repeated measures ANOVA revealed that blood glucose levels also varied as a function of the Treatment x Sample interaction ($F_{12,124} = 5.31$; $p<0.0001$). Newman-Keuls multiple comparisons of the simple effects for this interaction indicated that under all drug treatment conditions, blood glucose levels were significantly elevated above baseline levels and peaked at the 30 min time interval. In the absence of drug administration, glucose levels were comparable for all drug treatment conditions. At the 15, 30, 60 and 120 min time intervals, blood glucose levels were significantly elevated from the saline condition following saline + BN administration. Pretreatment with αh-CRF attenuated the BN-induced rise in blood glucose levels (at the 15, 30 60 and 120 min time intervals) as indicated by the significant difference in blood glucose levels between the saline + BN and αh-CRF + BN conditions.

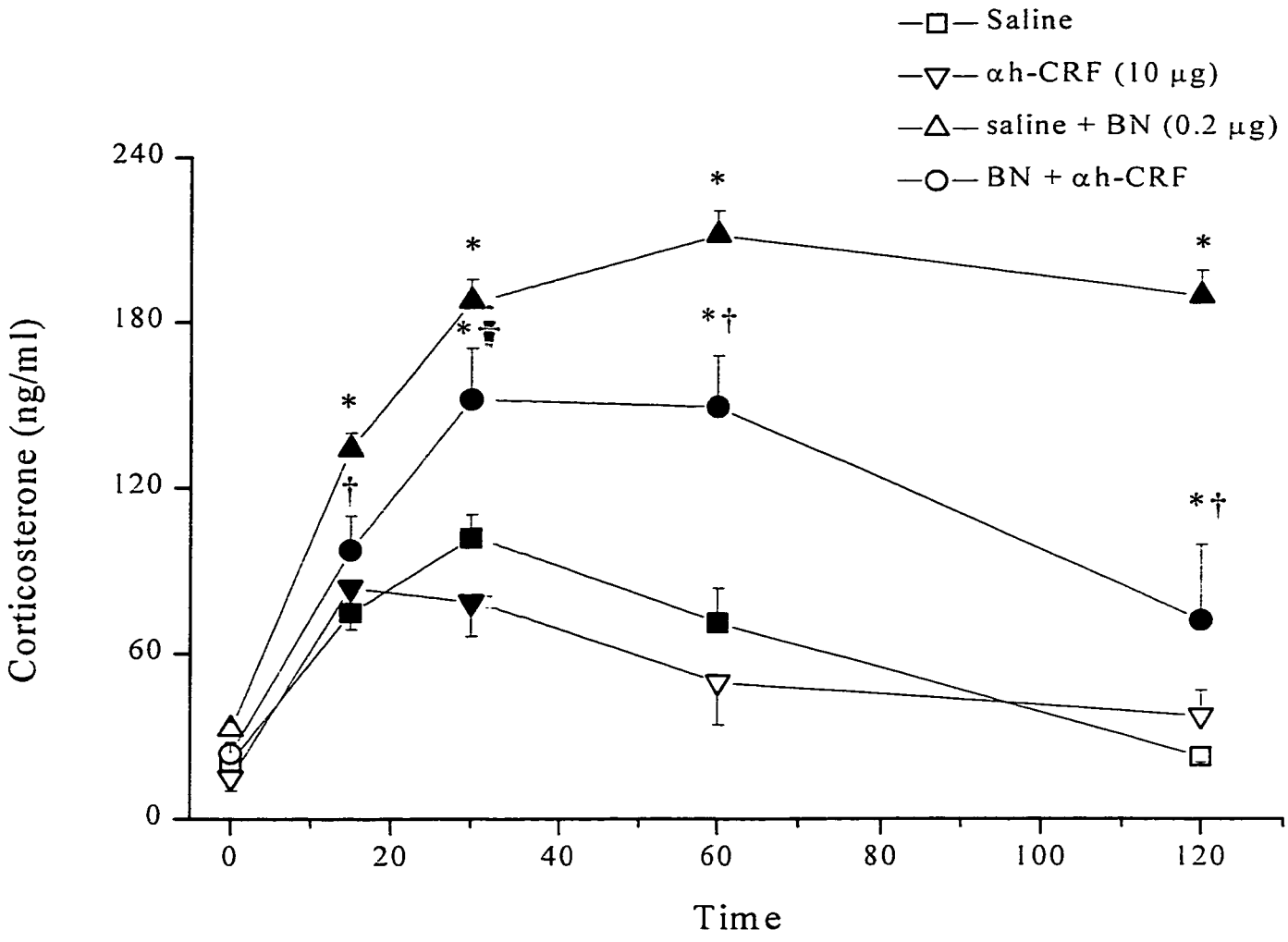

Fig. 3 Blood concentrations of corticosterone (mean ± S.E.M.) at the 0, 15, 30, 60 and 120 min time intervals (following drug administration) in rats that received central injections of either vehicle (saline), αh-CRF (10 μg/kg), saline + BN (0.2 μg; i.c.v.) or αh-CRF (10 μg/kg) + BN (0.2 μg; i.c.v.). Closed symbols represent points that are significantly different from (within-treatment condition) baseline values at $p<0.05$. * significantly different from (between-treatment condition) time-point matched saline values at $p<0.05$. † significantly different from (between-treatment condition) time-point matched saline + BN values at $p<0.05$.

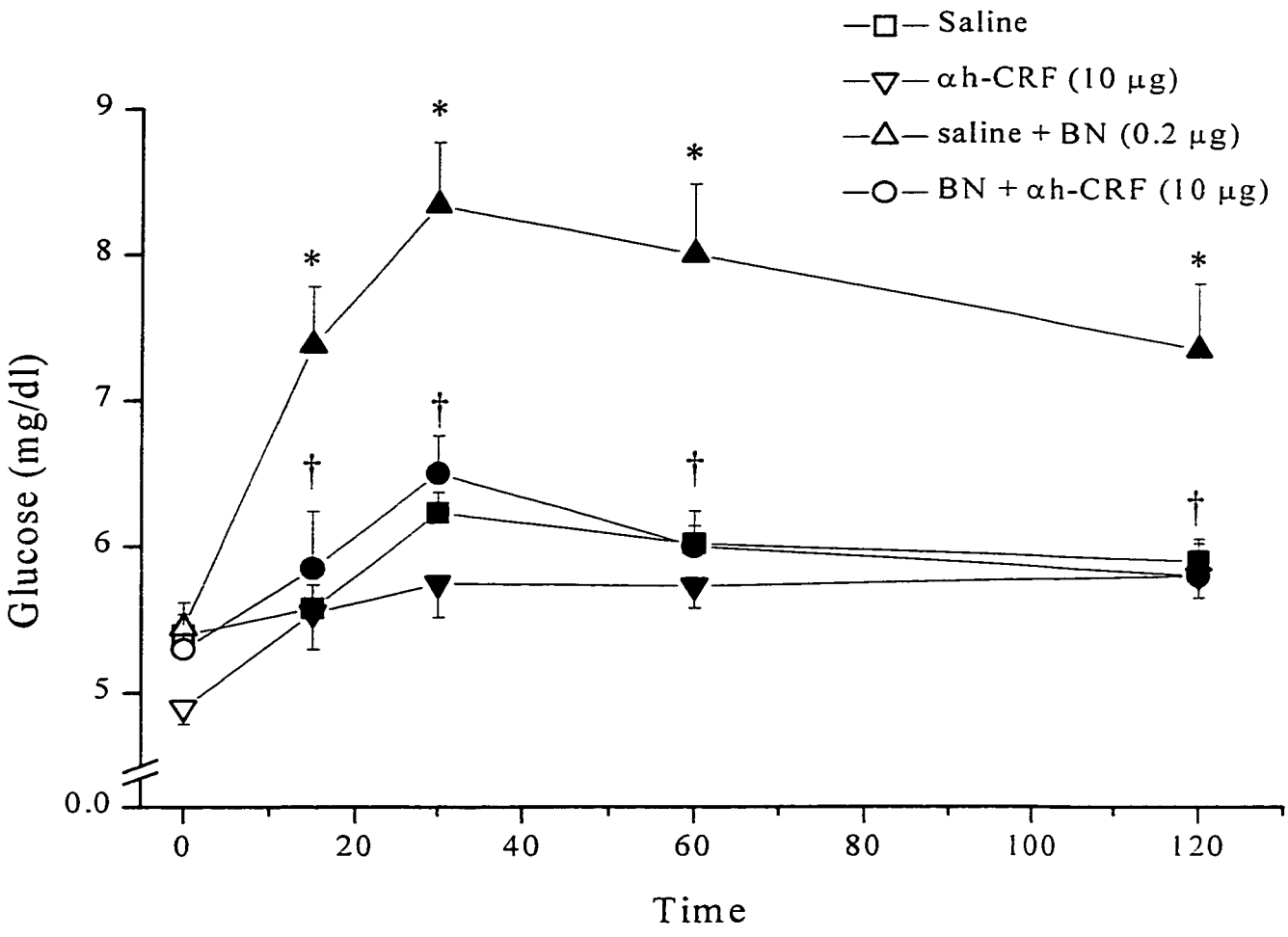

Fig 4. Blood glucose levels (mean ± S.E.M.) at the 0, 15, 30, 60 and 120 min time intervals in rats that received central injections of either vehicle (saline), αh-CRF (10 μg/kg), saline + BN (0.2 μg; i.c.v.) or αh-CRF (10 μg/kg) + BN (0.2 μg; i.c.v.). Closed symbols represent points that are significantly different from (within-treatment condition) baseline values at $p<0.05$. * significantly different from (between-treatment condition) time-point matched saline values at $p<0.05$. † significantly different from (between-treatment condition) time-point matched saline + BN values at $p<0.05$.

Discussion

CRH is well established as the primary endogenous regulator of ACTH secretion from the anterior pituitary.[105,350,402] There is also considerable evidence demonstrating the involvement of CRH in autonomic activation.[55,216] It is known that many different stimuli elicit CRH release including systemic (infections) and processive (psychological) type stressors, changes in blood pressure as well as appetitive events, however, the neurochemical mechanism(s) mediating this peptidergic release remain largely unknown.[278,339,419] Given the considerable functional and anatomical overlap between BN and CRH, the present study aimed to elucidate whether BN-induced pituitary-adrenocortical and autonomic activation are mediated via CRH neurons. In support of this contention, results from the present set of investigations demonstrated that 1) central BN administration elicited a significant dose-dependent increase in plasma levels of ACTH, corticosterone, norepinephrine, epinephrine and blood glucose levels and that 2) pretreatment with αh-CRF blocked or attenuated the BN-induced increases in plasma levels of ACTH, norepinephrine, epinephrine and blood glucose levels.

Since in the initial experiment αh-CRF attenuated a variety of effects with the exception of the BN-induced elevation of plasma corticosterone levels (measured at 30 min post BN injection), we decided to explore the possibility that this may have been due to the time-course of the effect. Specifically, it is possible that αh-CRF did exert an effect, however, it may have preceded or followed the 30 min time point selected. Thus, in the second experiment, blood samples were collected at different time points to assess the effects of drug administration on blood corticosterone and glucose levels. Results

revealed that following all drug injections (saline, αh-CRF, saline + BN and αh-CRF + BN). levels of corticosterone and glucose where significantly elevated. It is likely that the rises in blood corticosterone and glucose levels observed following the control injections (saline or αh-CRF) were attributable to the stress associated with the tail lance procedure. It should be noted however, that the elevations in corticosterone levels observed following both saline + BN and αh-CRF + BN administrations were significantly higher than those observed following the control injections. Similarly, blood glucose levels were significantly greater following saline + BN condition than following all other treatment injections. These findings suggest that the stress associated with the tail lance did not mask the drug-induced effects. Finally, in contrast to the result in our first experiment, when blood samples were taken at various time points relative to drug administration, αh-CRF significantly attenuated the stimulatory effect that BN (0.2 μg) had on corticosterone secretion. Similarly, αh-CRH attenuated the BN-elicited rise in blood glucose levels. This observation is consistent with results from our first experiment demonstrating that pretreatment with αh-CRF significantly attenuated the BN-induced rise in blood glucose levels at 30 min post injection.

Garrido et al. 1998[133] recently demonstrated that central αh-CRF administration completely blocked the stimulatory effect that GRP had on plasma ACTH and corticosterone levels (at 30 min post injection). Although we found a similar effect with ACTH, the ability of αh-CRF to attenuate the BN-induced rise in plasma corticosterone levels was only demonstrable in our second experiment using a slightly lower dose of BN than what was used in our first experiment (0.2 μg instead or 0.25 μg). A likely

explanation for this discrepancy is that Garrido and his colleagues used a much lower dose of GRP (0.01 μg) as compared to the doses of BN (0.2 or 0.25 μg) that we used in our experiments. Although BN and GRP share an almost identical active C-terminal decapeptide region (differing in only 1 amino acid), GRP is in fact less potent than BN.[383] Not only does BN stimulate both GRP (BB_2) and neuromedin B (BB_1) receptors (whereas GRP is active almost exclusively at BB_2 receptors), but BN is also thought to be more resistant than GRP to enzymatic degradation.[223,277,413]

Not only was αh-CRF able to attenuate the stimulatory effect that BN had on pituitary-adrenocortical activity, but pretreatment with this CRH antagonist significantly inhibited the autonomic activation induced by BN injection. To our knowledge, there is only one other study which has investigated the effects of αh-CRF on the BN-induced rise in plasma concentrations of norepinephrine, epinephrine and glucose.[56] Contrary to our results, they found that pretreatment with αh-CRF had no effect on the BN-induced elevations in plasma concentrations of norepinephrine, epinephrine and glucose. It should be noted however that the doses of BN and αh-CRH used in that experiment were substantially larger, 1 μg and 50 μg respectively, than the doses used in the present experiment. Indeed, the BN-induced increases in plasma levels of norepinephrine, epinephrine and glucose in that study were more than double to those observed in the present investigation.

While our results suggest that BN may be mediating its effects, at least in part, via activation of central CRH neurons, they do not reveal the site(s) of action for these

peptidergic interactions. It is well established that CRH produced in the paravocellular division of the PVN is released from the median eminence (ME) to regulate ACTH secretion from the anterior pituitary.[160,185] It is possible that BN activates the HPA axis by interacting with receptors located on CRH-expressing neurons at the PVN. This nucleus contains high levels of both immunoreactivity as well as binding sites for both BN-like peptides and CRH.[79,88,295,322] Moreover, microinjection of BN into the PVN (but not other hypothalamic sites) elicits the release of plasma corticosterone.[150] In addition, we have recently shown using *in vivo* push-pull perfusion that central BN administration elicits the release of CRH from the ME (unpublished findings).

In terms of autonomic activation, it has previously been observed that direct microinjection of BN into the NTS, (but not other brain structures) elicits a dramatic increase in plasma catecholamine levels.[53,64] It is possible that BN mediates these effects by interacting with receptors located on CRH neurons at the NTS or other brain stem structures. Indeed, high levels of both BN-like peptide and CRH immunoreactivity as well as high densities of binding sites for both of these peptides have been observed at the NTS.[79,88,295,322] Moreover, we have recently shown that central BN administration elicits a significant reduction in levels of immunoreactive CRH at the NTS (unpublished findings).

Conclusion

In summary, results from the present investigation demonstrate that pretreatment with a CRH receptor antagonist (αh-CRF) can block or attenuate BN-induced HPA axis

and sympathetic activation. These findings provide further support for the contention that BN-like peptides mediate their effects, at least in part, through activation of CRH neurons. In conjunction with our previous studies where we have demonstrated the release of both BN-like peptides and CRH, in response to appetitive as well as stressful events[278], it would appear that these two peptidergic systems may act in concert to prepare organisms to deal with biologically significant events.

Preface to Chapter V

In the previous chapter, we presented clear evidence supporting the notion that BN-like peptides mediate their endocrine and autonomic effects, at least in part, through the activation of CRH-sensitive circuits. In this chapter, we will focus on experiments aimed at identifying the potential locus (or loci) where such peptidergic interactions occur. To attain this objective, we injected exogenous BN into the brain (via 3rd ventricular infusion) and monitored regional changes in the endogenous levels of ir-CRH (and ir-AVP). In addition, at suspected target sites, we also monitored the *in vivo* release of these peptides, following BN administration.

CHAPTER V

Central Bombesin Activates the Hypothalamic-Pituitary-Adrenal Axis: Effects on regional levels and release of corticotropin-releasing hormone and arginine-vasopressin.

Abstract

While corticotropin releasing hormone (CRH) is a primary regulator of the hypothalamic-pituitary-adrenal (HPA) axis, the mechanism(s) triggering its release remain unknown. Stressful and appetitive events evoke the release of not only CRH but also of bombesin (BN)-like peptides. Furthermore, CRH antagonists attenuated the endocrine and behavioral effects of BN, suggesting that BN-like peptides may mediate their effects via CRH release. The initial (mapping) study revealed that central BN (0.25 or 0.5 μg; i.c.v.) administration increased circulating corticosterone and ACTH levels and decreased immunoreactive (ir)-CRH at the nucleus of the solitary tract, ventromedial (VMH) and anterior hypothalamic nuclei, and the central amygdaloid nucleus. Whereas BN treatment decreased ir-vasopressin (AVP) at the VMH, it elevated levels of this peptide at the hypothalamic paraventricular and median eminence/arcuate (Me/Arc) regions. Subsequent, more direct release experiments (using push-pull perfusion) revealed that the availability of ir-CRH and ir-AVP were markedly enhanced at the Me/Arc and at the anterior pituitary. These results suggest that BN-like peptides may regulate certain hypothalamic and extrahypothalamic circuits, including the HPA axis, as they influence tissue levels of ir-CRH and ir-AVP at discrete nuclei and stimulate the release of CRH and/or AVP at the Me/Arc and their availability downstream at the anterior pituitary, resulting in elevated circulating corticosterone levels.

Introduction

While CRH is a primary regulator of the hypothalamic-pituitary-adrenal (HPA) axis, the mechanism(s) modulating the release of this secretagogue remain obscure. Release of CRH is evoked not only by a wide variety of stressors including systemic (e.g. infections) and processive (e.g. psychological) stressors, but also by appetitive events.[278] It is possible that these differing stimuli activate different neural circuits and/or transmitter systems, eventually converging upon and modulating the relevant PVN neurons containing CRH.[160] In addition to the relatively well established role of classical neurotransmitters, such as norepinephrine, in the mediation of the stress response[217], there is reason to believe that the bombesin (BN) family of peptides also contribute to the integration and/or mediation of these responses. Like stressors, BN and related peptides, including gastrin-releasing peptide (GRP) and neuromedin B (NMB), are capable of eliciting the coincident activation of the hypothalamic-pituitary adrenal (HPA) axis[257,282,313] and the sympathetic division of the autonomic nervous system.[53] As well, central BN administration produces many of the behavioral effects typically associated with the stress response, such as suppression of food intake, increased grooming[214], and elicitation of anxiety-like behaviors (such as decreased exploration in a novel environment and increased locomotor activation in a familiar environment).[180] Recently, we demonstrated that acute immobilization stress induced brain-region specific alterations of endogenous levels of BN-like peptide as well as in the density of BN binding sites.[195] While the neural mechanism(s) through which BN-like peptides come to affect the HPA activity remain largely unknown, it appears likely that stress-relevant neuropeptides, including CRH and/or AVP, may be involved in the mediation of these

effects. Indeed, CRH, AVP and BN-like peptides share considerable anatomical overlap, and are present in significant concentrations within various aspects of the HPA axis, as well as in other stress-relevant extrahypothalamic nuclei, including the hippocampus, central amygdaloid nucleus (CeA) and the nucleus of the solitary tract (NTS).[10,79,138,153,183,232,238,322,377,394] Similarities also extend to the distribution of receptors for CRH, AVP and BN-like peptides. The binding sites for all three peptides being present at the arcuate nucleus (Arc), the anterior pituitary, NTS and CeA.[23,88,168,182,295] Functional interactions between these peptides have also been noted. Thus, GRP potentiated both CRH- and AVP-stimulated ACTH release[313], whereas pretreatment with either CRH or AVP antiserum attenuated GRP-induced ACTH secretion. Furthermore, the combination of CRH and AVP antisera completely blocked this effect.[313] Likewise, pretreatment with the CRH antagonist, αh-CRF, attenuated BN-induced endocrine (increased circulating ACTH, norepinephrine, epinephrine and glucose levels) and behavioral effects (suppression of food intake and increased grooming behavior).[282]

While evidence suggests that BN-like peptides may mediate their stress-relevant effects through activation of CRH and/or AVP neurons, the anatomical locus (or loci) for these interactions remain unknown. Thus, in the initial mapping study, we used brain micropunch technique followed by measurement of ir-CRH and ir-AVP levels in order to identify some of the brain sites where such peptidergic interactions might occur. Central BN microinjections did indeed elicit site-specific changes in concentrations of ir-CRH and ir-AVP at various elements of the HPA axis as well as at a variety of other stress-relevant nuclei. However, the nature of such interactions remained obscure owing to the fact that tissue stores can be influenced by a variety of factors including the rates of

synthesis, release and degradation. In order to assess the functional relevance of the observed tissue level alterations, more direct push-pull perfusion experiments were performed to assess the dynamics of the *in vivo* release of CRH and AVP from selected brain sites following central BN administration. Thus, the release of CRH and AVP from the median eminence/arcuate nucleus region (Me/Arc) (given the pivotal role this region plays in HPA activation) as well as the availability of these secretagogues downstream at the anterior pituitary, were assessed in the second set of experiments.

Materials and Methods

Animals

Male Sprague-Dawley rats (weighing between 325 and 400 g), obtained from Charles River (St. Constant, Quebec), were used in all experiments. Animals were individually housed and maintained on a 12-h light/dark cycle (with lights on at 6:00 a.m.), in a temperature (23°C) and humidity (60%) controlled room. Animals had free access to food and water.

Experiment 1. Effect of central BN on regional ir-CRH and ir-AVP levels

Surgical procedure

All rats were anesthetized with pentobarbital (60 mg/kg; i.p.), and stereotaxically implanted with permanent guide cannulae (Plastics One, VA, U.S.A.) aimed at the 3rd ventricle. The placement coordinates[324] were 4.4 mm posterior to bregma, 0 mm lateral, and 4.4 mm ventral to the skull surface. After a 7 day recovery period, rats were randomly divided into 3 groups (n = 7-10/group) which received microinjections of either vehicle (saline) or one of two doses of BN (0.25 or 0.5 μg; i.c.v.). Bombesin (Bachem,

California) was freshly dissolved in saline prior to administration. The microinjections were delivered in a volume of 3 μl infused over 60 s via an injection cannula (0.5 mm longer than the guide cannula) connected by polyethylene tubing to an infusion pump (Harvard Apparatus, Canada). Rats were sacrificed 20 min post injection, trunk blood was collected for corticosterone and ACTH analysis and the brains were rapidly excised and processed for radioimmunoassay (RIA).

Plasma ACTH and Corticosterone

Trunk blood was collected in tubes containing EDTA, centrifuged and the plasma was frozen at -70°C. Plasma ACTH and corticosterone levels were determined using commercial RIA kits (ICN Pharmaceuticals, Canada).

Tissue Preparation for RIA

Rats were sacrificed by decapitation and their brains rapidly extracted and frozen in a rat stainless steel brain matrix (ASI instruments, MI, U.S.A.) resting on dry ice. Prior to freezing, a razor blade was inserted through the brain to separate the cerebrum from the cerebellum and more caudal brain regions, and sectioned coronally (600 μm thick sections). The following 9 discrete nuclei were dissected, using a modification of the micropunch method described by Palkovits and Brownstein[320]: the median eminence/arcuate nucleus (Me/Arc), paraventricular hypothalamic nucleus (PVN), lateral (LH), anterior (AH), ventromedial (VMH) and dorsomedial (DM) hypothalamic nuclei, central amygdaloid nucleus (CeA), hippocampus (Hipp) and frontal cortex (Fc). The final (10^{th}) region, the nucleus of the solitary tract (NTS), was micropunched from a

horizontal section (600 μm thick) of the brain stem. All nuclei were derived bilaterally (Micro Punch MP-600, ASI instruments, U.S.A.) using a 1mm diameter punch, except for the Me/Arc, which was obtained using a single medial 1 mm diameter micropunch. The tissue punches were placed in microcentrifuge tubes containing 200 μl of 0.2 M acetic acid and heated to 80°C for 1 h. The tissue was then sonicated (Kontes Micro-ultrasonic Cell Disruptor, Kontes, NJ, U.S.A.) and 40 μl of the homogenate was removed for protein content determination, using a BCA Protein Analysis kit (Chromatographic Specialties, Canada) and a PC colorimeter (Brinkmann, Canada). The remainder of the homogenate was centrifuged at 10 000 x g for 4 min and the supernatant of each region was then divided into two 75 μl aliquots, lyophilized and stored at -20°C.

Experiment 2. Effect of central BN on the in vivo release of CRH, AVP and corticosterone at the Me/Arc region.

Animals were anesthetized (60 mg/kg pentobarbital; i.p.) and placed in a stereotaxic apparatus to implant guide cannulae (20 gauge) aimed at the Me/Arc (for push-pull assembly) and at the 4th ventricle (for microinjection cannula). In contrast to the preceding experiment, the ventricular cannula was positioned into the 4th rather than the 3rd ventricle, as the latter placement would have interfered (physically) with concomitant implantation of the push-pull cannula. The coordinates for the 4th ventricle were 2.5 mm anterior to the occipital crest, 0 mm lateral to the midline and 5.5 mm ventral from the dura. The coordinates for the Me/Arc were 2 mm posterior to bregma, 0.4 mm lateral to the midline and 9.5 mm ventral from the dura. The guide cannulae for push-pull perfusion (custom made Derelin™ pedestals) and microinjections (Plastics

One, VA, U.S.A.) were anchored to the skull surface using 4 stainless steel screws and acrylic dental cement and plugged with removable stainless steel stylettes. After a 7 day recovery period, animals were transferred to individual test cages 48 h prior to the experiment, to acclimate subjects to the test environment.

Pull-Pull Perfusion

On the test day, the stylet within the guide cannula was replaced with a push-pull probe aimed at the Me/Arc. The push-pull probe consisted of an outer (or pull) stainless steel cannula (protruding 0.7 mm beyond the end of the permanent guide cannula) and an inner (or push) cannula (glass silica) protruding 0.3 mm beyond the end of the pull cannula and into the Me/Arc. Probes were connected by polyethylene tubing (PE-20 and PE-10) to two independent, pre-equilibrated peristaltic pumps (Minipuls 3, Gilson, WI, U.S.A.), via a dual channel swivel assembly (Instech Laboratories, Horsham, PA). Kreb's ringer phosphate (KRB) solution (consisting of (in mM): 145 Na^+, 2.7 K^+, 1.35 Ca^+, 1.0 Mg^{2+}, Cl^- and 0.1% BSA) was perfused through the push cannula at the rate of 10 µl/min. Samples were collected every 15 min between 9:00 and 15:00 h. Five sequential samples were collected after each of the following manipulations: baseline (no treatment), central saline (control injection), and central BN injection (0.1 µg/3 µl). Each sample (150 µl) was immediately frozen on dry ice and stored at –80°C until RIA analysis.

Experiment 3. Effect of central BN on interstitial CRH, AVP and corticosterone levels at the anterior pituitary.

The design of this experiment was identical to that of experiment 2, with the exception that the guide cannula for push-pull perfusion was aimed at the anterior pituitary (placement coordinates: 5.0 mm posterior to bregma, 0.9 mm lateral to the midline and 0.5 mm dorsal to the sphenoid bone). The probe length was also slightly modified to reach the anterior pituitary with the outer (pull) cannula measuring 0.4 mm beyond the end of the permanent guide cannula and the inner (push) cannula measuring 0.2 mm beyond the end of the pull cannula.

Histology

Following completion of the experimental procedures, rats received an overdose of pentobarbitol and India ink (1 μl) was delivered through the push-pull probe (into the perfusion site) and the injection cannula (into the 4^{th} ventricle for experiments 2 and 3), for verification of probe or cannula placements. Brains were removed and frozen in a rat stainless steel brain matrix (ASI instruments, MI, U.S.A.) resting on dry ice, and subsequently sectioned using a cryostat and stained with thionine for histological identification and verification. For experiment 3, the pituitary gland was removed and visually inspected under a dissecting microscope, to verify that the tip of push-pull cannulae was correctly positioned within the anterior pituitary.

Radioimmunoassay (RIA)

A solid-phase RIA procedure was employed for the detection and quantification of CRH and AVP.[253,278] Corticosterone was measured in the perfusates using a micro-

adaptation[39] of the commercial RIA kit (ICN pharmaceuticals, Canada). Briefly (for CRH and AVP detection), well plates (Immulon-4, Dynatec Laboratories Inc., Chantilly, VA) were coated with protein A/G (Calbiochem Corp; La Jolla) at least 24 h before use and stored at 0-4°C. On the day of the RIA, the plates were rinsed with wash buffer (0.15 M phosphate buffered saline pH 7.4) and then with RIA buffer (0.15 M phosphate buffered saline with 0.1 % gelatin). The plates were then coated with either CRH or AVP antibody (1:100,000 final dilution) for 2 h at room temperature, and rinsed with wash buffer. The standards (reconstituted in KRB solution; ranging from 0.61 to 625 pg for CRH and 0.075 to 156 pg for AVP), blanks and samples were then pipetted into the designated wells and incubated for 24 h at 0-4°C. Next, ^{125}I-labelled CRH or AVP (Amersham, Canada; 5000 cpm) was added. Following a 24 h incubation period (at 0-4°C), the plates were rinsed with wash buffer and the individual wells counted for residual radioactivity using a gamma-counter (Canberra Packard, Cobra II Autogamma, model D5002).

The specific anti-CRH serum (rC70, kindly provided by W. Vale) recognizes CRF_{1-41} and has been shown to cross-react negligibly with other related peptides including urotesin 1 and urocortin.[403] The specific anti-AVP serum (Pheonix Pharmaceuticals, U.S.A) used in this study recognizes [Arg^8]-Vasopressin and cross-reacts 100% with [Arg^8]-vasotocin, 38% with [Lys^8]-vasopressin, and negligibly with other peptides.

Statistical Analysis

All results are expressed as means ± S.E.M. In the first experiment, plasma ACTH and corticosterone values were analyzed independently using a one factor (BN dose) ANOVA followed by Tukey post hoc comparisons. CRH and AVP concentrations were analyzed independently by one factor (BN dose) ANOVAs for individual brain regions followed by Tukey post hoc comparisons. A probability of less than 5% was considered statistically significant. In the second experiment, the basal CRH, AVP and corticosterone values for each subject were averaged individually, over the 5 baseline samples and defined as 100%. Values were then expressed as a percentage of that baseline. CRH, AVP and corticosterone values were analyzed independently for each experiment using a repeated measures ANOVA with treatment blocks (baseline, saline and BN (0.1 μg)) and samples nested within each block, treated as within measures. Post hoc comparisons were conducted using Tukey's test. A value of $p<0.05$ was considered statistically significant.

Results

Experiment 1. Effect of central BN on plasma ACTH and corticosterone levels and on regional ir-CRH and ir-AVP levels.

Plasma ACTH and corticosterone levels in rats that received central injections of either saline, or one of two BN doses (0.25 or 0.5 μg; i.c.v.) are depicted in Figure 1. ANOVA revealed a significant effect of BN Treatment on plasma levels of ACTH ($F_{2,21}=20.42$; $p<0.0001$). Post-hoc analyses revealed that the effects of BN on circulating levels of ACTH were dose-dependent. Similarly, BN significantly affected circulating

corticosterone levels ($F_{2,21}$=49.62; $p<0.0001$). Tukey post hoc comparisons indicated that the lower dose of BN significantly elevated plasma corticosterone levels and BN-elicited rise was significantly greater with the higher dose of BN.

As illustrated in Figure 2, central BN administration altered the levels of ir-CRH in a regionally specific manner. Concentrations of ir-CRH for the 10 brain regions are depicted in a descending order (from left to right). In agreement with previously published immunohistochemical findings, the highest concentration of ir-CRH was detected at the Me/Arc.[79] This finding is also concordant with the established role of CRH as the principal mediator of the HPA axis.[160,381] Moderate levels of ir-CRH were detected at the majority of the other brain regions sampled including other hypothalamic nuclei, the CeA and the NTS. Analyses of individual brain regions revealed significant effects of BN Treatment at the NTS ($F_{2,21} = 3.69$; $p<0.044$), VMH ($F_{2,20} = 13.45$; $p<0.000$), AH ($F_{2,22} = 6.18$; $p<0.008$) and CeA ($F_{2,22} = 5.64$; $p<0.011$). Tukey's post hoc comparisons revealed that both doses of BN (0.25 and 0.5 μg) decreased ir-CRH at the NTS ($p<0.05$) and the VMH ($p<0.01$). At the higher dose, BN also reduced ir-CRH levels at the AH ($P<0.01$) and at the lower dose, BN reduced ir-CRH at the CeA ($p<0.01$). In contrast, central BN administration failed to affect ir-CRH at several other regions including the PVN, LH, DM, Hipp and Fc.

As illustrated in Fig. 3, central BN administration altered ir-AVP levels in a regionally specific manner. Regional concentrations of ir-AVP are depicted in a descending order (from left to right). The highest levels of ir-AVP were observed at the

ME/Arc and the PVN. This is consistent with the literature suggesting that the principal site of AVP synthesis is within the cell bodies of the PVN (both the magnocellular and parvocelluar divisions) which project to the ME (both the internal and external laminae).[15,377] Moderate concentrations of ir-AVP were observed at several other hypothalamic regions including; the AH, VMH and LH. Relatively low levels of ir-AVP were observed at the DM as well as certain extra-hypothalamic sites. ANOVA revealed a significant effect of BN Treatment at the Me/Arc ($F_{2,22} = 5.30$; $p<0.014$), the PVN ($F_{2,22} = 4.65$; $p<0.021$) and the VMH ($F_{2,21} = 7.15$; $p<0.004$). Tukey's post hoc comparisons revealed that at the higher dose, BN significantly increased ir-AVP at the Me/Arc ($p<0.01$) and the PVN ($p<0.01$) but reduced levels of his peptide at the VMH (at the 0.25 and 0.5 μg doses; $p<0.01$). No significant alterations in ir-AVP were noted at the other brain regions sampled.

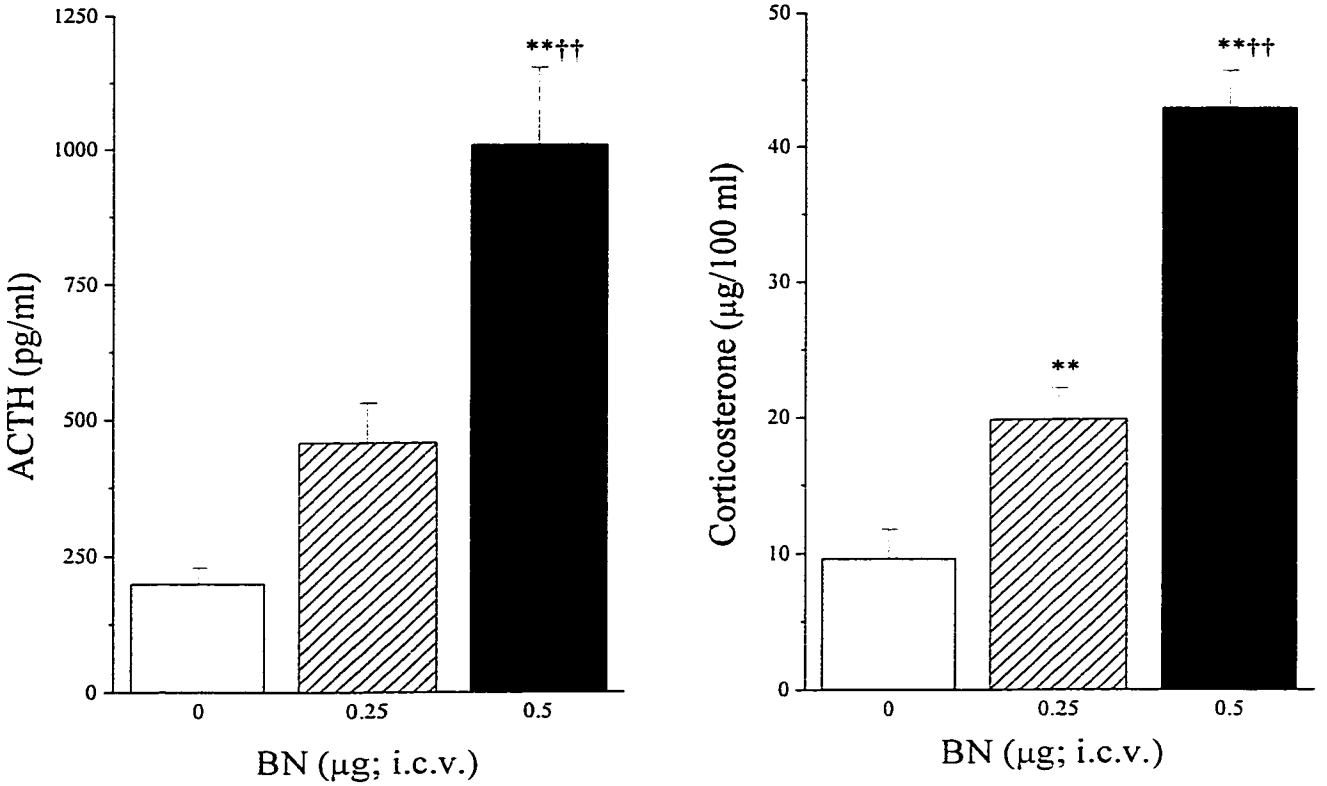

Fig. 1 Plasma levels of ACTH and corticosterone (mean ± S.E.M.) following central BN administration. The left panel represents ACTH levels expressed as pg/ml (mean ± S.E.M.). Whereas the right panel represents the circulating corticosterone levels expressed as μg/100 ml. The open columns represent control animals injected with vehicle (saline, 3 μl; i.c.v.) whereas the hatched and the solid columns represent animals injected with BN at 0.25 and 0.5 μg (i.c.v.), respectively. *,** significantly different from saline values at $p<0.05$ and $p<0.01$, respectively. †, †† significantly different from low dose BN (0.25 μg) values at $p<0.05$ and $p<0.01$, respectively.

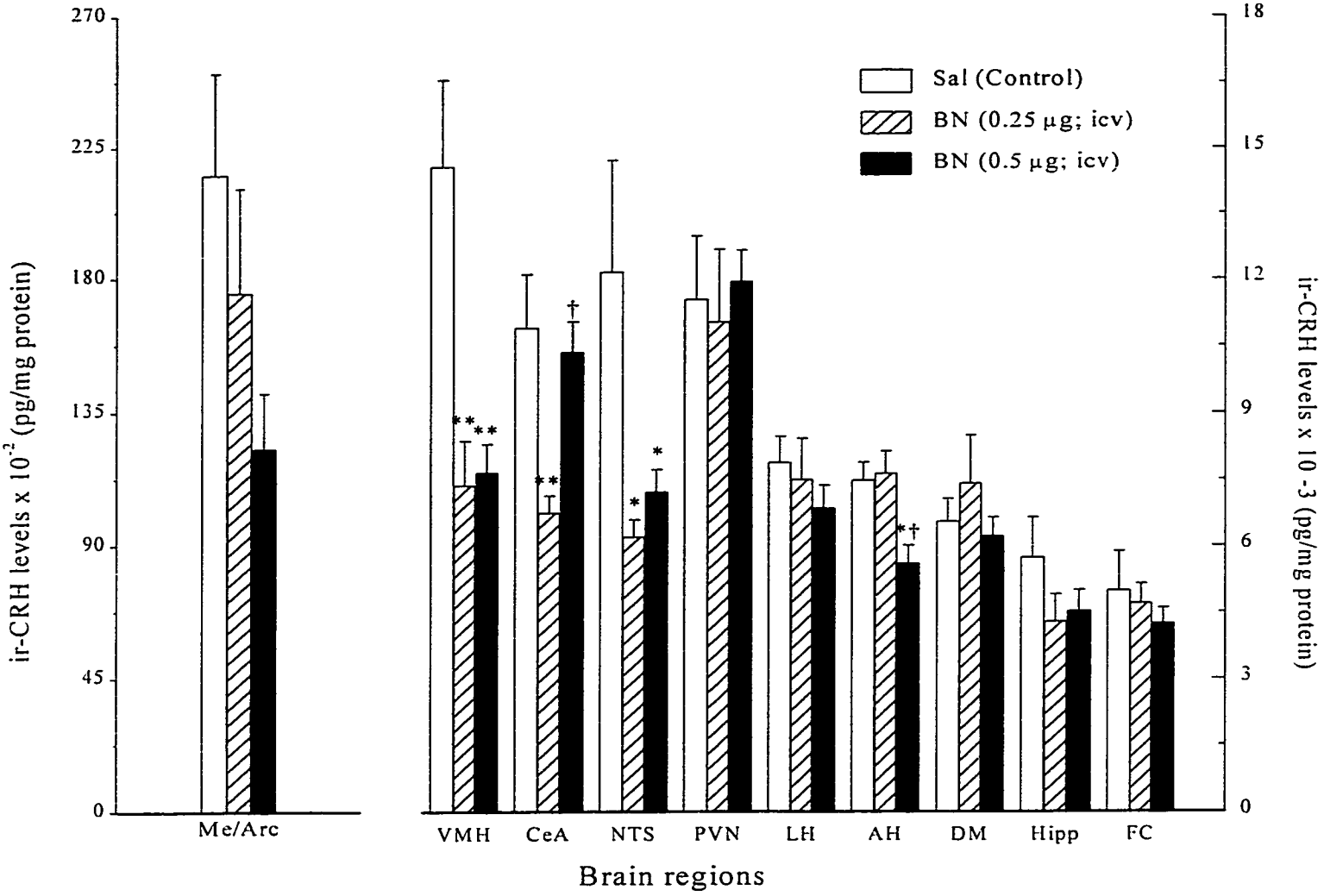

Fig. 2 Regional ir-CRH levels (mean ± S.E.M.) following central BN administration. Each column represents the concentration of ir-CRH expressed in pg/mg protein (mean ± S.E.M.). The open columns represent control animals injected with vehicle (saline, 3 μl; i.c.v.) whereas the hatched and the solid columns represent animals injected with BN at 0.25 and 0.5 μg (i.c.v.), respectively. Brain regions include: median eminence/arcuate nucleus (ME/Arc), ventromedial hypothalamus (VMH), central amygdaloid nucleus (CeA), nucleus of the solitary tract (NTS), paraventricular hypothalamic nucleus (PVN), lateral hypothalamus (LH), anterior hypothalamus (AH), dorsomedial hypothalamus (DM), hippocampus (Hipp) and frontal cortex (Fc). *, ** significantly different from saline values at $p<0.05$ and $p<0.01$, respectively. †,†† significantly different from low dose BN (0.25 μg) values at $p<0.05$ and $p<0.01$, respectively.

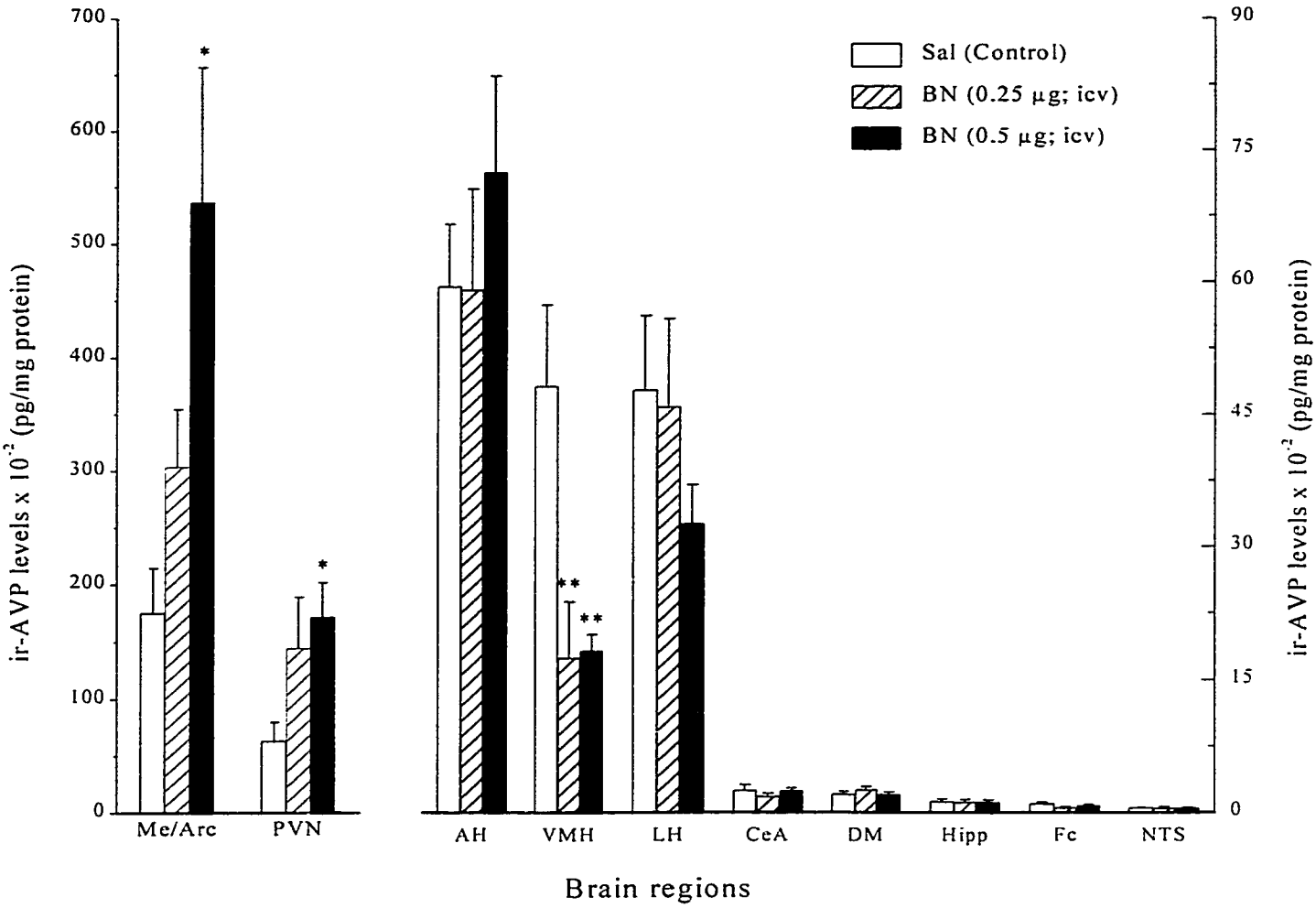

Fig. 3 Regional ir-AVP levels following central BN administration. Each column represents levels of ir-AVP expressed in pg/mg protein (mean ± S.E.M.). Open columns represent the control animals, injected with vehicle (saline, 3 µl; i.c.v.), whereas the hatched and solid columns represent animals injected with BN at 0.25 and 0.5 µg (i.c.v.), respectively. See Fig. 2. legend for abbreviations of the brain regions analyzed. *, ** Significantly different from saline values at $p<0.05$ and $p<0.01$, respectively.

Experiment 2. Effect of central BN on the in vivo release of CRH, AVP and corticosterone at the ME/Arc region.

Figures 4, 5, and 6 show BN (0.1 μg)-elicited changes in the interstitial levels of CRH, AVP and corticosterone, respectively, at the Me/Arc. All values are expressed as a percent of their respective baselines. ANOVA indicated that interstitial concentrations of both CRH and AVP varied as a function of the interaction between Treatment (Saline vs. BN) x Sample (5 repeated samples), $F_{8,48}$ =3.34; $p<0.004$ and $F_{8,40}$ = 7.81; $p<0.000$, respectively. Tukey's post hoc comparisons revealed that these interactions were attributable to the differential effect of the treatments on the release of both CRH and AVP. Tukey's post hoc comparison's further revealed that BN elicited a significant increase in the release of both CRH and AVP at the Me/Arc as compared to both baseline- and saline-control conditions. The release of CRH and AVP occurred soon after BN administration, being evident within the first 2 samples post-BN sampling intervals, and peaked at the 3rd sample. ANOVA also revealed a significant Treatment effect for corticosterone levels ($F_{2,10}$ = 18.26; $p<0.0004$). Tukey's post hoc comparisons indicated that BN administration significantly increased interstitial levels of corticosterone at the Me/Arc as compared to both the non-injected and saline-injected control conditions. Again, the corticosterone rise occurred rapidly and was still climbing at the 5th sample. In animals with off- target 4th ventricular placements (n=5), BN failed to alter the levels of CRH, AVP or corticosterone, compared to their respective baseline levels (data not shown).

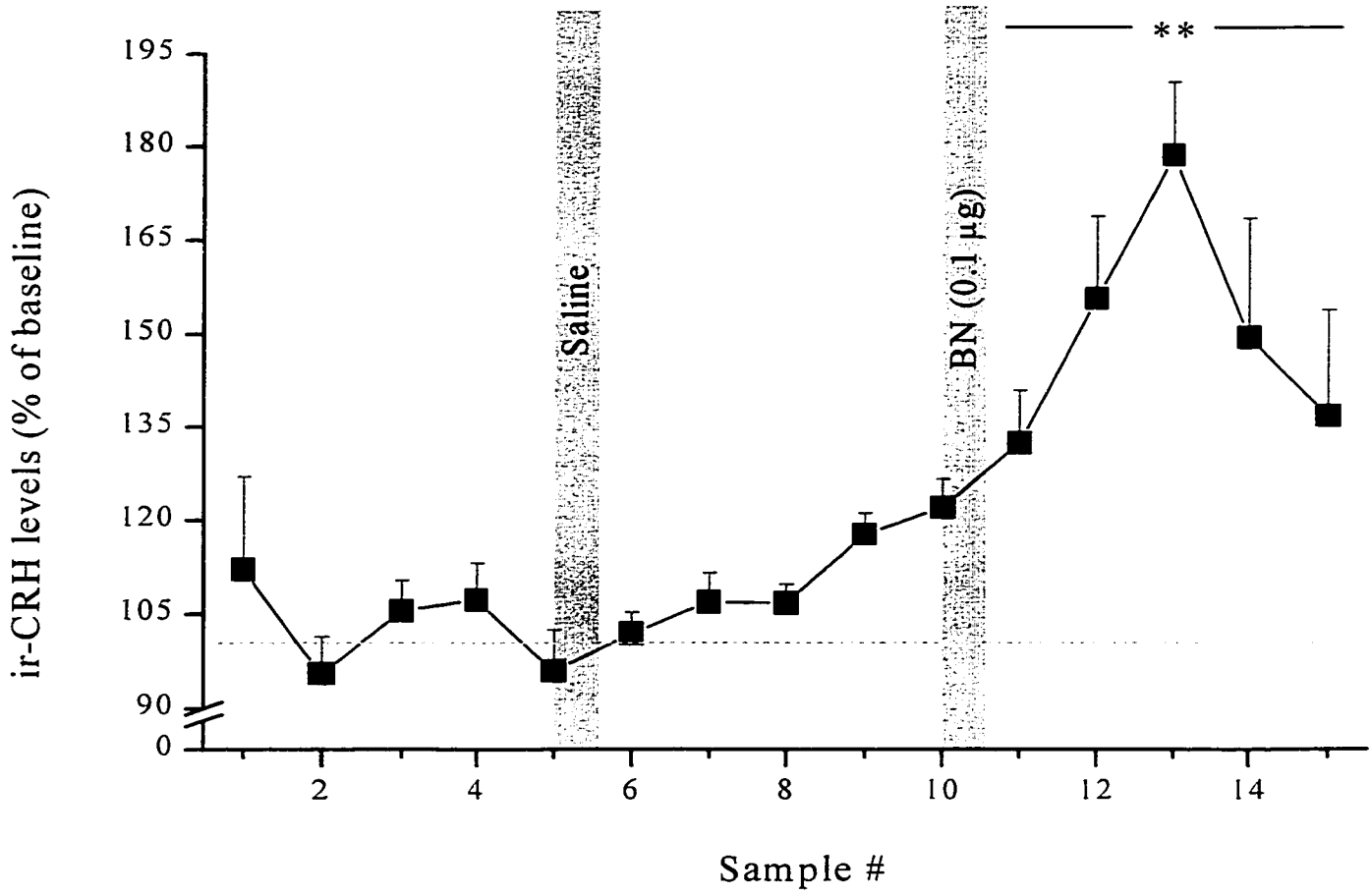

Fig. 4 Levels of CRH at the Me/Arc under baseline condition, and following central administration of saline and BN (0.1 µg). The basal values for each subject were averaged over the 5 baseline samples and defined as 100%. All values were then expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

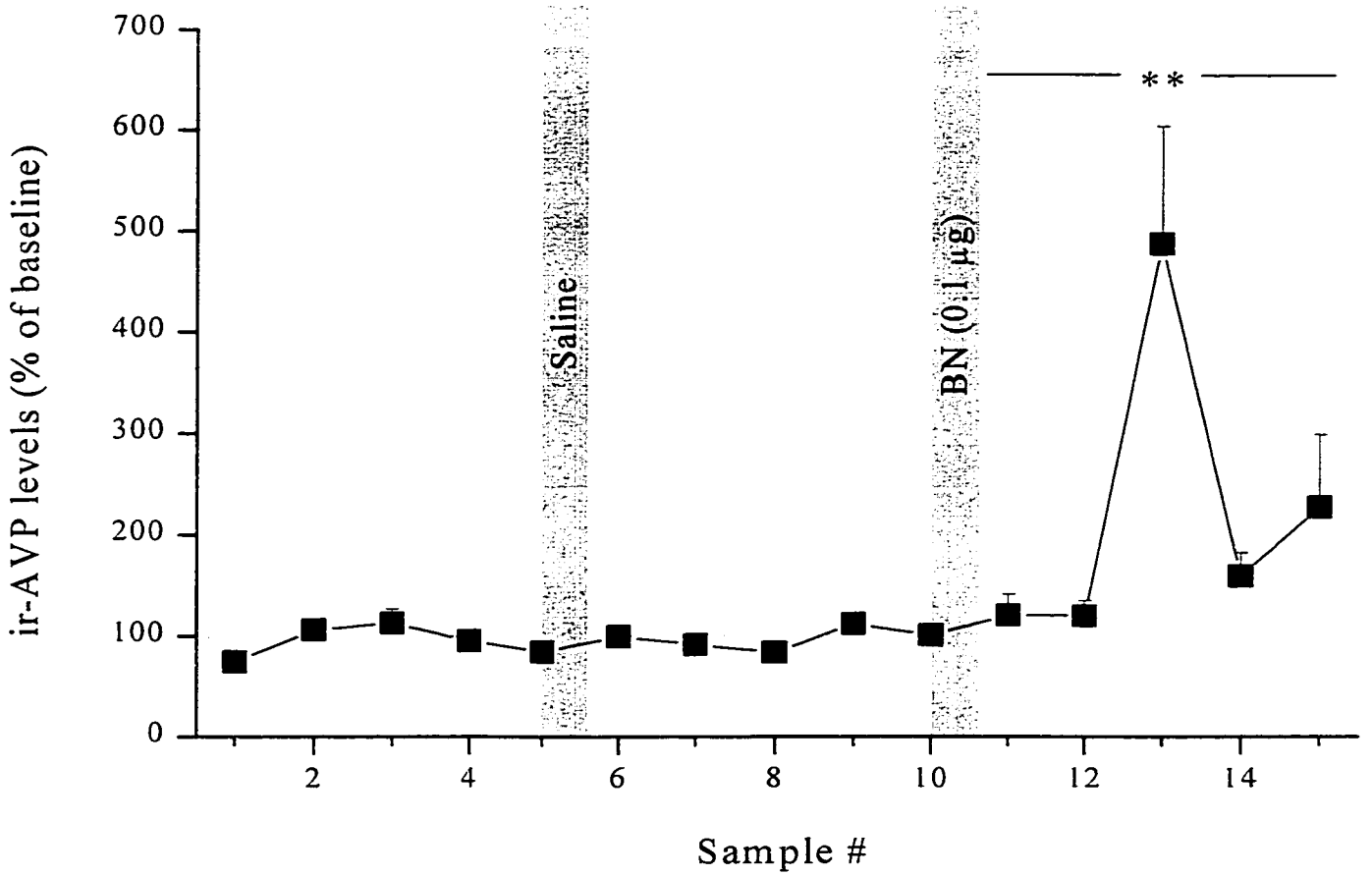

Fig. 5 Levels of AVP at the Me/Arc under baseline condition, and following central administration of saline and BN (0.1 μg). The basal values for each subject were averaged over the 5 baseline samples and defined as 100%. All values were then expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

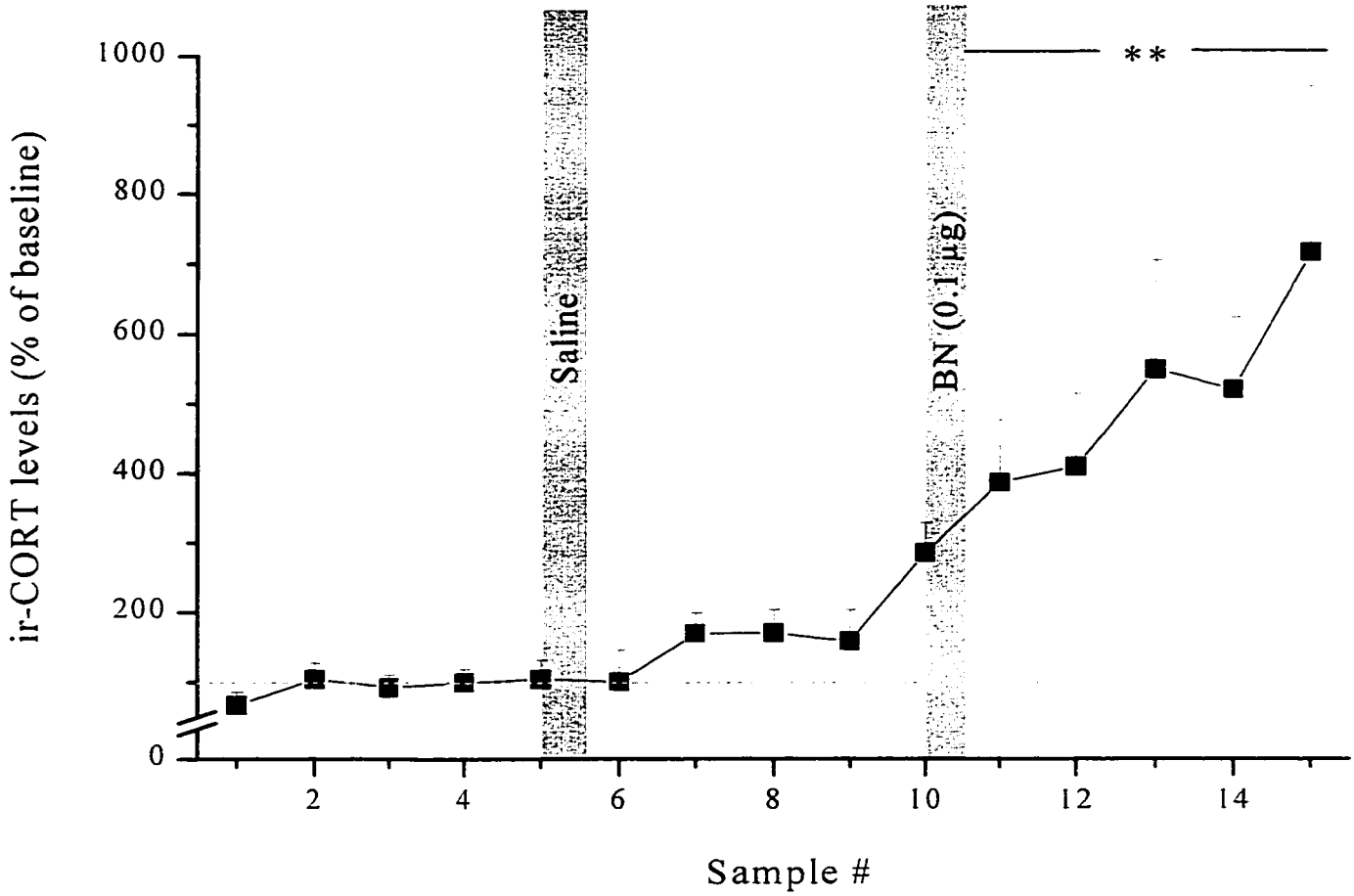

Fig. 6 Levels of corticosterone (CORT) at the Me/Arc following central administration of saline and BN (0.1 µg). The 5 baseline sample values were averaged and defined as 100%. All values are expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

Experiment 3. Effect of central BN on interstitial CRH, AVP and corticosterone levels at the anterior pituitary.

The influence of central BN on interstitial levels of CRH, AVP and corticosterone at the anterior pituitary are illustrated in figures 7, 8 and 9, respectively. The values are depicted as a percent of non-injected baseline condition following the control (saline) injection, and then following BN (0.1 μg) administration. The analysis revealed that BN administration significantly elevated CRH levels in comparison to control (saline) injection ($F_{2,14} = 8.76$; $p<0.003$). Furthermore, AVP levels varied as a function of the Treatment x Samples interaction ($F_{8,80}=2.26$; $p<0.03$). Tukey's post hoc comparisons revealed that BN administration elicited a significant increase in the availability of CRH and AVP at the anterior pituitary as compared to the saline injection condition. Relative to the saline controls, the increase of interstitial CRH and AVP levels was evident by the 2nd sample following BN injection, and peaked at the 4th sample. It was further observed that corticosterone levels varied as a function of the Treatment x Sample interaction ($F_{8,72} = 3.57$; $p<0.001$). Tukey's multiple comparison's revealed that BN administration significantly increased the availability of corticosterone at the pituitary as compared to both baseline conditions (non-injected and saline-injection). The increase of corticosterone availability occurred rapidly, being evident by the 2nd sample post BN injection, and was still increasing at the 5th sample.

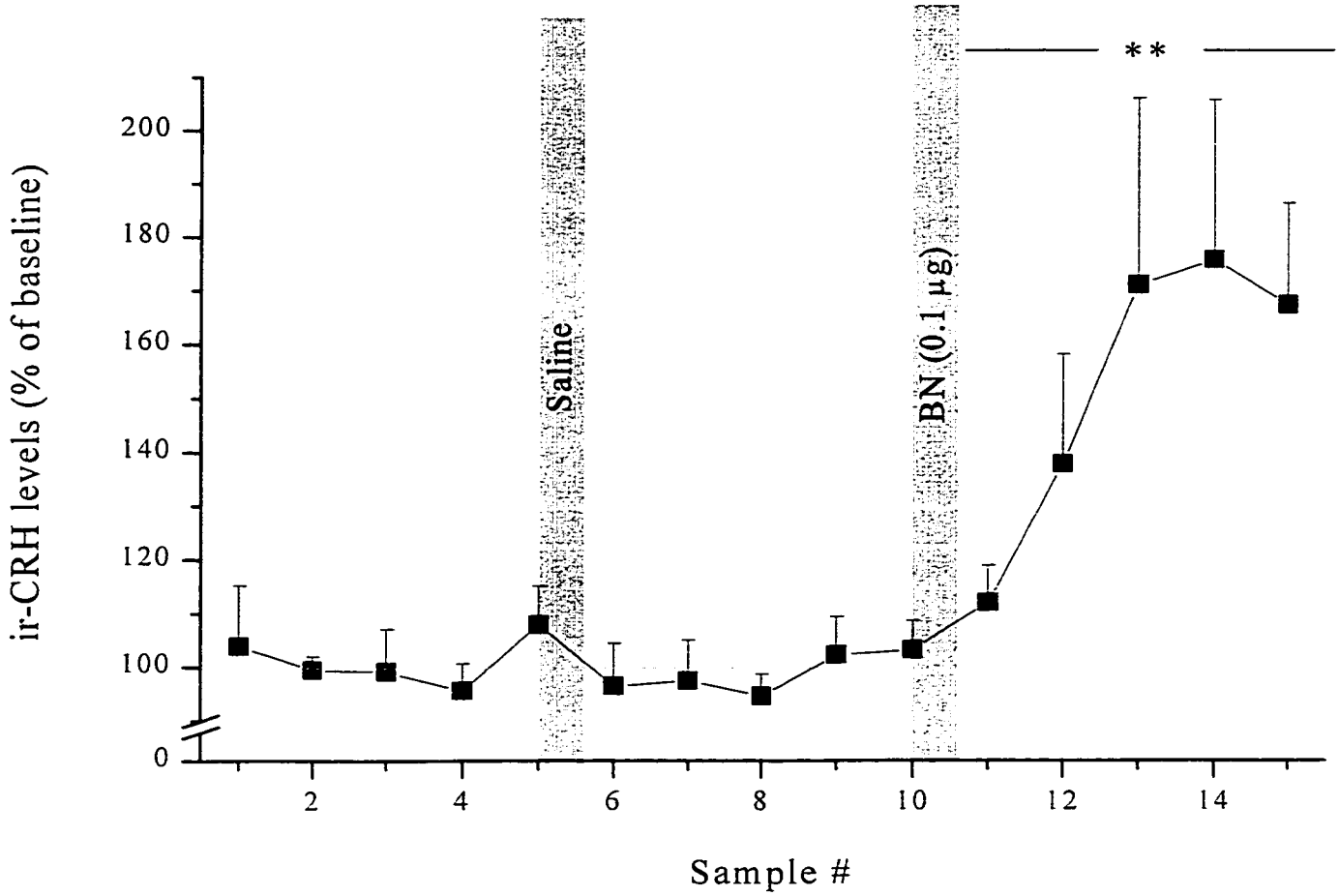

Fig. 7 Effects of central BN (0.1 μg) administration on interstitial CRH levels at the anterior pituitary. The 5 baseline values for each subject were averaged and defined as 100%. All values are expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

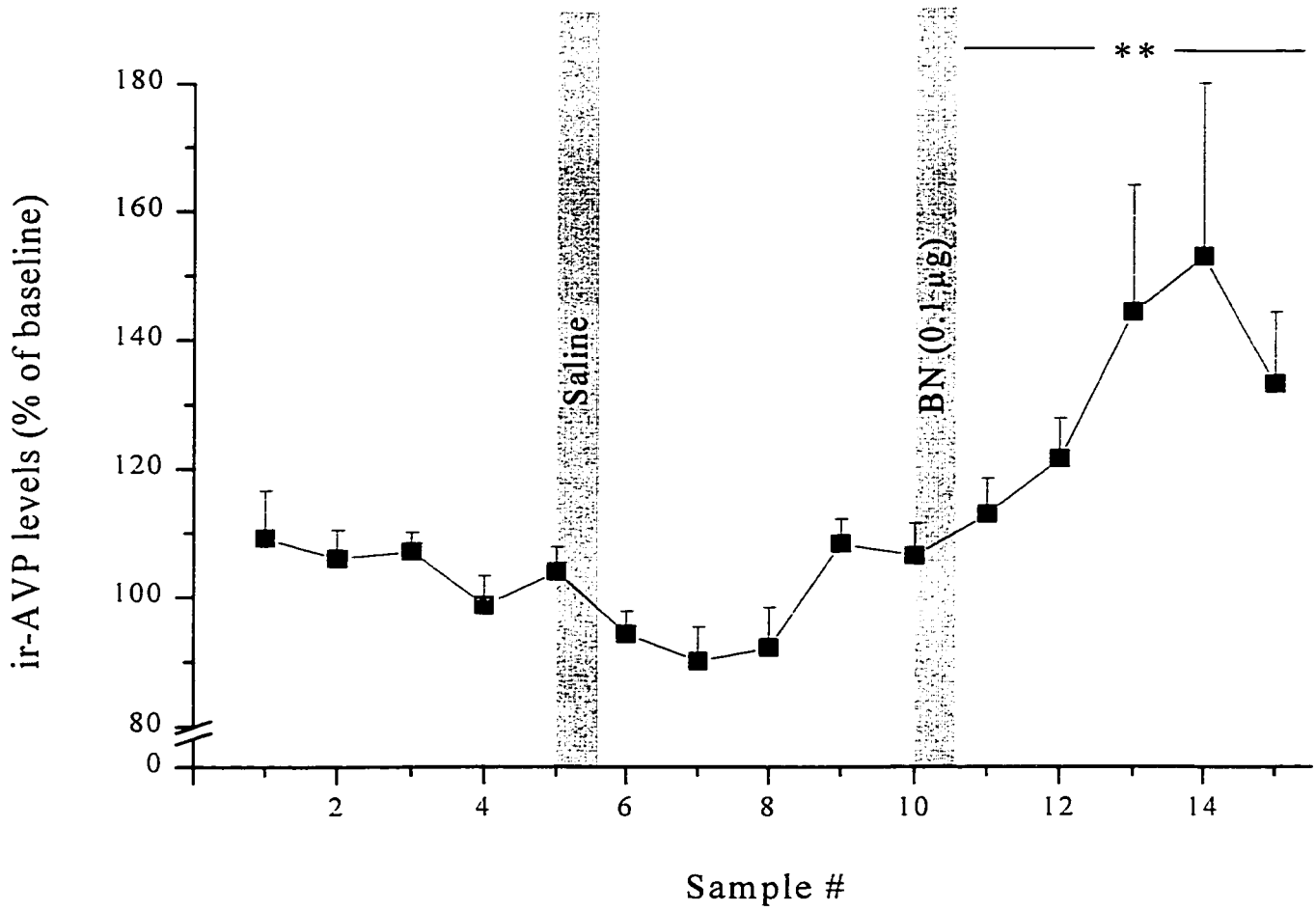

Fig. 8 Effects of central BN (0.1 μg) administration on interstitial levels of AVP at the anterior pituitary. The 5 baseline values for each subject were averaged and defined as 100%. All values are expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

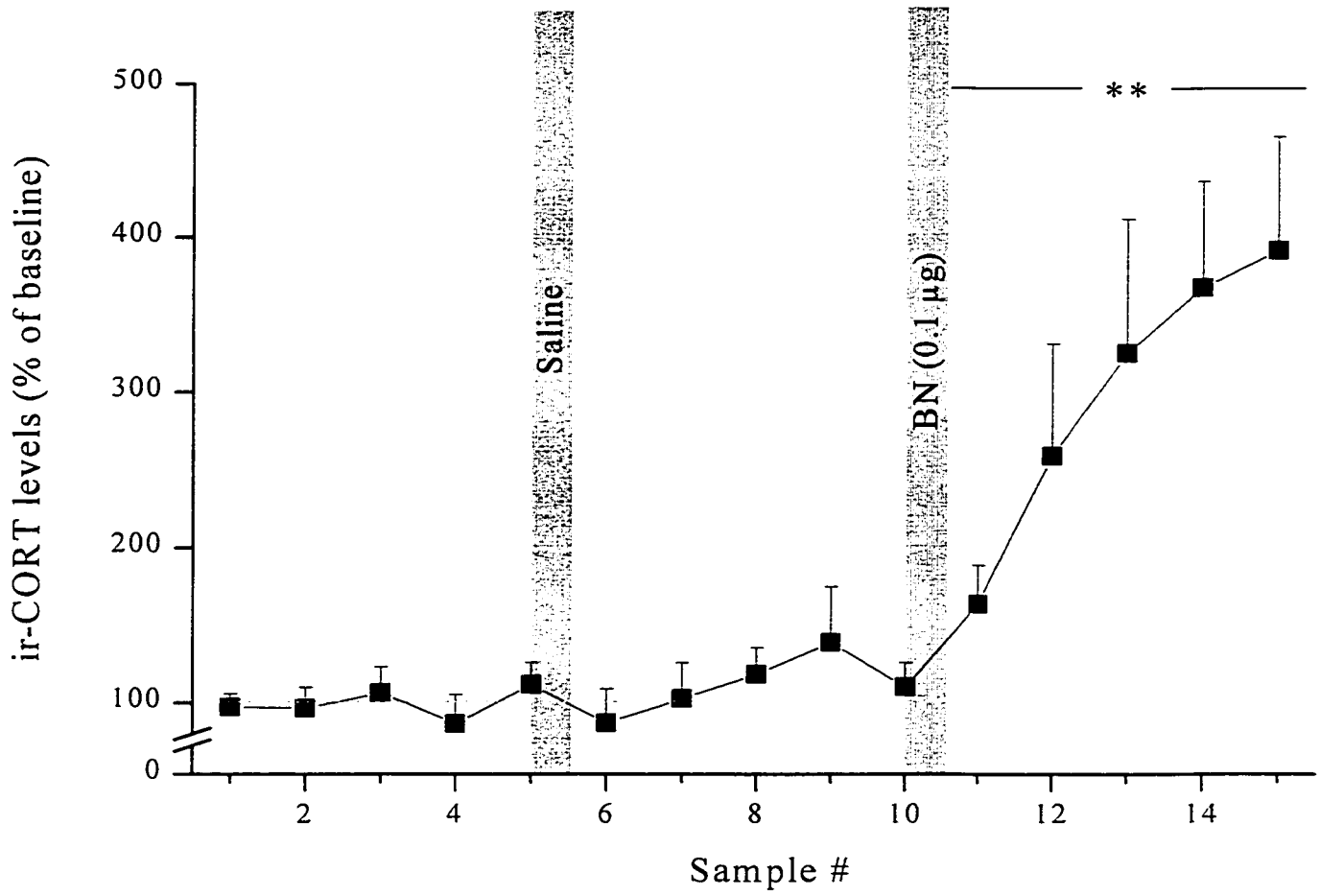

Fig. 9 Interstitial levels of anterior pituitary corticosterone (CORT) following central BN (0.1 µg) administration. The 5 baseline values were averaged and defined as 100%. All values are expressed as a percentage of that baseline. *, ** significantly different from baseline condition at $p<0.05$ and $p<0.01$ respectively.

Discussion

The HPA axis plays a critical role in preparing the body to deal with actual as well as anticipated homeostatic perturbances. Amongst the most potent activators of this axis are stressors of various sorts including systemic and processive-type stressors.[160] Although it is well established that the activation of CRH and/or AVP neurons, emanating from the PVN hypothalamic neurons and terminating at the ME, represent one of the key events in the activation of the HPA cascade[160,211,231,349], the mechanism(s) triggering CRH activation remain less well characterized. Furthermore, although the actions of CRH are known to be potentiated by AVP[15,136] (which may be co-released with CRH), the potential role of other peptides in such synergistic or additive actions needs to be explored. We have recently proposed a working hypothesis suggesting that BN and related peptide(s) may contribute to the activation of the HPA axis by promoting the release of CRH and/or AVP. If BN related peptides do indeed affect the activity of CRH and/or AVP containing neurons, then exogenous administration of such ligands might be expected to influence the endogenous levels CRH and/or AVP. The initial mapping study (using brain micropunch approach) revealed that BN administration did indeed influence the levels of ir-CRH and ir-AVP at various hypothalamic and extrahypothalamic brain regions, many of which are thought to play a role in the stress response. Within the hypothalamus, both doses of BN (0.25 and 0.5 μg) significantly decreased ir-CRH and ir-AVP at the VMH. At the higher dose, BN decreased ir-CRH at the AH and increased ir-AVP at the Me/Arc and PVN. These findings are consistent with other lines of evidence indicating that hypothalamic nuclei are likely sites for interactions between BN and CRH and/or AVP. For instance, high levels of immunoreactivity as

well as receptor density for all three peptides were found within many of the hypothalamic nuclei.[23,79,88,295,377] In addition, while CRH and AVP are co-localization within some PVN neurons terminating at the ME[397,422], ir-BN has also been reported to be co-localized with ir-CRH within the external zone of the ME.[138] Recent studies have also revealed strong c-Fos activation at the PVN following central administration of BN.[240] Microinfusions of BN at the PVN dose-dependently increased blood corticosterone.[150] In addition, we have recently demonstrated that acute immobilization stress is associated with fluctuations in ir-BN levels within the hypothalamus as well as alterations in the density of BN binding sites at specific hypothalamic nuclei including the PVN and Arc.[195] Given that much of this evidence points towards the PVN and/or ME as likely sites for possible interactions between BN-like peptides, CRH and/or AVP, the lack of fluctuations in ir-CRH at these regions in response to BN infusion was somewhat surprising. It is conceivable that post-mortem peptide content may remain unchanged despite increased release or utilization of the peptide, in cases where the rate of release is matched by increased rate of peptide synthesis. Although a significant increase in ir-AVP was noted at the PVN and Me/Arc, similar ambiguity exists for interpretation of these results, as underlying processes are not revealed from alterations in the tissue peptide content. Thus, a second set of experiments was performed using a more dynamic assessment of CRH and AVP release (in contrast to "snap shot" tissue level assessment) at selected sites. Since the Me/Arc plays a pivotal role in HPA activity (being the primary source of CRH release), push-pull perfusion was performed at this region and also downstream at the anterior pituitary, where these peptidergic messages influence ACTH release.

Push-pull perfusion revealed that central administration of BN elicited the release of CRH and AVP from the Me/Arc and increased the availability of these peptides downstream at the anterior pituitary. It should be noted that a lower dose of BN was used in this experiment (0.1 μg) as compared to the doses used in the initial study (0.25 and 0.5 μg) because BN effects have previously been shown to be more potent at the 4th ventricle as compared to the 3rd or lateral ventricles.[126,280] The time-line for the increase of interstitial levels of CRH and AVP at the Me/Arc and anterior pituitary were very similar, supporting the view that there was a tight temporal and anatomical association regarding the BN effects on these peptides. This finding is consistent with the contention that AVP is co-secreted with CRH from nerve terminals in the external zone of the ME, to affect ACTH secretion. There is significant co-localization of CRH and AVP within the nerve terminals at the ME.[422] In fact, some studies have shown that AVP in the external zone of the ME is located primarily in CRH-containing nerve terminals, and that AVP and CRH are co-released from this region.[396] Using a different stimulus, namely intrahypothalamic infusion of interleukin-1β, Watanoboe & Takebe (1993)[418] observed the coincident release of CRH and AVP at the PVN and the ME, not unlike those elicited by BN at the Me/Arc and the pituitary, in the present investigation. Although it is commonly assumed that CRH and AVP act conjointly (or synergistically) at the pituitary, the concomitant release of these two peptides at the anterior pituitary have, to our knowledge, never been assessed directly. Given the intimate relationship between the ME and anterior pituitary, a similar temporal profile would be expected at these two sites with respect to CRH and AVP availability. As the ME is the main site of CRH and AVP release, it is possible that our peptide 'pick up' at the anterior pituitary reflects CRH and

AVP released upstream. However, NMB and GRP receptor mRNA have been demonstrated in the anterior pituitary[169], and BN has been shown to potentiate CRH-induced ACTH secretion in mixed anterior pituitary cell cultures.[19] These findings suggest that BN is capable of eliciting the local release of ACTH from the anterior pituitary.

It is now technologically feasible to measure interstitial levels of corticosterone within various brain structures, using *in vivo* microdialysis.[39] Using push-pull perfusion technique in the current study, we demonstrated that central BN administration markedly increased interstitial levels of corticosterone at the ME/Arc and the anterior pituitary. Likewise, central BN administration increased plasma corticosterone and ACTH levels. These findings are concordant with past research reporting BN-elicited increases in circulating corticosterone levels.[257,282] In the current study, perfusates were collected at 15 min intervals, and hence the temporal resolution was inadequate to determine whether the CRH and AVP variations preceded the rise of corticosterone. However, the decline in the levels of the CRH and AVP did precede those of corticosterone.

In addition to the changes at the hypothalamic level, the tissue-punch study revealed that BN induced a marked reduction in ir-CRH at the NTS. Certain brainstem nuclei are known to play a prominent role in the regulation of the HPA axis.[131,160] Mapping studies have shown that the NTS receives direct sensory input from the vagal and glossopharyngeal nerves and gives rise to noradrenergic projections that extend to the parvocellular division of the PVN as well as other basal forebrain nuclei.[347] In addition

to peptidergic interaction within the HPA axis, other findings also suggest BN-like peptide-CRH interactions at the NTS. For instance, like certain hypothalamic nuclei, the NTS contains both ir-CRH and ir-BN as well as CRH and BN binding sites.[79,88,295,322] In addition, both BN and CRH have been shown to stimulate neuronal activity at the NTS, as induction of c-fos mRNA expression is noted following central administration of either of these peptides.[176,240] Moreover, we have recently reported that acute immobilization stress is associated with significant alterations in the density of BN binding sites at the NTS.[195]

Finally, central BN administration (0.25 μg) also decreased ir-CRH levels at the CeA. This is particularly interesting, as the CeA is believed to be involved in the stress response, and may influence HPA axis activity through direct or indirect projections to the PVN.[145,160] As in the case of other sites where BN affected ir-CRH level alteration, the CeA is also rich in ir-BN and ir-CRH and is endowed with receptors for BN-like peptides and CRH, making this nucleus another likely site for functionally important BN-like peptide-CRH interactions.[79,88,295,322] Indeed, the CeA was recently implicated in the mediation of BN's stress-like effects as exposure to restraint stimulated the release of ir-BN from the CeA.[278] It was somewhat surprising however, that only the lower dose of BN altered ir-CRH content. This anomaly might be related to greater release with the higher dose and a greater compensatory increase in synthesis at the time of sampling. i.e. these changes may have occurred faster and returned to normal levels at the time of sacrifice. Alternatively, this may have been due to other phenomena, such as an inverted-U dose response at this nucleus. Indeed, previous findings have shown that

microinjection of increasing doses of BN (50 to 150 ng/rat) into various hypothalamic nuclei had varying effects on serum free fatty acids (i.e. stimulatory or inhibitory), depending on the site or dose of peptide administration.[150]

It is conceivable that the peptidergic changes noted after BN administration may have been indirect rather than direct (or pharmacological) effects. For instance, central BN is known to elicit behavioral changes, which may indirectly influence other peptidergic systems. The doses utilized have varied widely (0.01 to 3 μg; i.c.v.), however, the more commonly used doses have ranged between 0.01 – 1 μg of BN (i.c.v.).[135,219,279,329] As the doses of BN used in the present study exceed the minimum necessary to induce a behavioral change, the observed BN-elicited central peptidergic alterations may have been secondary to BN-elicited behavioral alterations. Furthermore, although stressors promote peptidergic changes similar to those evoked by central BN administration, it is possible that these doses may be in a pharmacological (rather than a physiological) range, and might not necessarily simulate endogenous fluctuations normally associated with environmental challenges. Finally, it can be argued that some of the BN-elicited regional changes in CRH and/or AVP may have occurred due to diffusion of BN from the ventricular system to brain regions not typically affected under physiological conditions, or for that matter, from leakage of the peptide into systemic circulation. Although the former possibility can not be refuted, the latter possibility appears unlikely given that systemic administration of BN at even higher doses (up to 4 μg/kg; i.p.), failed to engender endocrine variations (unpublished results). This is also

congruent with the observation that central blockade of GRP receptors attenuates stressor-elicited rise in ACTH and corticosterone.[133]

In conclusion, results from the present study demonstrate that central BN administration induces site-specific alterations in the turnover of CRH and AVP at several brain sites associated with the HPA axis. Specifically, these experiments are the first to demonstrate 1) BN-induced fluctuations in tissue levels of ir-CRH and ir-AVP at discrete brain nuclei and 2) BN's stimulatory action on *in vivo* release of CRH and AVP at the Me/Arc and the consequent increased availability of these ACTH secretagogues at the anterior pituitary. These findings support our contention that BN-like peptides are important in the mediation or modulation of the HPA activity observed in response to stressful or appetitive stimuli, and may involve circuits releasing CRH and/or AVP.

General Discussion

In preparation to deal with actual (or anticipated) homeostatic disturbances, the body responds by initially activating the sympathetic nervous system (reflected by the release of epinephrine and norepinephrine from the adrenal medulla) and the activation of the HPA axis (reflected by sequential release of CRH from the hypothalamus (into the portal circulation), ACTH from the anterior pituitary and finally corticosterone (or cortisol) from the adrenal cortex). Amongst the most potent activators of these systems are stressors of various sorts including systemic (infections)-type and processive (psychological)-type stressors.[160] It is possible that these differing stimuli activate different neural circuits and/or transmitter systems, eventually converging upon and activating CRH and/or AVP neurons in the parvocellular division of the PVN. In addition to the relatively well established role of classical neurotransmitters, such as norepinephrine, in the mediation of the stress response, there is reason to believe that the BN family of peptides may also contribute to the integration and/or mediation of these responses. Indeed, BN-like peptides share an anatomical distribution pattern that is similar to that of other stress relevant peptides including CRH and AVP.[79,297,377] In addition, central BN administration produces endocrine, autonomic and behavioral effects which resemble those elicited by stressor exposure as well as by exogenously administered CRH or AVP.[53,133,134,180,215,275,313] The striking similarities between BN-like peptides, CRH, AVP and stressor exposure in terms of their anatomical distributions, endocrine, autonomic and behavioral effects led us to hypothesize that i) BN-like peptides may be important in the mediation and/or integration of the stress response and ii) that BN-like peptides may in part, mediate their stress-relevant effects through

activation of CRH and/or AVP circuits. The overall objective of this research project was to test these working hypotheses from a set of 5 major studies addressing specific questions from both physiological and pharmacological perspectives.

The thrust of the physiological approach was based on the contention that stressor exposure should evoke endogenous fluctuations in the utilization of stress relevant neurochemicals. Thus, techniques such as gross brain dissection, receptor autoradiography, brain micropunch, *in vivo* microdialysis and push-pull perfusion, were employed to determine if exposure to stressors was associated with alternations in the endogenous system(s) utilizing BN-like peptides. Using such an approach, we demonstrated that stressor exposure did indeed evoke changes in post-mortem tissue levels of BN-like peptides, the density of BN binding sites (chapters I and II) and in the availability of these peptides at the anterior pituitary (chapter III). Another approach to test our working hypotheses was to utilize pharmacological tools. From this perspective, we investigated whether BN-like peptides mediated their stress relevant effects through activation of CRH and/or AVP neurons. Specifically, we assessed whether the blockade of CRH receptors attenuated BN-induced autonomic and endocrine activation. Next, we examined the effects of centrally administrated BN on endogenous system(s) utilizing CRH and/or AVP (using brain micropunch and *in vivo* push-pull perfusion). Our results showed that blockade of CRH receptors with αh-CRF significantly attenuated BN-induced HPA and autonomic activation (chapter IV), and that exogenously administered BN elicited site-specific alterations in both ir-CRH and ir-AVP, as well as the release of CRH and AVP from the Me/Arc translating into an increased availability of these ACTH

secretagogues at the anterior pituitary (chapter V). Together, these data provide evidence for the involvement of BN-like peptides in the mediation and/or modulation of the stress response and suggest that BN-like peptides may mediate their stress relevant effects, at least in part, via CRH- and/or AVP-sensitive circuits.

Physiological Approach

Prior to the initiation of this thesis project, evidence supporting the involvement of BN-like peptides in the mediation and/or modulation of the stress response was minimal and indirect. In particular, there were a few studies demonstrating that exogenous administration of BN or related peptides (GRP or NMB) elicited endocrine, autonomic and behavioral effects, some of which resembled those induced by stressor exposure or CRH administration.[53,133,134,180,215,275,313] What was lacking, however, was *direct* evidence functionally linking the BN family of peptides to the mediation and/or modulation of the stress response. One approach to establish an association between BN-like peptides and stress response would be to assess whether stressor exposure functionally affected the endogenous system(s) utilizing BN-like peptides. For instance, does stressor exposure alter a) the release of BN-related peptides, b) the tissue levels and/or turnover of these peptides, c) their mRNA expression, and/or d) the receptor function of these peptides? A large portion of this thesis was devoted to addressing questions of this nature. In chapter I, we reported that acute immobilization stress was associated with site-specific alterations in endogenous levels of BN-like peptides at the hypothalamus and medulla and that the density of BN binding sites at the NTS, arcuate nucleus and PVN was altered in parallel. We were then interested in exploring whether

the stress-related peptidergic alterations were dependent upon a) the nature of the stressor, and b) genetic differences in stressor vulnerability. Subsequently, in chapter II, we observed that in 2 lines of rats with differential sensitivities to stressors (Fast and Slow seizing), acute exposure to a natural predator (i.e. ferret) or restraint induced alterations in ir-BN levels at the anterior hypothalamus and NTS. Collectively, these findings provided the first accounts of alterations in the BN-like peptide system in response to stressor exposure, confirming a functional relationship between this family of peptides and the stress response. It is interesting to note that despite differences in the genetic makeup and the nature of the stressors, the regionally specific pattern of stress-related changes in the content of BN-like peptide and the density of BN binding sites was quite similar. In particular, alterations in the BN-like peptide system(s) were observed within the hypothalamus and medulla. Unfortunately, in the earlier studies (chapter I), the gross dissection procedure was used which precluded identification of the specific nuclei within the hypothalamus and medulla, that may have been responsive to stressor exposure. The higher anatomical resolution provided by receptor autoradiography (chapter I) and the subsequent brain micropunch studies (chapter II) revealed that specific sites within the hypothalamus (such as the arcuate, paraventricular, and anterior nuclei) and within the medulla (such as the NTS), may be involved in BN's mediation of the stress response. These results are consistent with *in situ* hybridization findings showing a strong induction of immediate early gene expression at the NTS and numerous hypothalamic nuclei following exposure to a variety of different stressors.[65,77,319] It is notable that the brain micropunch and autoradiographic experiments did not reveal any stressor-induced alterations in ir-BN or BN binding sites at limbic structures such as the

amygdala or hippocampus. The stressors employed in chapters I and II (immobilization/restraint stress and ferret exposure) are considered psychological or "processive" in nature, and are thought to require significant sensory processing by the limbic structures in the brain in order to take on physiologic meaning.[160] It is possible that the BN family of peptides is involved with generalized reactivity to stressors (involving direct HPA axis activation) as opposed to higher order interpretation of the stressor (which would involve activation of limbic brain structures). In contrast to the lack of peptidergic alterations at limbic structures for BN-like peptides, we observed (in chapter II) significant fluctuations in ir-CRH content and the endogenous release of CRH at the CeA following restraint stress and ferret exposure. Based on these results, it would seem that BN-like peptides and CRH play distinctive roles in the stress response, with CRH being more involved with higher order sensory processing in addition to direct HPA activation. This interpretation, is however, quite tentative particularly as the *in vivo* release of BN-like peptides from the CeA was not assessed in experiments presented in chapter II. Although no change in ir-BN at the CeA was observed, it is conceivable that this "snap shot" assessment of post-mortem tissue peptide levels may has missed detecting changes in turnover, if for instance, the alterations in peptide utilization were offset by compensatory changes in peptide synthesis or degradation. Indeed, in a subsequent *in vivo* microdialysis study, we observed that acute restraint stress elicited the release of both CRH and BN-like peptide from the CeA.[278] This demonstrates that these peptides may be involved in the physiological manifestation of the stress response and that the lack of peptide level changes (post-mortem) does not necessarily reflect lack of effect on the peptide utilization.

Taken together, these studies demonstrate that exposure to a variety of different stressors induced site-specific changes in the endogenous levels of BN-like peptides. While these results give some indication of key structures where BN-related peptides might be acting to mediate and/or modulate the stress response, they do not give any insight into the underlying mechanism(s) of action. This is especially true because alterations in post-mortem tissue content (or lack there of) are difficult to interpret. For example, increased tissue peptide levels of ir-BN may reflect a) an increased synthesis of the peptide and/or b) decreased release of the peptide, or c) increased peptide release accompanied by even greater rate of its synthesis. Similarly, decreased tissue levels of ir-BN may indicate a) decreased synthesis and/or b) increased release or c) increased synthesis accompanied by an even greater rate of release. Moreover, a lack of fluctuations in tissue peptide levels may be ambiguous as this could reflect either a) no change in the rate of synthesis and/or release or b) a rate of release matched by increased rate of peptide synthesis. Due to the limitations in the interpretation of regional peptide level changes, in chapter III, we set out to monitor, using push-pull perfusion technique, the dynamics of the *in vivo* release of BN-like peptides (GRP and NMB), in addition to other stress-relevant peptides including CRH and AVP, from the anterior pituitary in response to stressor exposure. We selected the anterior pituitary as the target site for these studies because it is an integral component of the HPA axis as the primary target of peptides released from the Me/Arc and the source of ACTH secretion during the stress response. In addition, in a recent micropunch experiment, we found striking alterations in ir-BN at the pituitary in response to audiogenic stressor exposure (data not shown). In that experiment the peptidergic alterations were protracted, as changes in ir-BN were

observable 24 h after exposure to the noise stress. To further explore the role of the BN family of peptides in long-term stressor-related changes, a second objective of chapter III was to assess whether prior stressor exposure had any long-lasting effects on the GRP and/or NMB systems (as well as on the CRH and AVP systems). Thus, animals were exposed to either no stressor, 14 sessions of daily restraint (chronic), or one session of restraint followed by the passage of time (14 days; acute) and then the *in vivo* release of GRP, NMB, CRH and AVP was assessed at the anterior pituitary. We observed that basal interstitial levels of GRP, NMB, CRH and AVP at the anterior pituitary were significantly elevated in animals previously exposed to either chronic or acute restraint stress as compared to naïve controls. In response to a subsequent novel stressor (air-puffs) exposure, the naïve rats showed a rapid and pronounced increase in the availability of ir-GRP, ir-NMB, ir-CRH and ir-AVP at the anterior pituitary. Although rats with previous stress experience also showed enhanced interstitial levels of GRP, NMB, CRH and AVP following air-puff exposure, the rise was more delayed and less pronounced as compared to the rise observed in the naïve control rats. These findings have several important implications. To begin with, although changes in the CRH and AVP systems following previous stressor experience have been documented in the past, using indirect measures, our results are the first to directly show that the availability of these secretagogues is altered by the prior stress history of an organism and that the stress history also differentially influences the GRP and NMB systems. The fact that basal levels of GRP and NMB were enhanced in rats previously exposed to acute or chronic restraint as compared to naïve rats suggests that these peptidergic systems may become up-regulated following conditions of prior stress experience. Thus, it is possible that BN-

like peptides may serve a permissive or modulatory role in the stress response over the short-term, which may change to a more dominant one, if the system has been primed by previous chronic or acute stressor exposure with adequate passage of time. More research is needed, however, to further explore this contention. Our results also demonstrated an increased availability of interstitial GRP, NMB, AVP and CRH levels at the anterior pituitary in response to an acute stress challenge. It was hoped that by measuring both GRP and NMB we might get some indication if individual members of the BN family of peptides are differentially activated in response to stressor exposure. Indeed, the expression of the mRNA for GRP or its receptors is more prominent than that of mRNA for NMB or its receptors at the hypothalamic structures (including the PVN).[26,222] In contrast, NMB mRNA expression predominates in the anterior pituitary whereas both NMB and GRP receptor mRNA expression are present at this structure.[169] Based on our findings however, it is difficult to draw any conclusions with respect to the relative contributions of GRP and NMB to the stress response. Although, it is notable that the stressor-induced rise in interstitial levels of NMB was slightly more rapid reaching statistical significance by the 1st post-stress sample (as compared to the 2nd post-stress sample for GRP). Thus, both of these BN-related peptides may be important from the perspective of the stress response.

Pharmacological Approach

While the above-mentioned physiological findings suggest a functional role for BN-like peptides in the mediation and/or modulation of the stress response, they provide little insight into the neural mechanism(s) by which this family of peptides comes to

affect HPA activity. Thus, the main objective of the experiments comprising the pharmacological approach was to investigate potential interactions between BN-like peptides and other stress relevant peptides. The similarities between BN-like peptides, CRH and AVP in terms of anatomical distribution, endocrine, autonomic and behavioral effects led us to hypothesize that BN-like peptides may mediate their effects at least in part via activation of CRH and /or AVP neurons. In chapter IV, we demonstrated that central blockade of CRH receptors significantly attenuated BN-induced increases in plasma ACTH, corticosterone, norepinephrine, epinephrine and glucose levels. While these findings support the contention that BN-like peptides may be mediating their effects through central CRH neurons, the anatomical locus (loci) for these peptidergic interactions remained unknown. Thus in chapter V, an initial mapping study revealed that central BN administration elicited a significant decrease in endogenous levels of CRH at the ventromedial and anterior hypothalamic nuclei, NTS and CeA. In contrast, central BN administration was associated with a significant increase in the endogenous levels of AVP at the PVN, Me/Arc and a significant decrease at the ventromedial hypothalamic nucleus. In order to assess the functional relevance of these tissue level alterations, more direct push-pull perfusion experiments (see chapter V) were performed to assess the dynamics of the *in vivo* release of CRH and AVP from the Me/Arc and downstream at the anterior pituitary, in response to central BN administration. Results showed that central administration of BN stimulated the release of CRH and AVP from the Me/Arc translating into an increased availability of these ACTH secretagogues at the anterior pituitary.

Collectively, the findings from Chapter IV and V provide support for the contention that BN-like peptides mediate their effects, at least in part via site-specific CRH and/or AVP neurons. Many questions, however, remain unanswered particularly with respect to the exact nature of these peptidergic interactions. In fact, several possible models could provide a framework to explain our pharmacological findings. Before describing these models, it should be emphasized that they are not mutually exclusive; that is, if one is correct, the others are not necessarily wrong. In fact, it has become increasingly clear that peptides often function in an overlapping and redundant manner.[380] Just as there appears to be multiple peptides capable of performing similar tasks, it would make good sense for a single peptide to be able to perform its functions through multiple neuronal mechanisms. This kind of overlap and redundancy would help to ensure the functioning of a psychological fail-safe system.

The first and probably most obvious model is that BN-like peptide receptors (BB_1 and/or BB_2) are located on the cell bodies and/or dendrites of CRH and/or AVP expressing neurons. Following stressor exposure (or exogenous BN or related peptide administration), BN-like peptides would bind to these specific receptors to provoke the release of CRH and/or AVP. It is well established that CRH-containing neurons in the parvocellular division of the PVN (that co-express AVP) comprise the final common pathway controlling the activity of the HPA axis.[160] Given that central BN administration elicits the release of CRH and AVP from the Me/Arc, a region where CRH neurons originating from the PVN, terminate (chapter V), it is likely that BN-like peptides exert an effect on the CRH containing neurons at the PVN. This interaction could occur

directly, through local BN-like peptide release at the PVN or through release originating from more distal BN-like peptide projections. Indeed, it is well established that a diverse set of inputs arise from brainstem, limbic and/or hypothalamic pathways to converge on the parvocellular CRH expressing neurons.[78,160] Like noradrenergic and adrenergic neurons that project from the NTS to synapse on the cell bodies and dendrites of CRH-containing neurons at the PVN[80,81], BN-like peptide neurons may also extend from brainstem structures such as the NTS to the PVN. Although tract tracing studies have revealed BN-like peptide fibers projecting from the PVN to the dorsal vagal complex (which in encompassed within the boundaries of the NTS), BN-like peptide neurons extending in the opposite direction have not yet been identified.[72,249] Interestingly, BN-like peptides are co-localized with tyrosine hydroxylase (the rate-limiting enzyme in the biosynthesis of norepinephrine) at the NTS.[249] This observation opens up the possibility that BN-like peptides may be co-secreted with norepinephrine and/or epinephrine at the PVN to stimulate CRH release. Alternatively, BN-like peptides may activate CRH neurons in the PVN via indirect routes. Consistent with this notion, direct microinjection of BN into the NTS stimulates an increase in circulating plasma norepinephrine and epinephrine levels.[64] These findings lead to the possibility that BN-like peptide neurons may synapse on catecholaminergic neurons of the NTS, projecting to the PVN. Alternatively BN-like peptide neurons may synapse on CRH and/or AVP expressing neurons directly at the NTS. The observation that central BN administration elicited a significant decrease in ir-CRH content at the NTS (chapter V) is in keeping with this notion. Activation of CRH neurons at the NTS may then stimulate the HPA axis, either directly through their own projections to the PVN or indirectly, through noradrenergic or

adrenergic fibers. Aside from the NTS, BN-like peptide neurons projecting from other stress-relevant limbic structures (CeA, hippocampus, BNST) or hypothalamic nuclei (ventromedial, anterior, and/or arcuate nuclei) may synapse with parvocellular CRH expressing neurons in the PVN. Although at the present time information is limited with respect to the location of BN-like peptide projections, moderate to high levels of ir-BN and BN binding sites are present at most of the above mentioned limbic and hypothalamic structures.[295,297,322] Alternatively, BN-like peptide neurons may synapse directly on CRH and/or AVP expressing neurons (or on neurons expressing other stress relevant neurotransmitters/neuromodulators) at these various limbic and/or hypothalamic sites. This notion is consistent with our findings from chapter V demonstrating a decrease in ir-CRH content at the CeA and anterior hypothalamus and a decrease in both ir-CRH and ir-AVP content at the ventromedial hypothalamus following central BN administration. Moreover, we have recently demonstrated in our laboratory that exposure to restraint stress produced a pronounced increase in CRH and BN-like peptide release at the CeA.[278] Irrespective of where BN-like peptides may be binding to specific receptors on CRH and/or AVP neurons, in order to validate this model, electron-microscopic studies are needed to analyze possible synaptic contacts between BN-like peptide axons and CRH-positive dendrites.

One of the assumptions made by this first model is that the relationship between BN-like peptides, CRH and AVP is unidirectional; i.e. BN-like peptides cause the release of CRH and/or AVP and not vice-versa. While this assumption needs to be further investigated it is not in keeping with the contention that peptides generally function in an

overlapping and redundant capacity. This being said, it is more likely that the relationship between BN-like peptides, CRH and AVP is bi-directional. The next two models that I will briefly discuss incorporate this concept. The first is modeled after the relationship between CRH and AVP. It is well established that most if not all immunodectable AVP in the ZEME is found in CRH containing terminals, implying that AVP is co-produced in the CRH-expressing neurons of the PVN.[422] Like AVP, BN-like peptides may also be co-produced in parvocellular CRH-expressing neurons and co-stored with CRH in the nerve terminals of the ZEME. Indeed there is some evidence that this in fact is the case, as co-localization of BN-like peptides and CRH has been observed in the ovine ME.[138] Although, the basic principle of the first model, namely that BN-like peptide receptors are located on CRH neurons, would still hold true for this second model, the difference is that when BN-like peptides bind to their receptors, CRH, AVP and/or BN-like peptides would be released. Similarly, if CRH binds to specific receptors on CRH neurons co-storing AVP and/or BN-like peptides, then CRH, AVP and/or BN-like peptides would be released. Evidence for or against this model could be provided by further characterization of the extent of co-localization of CRH and BN-like peptides at the ME (as well as in other regions) in species other than sheep. In addition, further studies characterizing the *in vivo* (or *in vitro*) peptidergic release from the ME are needed. Interestingly, in chapter III we demonstrated an increased availability of CRH, AVP, GRP and NMB at the anterior pituitary (located downstream from the ME) in response to air-puff exposure. Unfortunately, the temporal resolution in this method is not sensitive enough to provide a clear indication as to whether or not these four peptides could have been co-released from the external zone of the ME.

Finally, the third model suggested is that BN-like peptides and CRH (and/or AVP) function as cooperative and interdependent parallel systems. This model may be similar to one proposed by Cooper and Dourish (1990)[71] to explain pharmacological interactions between cholecystokinin (CCK) and 5-HT in the regulation of satiety. In this model, the relationship between CCK and 5-HT is cooperative in that the release of one would enhance the release of the other and vice-versa. Moreover, the model further suggests that CCK and 5-HT act as parallel systems, in that they perform separate actions at distinct receptor sites. However, the systems are interdependent as they both are necessary for the full expression of satiety as their respective receptors are interfaced through a common gate. Thus, blockade of one system will affect the ability of the other to induce satiety. Although we have shown that BN-like peptides cause the release of CRH (chapter V), we have not assessed whether CRH can elicit the release of BN-like peptides. This observation would be necessary in order to satisfy the assumption that the relationship between BN-like peptides and CRH is cooperative. Moreover, although we have shown that blockade of central CRH receptors attenuates BN-induced HPA and sympathetic activation (chapter IV), as well as BN-induced behavioral effects[335], studies on the effects of BN receptor blockade on CRH-induced endocrine, autonomic and behavioral effects are currently lacking. Again, observations such as these would be necessary to satisfy the interdependence assumption.

In summary, although our findings from the pharmacological perspective provide evidence that BN-like peptides mediate their stress-relevant effects, at least in part, via activation of CRH and/or AVP neurons, the underlying neuronal mechanism(s) mediating

these peptidergic interactions is (are) currently not known. While the three models previously discussed may provide a framework to explore these peptidergic interactions, more research is needed to determine if any (or all) of these models are applicable to our findings.

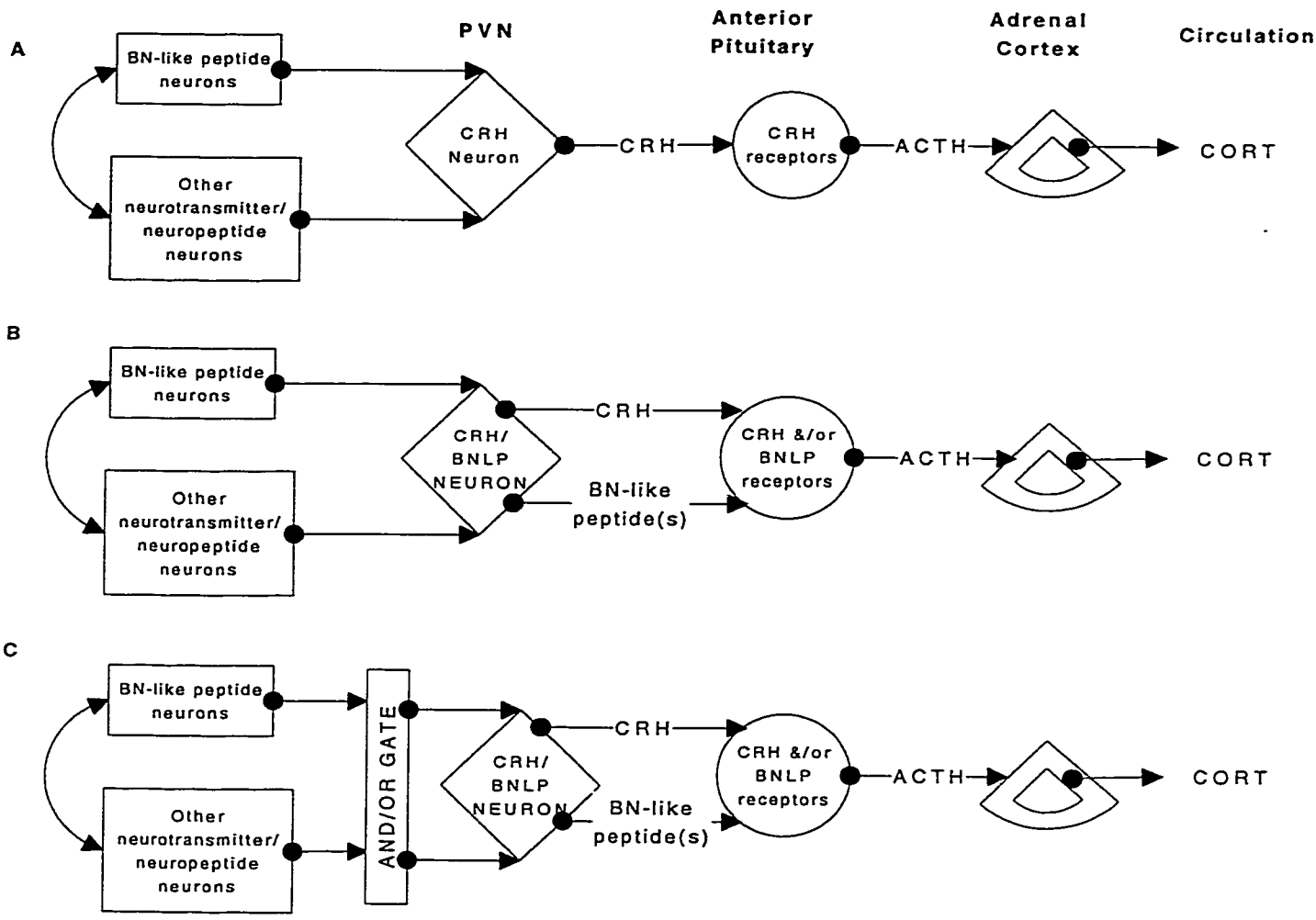

Fig. 1 Three schematic models illustrating potential mechanisms through which BN-like peptides might interact with CRH (AVP not shown). Model *a.* depicts a scenario where receptors for BN-related peptides are located on the cell bodies and/or dendrites of CRH-expressing neurons. Model *b.* is similar to model *a.* with the added possibility that BN-like peptides are co-localized in CRH-expressing neurons. Model *c.* illustrates a situation where BN-like peptides and CRH may function in cooperative and interdependent parallel systems.

Implications

The present findings that a) BN-like peptides may be involved in the mediation and/or modulation of response to stress and b) that BN-like peptides may mediate their effects at least in part via activation of CRH and/or AVP neurons, have several potential neuroscientific and heuristic implications. In recent years, a large body of evidence has emerged linking stressful life events with an increased vulnerability to pathological conditions such as depression and anxiety disorders[338] For example, stressful life events often precede the onset of depression.[50,58,192] In addition, stressful events in childhood such as abuse, neglect, or loss of a parent are identified as predictors for depression, generalized anxiety disorder or posttraumatic stress disorder.[42,57,193,194] These and other studies have spawned research into elucidating the underlying mechanisms mediating stress-related psychopathology. Much of this research has focused on CRH, the primary mediator of the stress response. Indeed, there is considerable evidence suggesting that CRH is hypersecreted from hypothalamic and extrahypothalamic neurons in depression. For example, increased concentrations of ir-CRH have been reported in the CSF of depressed patients and in suicide victims (who presumably suffered from depression).[17,21,305] Moreover, an increase in CRH expressing neurons in post-mortem PVN tissue of depressed patients and a down regulation of CRH receptors in the frontal cortex of suicide victims have been observed.[304,343]

More recently, the role of other stress relevant peptides, such as AVP, in stress-related psychopathology has begun to be explored. Much of the interest in AVP was generated from animal findings demonstrating that exposure to repeated stressors or a

single stressor exposure followed by an adequate passage of time, caused an increase in the co-expression of AVP in CRH neurons at the external zone of the ME.[92,94,366,396] This action appears to lead to a shift in the composition of the signal driving ACTH secretion from predominantly CRH-driven to predominantly AVP-driven, suggesting that AVP may play an important role in the maintenance of a strong stress response following exposure to certain types of stressors.[396] Although human data is limited, consistent with these animal findings, Purba et al. (1996)[342], observed an increased number of AVP-expressing neurons in post-mortem PVN tissue of depressed patients. Taken together, these findings are suggestive of an up-regulation of the AVP system in depression.

The present data from this thesis suggest that along with CRH and AVP, the BN family of peptides are also involved in the integration of the stress response and deserve to be recognized as 'stress peptides'. As such, it is quite likely that the BN family of peptides play a role in the pathophysiology of stress-related disorders. In support of this contention, we have recently shown that in the brains of human suicide victims, there are site-specific alterations in the endogenous levels of CRH, AVP and BN-like peptides (manuscript in preparation). Expansion of the realm of 'stress peptides' to include the BN family of peptides could open up new avenues for therapeutic intervention of stress-related disorders. Just as CRH neurons and/or receptors are targets for prevention and therapy for depression and anxiety related disorder, GRP and/or NMB neurons and/or their respective receptors may also be considered potential therapeutic targets.

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