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Characterization of the Role of Neuromedin B and Gastrin-releasing Peptide in the Mediation of Stress and Anxiety-related Responses

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Characterization of the role of Neuromedin B and Gastrin-releasing peptide in the mediation of stress and anxiety-related responses

By

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Abstract

The perception of an event as being potentially harmful or stressful to the individual sets off a cascade of responses, including the activation of neurons located in the hypothalamus. Despite the extensive efforts in understanding the various neurochemical systems regulating this cascade of events, many questions remain unanswered concerning the underlying mechanisms involved. Evidence from our laboratory suggests involvement of bombesin (BB; a peptide of amphibian origin), in the mediation and/or modulation of the stress response. Two BB-like peptides have been identified in mammals, gastrin-releasing peptide (GRP) and neuromedin B (NMB). Experimental evidence acquired thus far seems to suggest the potential involvement of BB-like peptides in the mediation of the stress response as well as other related responses, such as fear and anxiety. However, very few experiments have focused on determining the specific roles of NMB and GRP in mechanisms underlying the integration of stress and/or anxiety responses. Thus, the overall objective of this thesis is to clarify the intrinsic role of each of these peptides in the hope of gaining a better understanding of the underlying mechanisms mediating stress-related responses. This extensive research project provides new evidence suggesting that both endogenous BB-like peptide systems, GRP and NMB, are significantly involved in the modulation/facilitation of stress-, anxiety- and/or fear-related behaviors. Their intrinsic roles appear to be different; whereas NMB seems to mediate both anxiety and fear, GRP seems to selectively alter more robust stress-related behaviors, such as fear. This thesis project provides important additional support for the involvement of this family of peptides in stress related responses. It also provides interesting insight into the different mechanism(s) of action that might underlie stress, anxiety and/or fear. This new evidence should help in the development of more specific, and therefore efficient, therapeutic agents in the prevention and treatment of stress-related disorders.

Dedicated to

My loving parents who devoted their life to our family and always supported me

Pierre and Odette Bédard

And in loving memory of my precious brother who I know would be so proud of me

Patrice Bédard
1973 - 1992

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Summary

A characteristic physiological response of the body following exposure to a stressful event involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis as well as the sympathetic nervous system. The perception of an event as being potentially harmful or stressful to the individual sets off a cascade of responses, including the activation of neurons located in the hypothalamus. More specifically, neurons of the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN), projecting to the external zone of the median eminence, become activated and release a cocktail of adrenocorticotropic hormone (ACTH) secretagogues, the most important of which are corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP). These chemical signals then travel to the anterior pituitary gland where they stimulate the release of ACTH, which in turn evokes the synthesis and release of corticosterone from the adrenal cortex. Simultaneously, norepinephrine and epinephrine are released from the adrenal medulla to activate or mediate the sympathetic arm of the nervous system response. Despite the extensive efforts in understanding the various neurochemical systems regulating this cascade of events, many questions remain unanswered concerning the underlying mechanisms involved.

Recent evidence from our laboratory suggests the involvement of bombesin (BB; a tetradecapeptide of amphibian origin) related peptides, in the mediation and/or modulation of the stress response. Indeed, it was demonstrated that central administration of BB dose-dependently increased plasma levels of ACTH, corticosterone, norepinephrine and epinephrine. Furthermore, BB administration also elicited behaviors associated with emotionality and arousal, which are commonly associated with the stress

response. These behaviors included altered locomotor activity, increased occurrences of grooming, as well as suppression of food intake. Furthermore, a wide variety of stressors have been reported to affect changes in tissue concentration of BB-like peptides (BLPs) as well as in the density of their receptors, particularly within some of the stress relevant brain regions.

Two BLPs have been identified in mammals, namely gastrin-releasing peptide (GRP), which has high affinity for BB receptor subtype 2 (BB_2) and neuromedin B (NMB), with high affinity for BB receptor subtype 1 (BB_1). These two peptides have striking structural and molecular homology with BB, which suggests that they could share similar biological effects with this amphibian peptide. In keeping with this contention, central GRP administration increases plasma levels of ACTH and corticosterone, an effect similar to that evoked by BB. Similarly, centrally administered NMB elicited behaviors akin to those seen with BB. Furthermore, blockade or functional deletion of NMB receptors has resulted in altered responses to stress, increased anxiety, and increased blood glucose levels in rodents. Furthermore, administration of a NMB receptor antagonist caused a decline in memory performance.

Experimental evidence acquired thus far seems to suggest the potential involvement of BLPs in the mediation of the stress response as well as other related responses, such as fear and anxiety. However, very few experiments have focused on determining the specific roles of NMB and GRP in mechanisms underlying the integration of stress and/or anxiety responses.

Thus, the overall objective of this thesis is to clarify the intrinsic role of each of these peptides in the hope of gaining a better understanding of the underlying mechanisms mediating stress-related responses. Summarized below are the six specific objectives of this research project and their key outcomes:

1. Our laboratory has shown that exogenously administered BB caused a dose-dependant increase in plasma levels of ACTH and corticosterone. Central administration of GRP is known to activate the HPA axis. It has also been shown that GRP antagonist administration completely blocked the GRP and stressor-induced increases of ACTH and corticosterone. While there is ample data demonstrating that exogenously administered BB or GRP cause elevations in plasma levels of ACTH and corticosterone, the evidence linking NMB to activation of the HPA axis is rather sparse. In order to further study the role of NMB and GRP in regulation of the endocrine response to stress we monitored changes in plasma levels of corticosterone and glucose in response to a central administration of these peptides and their receptor antagonist. Results showed that glucose and corticosterone levels increased above baseline levels following stressor exposure in all treatment groups (vehicle, GRP antagonist (GRPa) and NMB antagonist (NMBa)). Whereas central administration of GRPa significantly potentiated the stressor-induced rise in corticosterone levels, the NMBa was without effect. These findings support the notion that BLPs can affect the outcome of stressor-elicited HPA axis and sympathetic activation.

2. Exposure to a variety of different stressors, including immobilization, ferret exposure, noise and social/crowding stress elicit regional specific alterations in endogenous levels of immunoreactive-BB. However, post-mortem regional analyses do not necessarily reflect dynamic changes that occur during exposure to a stressful event. In order to determine whether these changes seen in previous studies translated into dynamic changes in peptidergic release, *in vivo* push-pull perfusion sampling technique was utilized at a specific brain region to allow for a clearer interpretation of the potential mechanism(s) of action of BLPs. The differential effect of stressor exposure on GRP and NMB was assessed. We found that basal levels of GRP and NMB at the anterior pituitary were significantly elevated in animals previously exposed to either chronic or acute restraint stress, suggesting that the role of the BLPs in the HPA axis activation is indeed influenced by prior stress history of an organism. The naïve control animals were most responsive to exposure to an acute novel stressor compared to the changes in rats previously subjected to a previous stressor (chronic or acute). It appears that the peptidergic release in response to an acute stress challenge was delayed in animals with previous stress experience. The release of NMB and GRP was similarly influenced following stressor exposure in all experimental groups

3. Central administration of BB induces behaviors normally associated with a state of stress and/or anxiety. In these experiments we wanted to assess the differential effects of activation and blockade of the distinctive BLP systems on fear and anxiety-related behaviors using different behavioral paradigms. We monitored the behavior of animals in the open field, the elevated plus maze (anxiety related paradigms) and in a fear

potentiated startle paradigm (a model of conditioned fear) following administration of GRP and NMB agonists and antagonists. Results revealed that the BB_1 receptor blockade and the mixed BB_1/BB_2 receptor antagonist elicited decreased anxiety in the open field and EPM, whereas BB_2 receptor blockade alone was without effect. In the fear potentiated startle paradigm, pretreatment with either the BB_1 receptor antagonist or the BB_2 receptor agonist attenuated the fear potentiated startle response. Taken together our data support the contention of the involvement of BLPs in the facilitation/modulation of stress-related responses. However, these peptides appear to act on different stress responses. NMB appear to play a role in both anxiety and fear responses, whereas GRP seemed to be selectively involved in fear responses.

4. The involvement of BLPs in stress-related responses is well established, however the neuronal mechanisms by which they mediate their stress and/or anxiety related effects remain unknown, this is particularly true for NMB. Evidence has demonstrated that serotonin neurons projecting from the dorsal (DRN) and median raphe nuclei to limbic structures are thought to play a role in the mediation of the stress response. In addition, administration of NMB was able to increase the firing rate of serotonergic cells at the DRN. Thus, we first wanted to assess the central and intra-DRN administration of a BB_1/BB_2 receptor antagonist on various anxiety-related behaviors. Results showed a reduction in anxiety-like behaviors in animals treated with the BB_1/BB_2 receptor antagonist, whether it was infused centrally or micro-infused directly in the DRN. Furthermore, using *in vivo* microdialysis sampling (followed by *ex vivo* radioimmunoassay), we set out to monitor changes in the interstitial levels of serotonin at the ventral hypothalamus following

administration of NMB or NMB receptor antagonist at the DRN. Our findings indicated that the *in vivo* release of serotonin at the ventral hippocampus was decreased following NMB antagonist (PD 176252), and increased following agonist (NMB-30) administration. These findings indicate that NMB appear to be acting through the serotonergic system in order to exert its effects on behavior during stressor exposure.

5. We have demonstrated thus far that BLPs, mainly NMB and GRP, are involved in the mediation of stress and/or anxiety type responses. We have also found in our previous research that GRP and NMB appear to differentially affect the startle response in the fear-potentiated startle paradigm. Whilst NMB affected both fear and anxiety responses, GRP seemed to be selectively involved in fear. One way to further differentiate the specific role of GRP and NMB in stress-related responses is through the use of specific receptor antagonists. To this end, we have designed and synthesized highly specific antisense oligodeoxynucleotide probes to selectively influence the BLP systems. These probes were chronically administered centrally and the behavioral and endocrine responses to different stressors assessed. Furthermore, we attempted to elucidate anti-anxiety type responses using various behavioral assays. Behaviorally, blockade of BB_2 receptors resulted in alterations in anxiety-type behaviors (higher frequencies in the punished conflict drinking test, increased frequencies in exploratory behavior in a novel environment, decreased grooming and scratching). Physiologically, it was also found to attenuate the BB-induced increases in blood glucose and corticosterone levels. Animals with a functional deletion of the BB_1 receptors displayed increased exploratory behavior in a novel environment. Whilst further investigations are needed, these data do support

the contention that BB_1 and BB_2 receptors yield differential roles in the mediation of the stress response. BB_2 receptors appear to exert a more prominent and direct role in grooming, conflict drinking as well as glucose and corticosterone regulation, than the BB_1 receptors.

6. One behavior which has been intimately linked to stress and anxiety-type responses is fear. Since NMB and GRP seem to have different roles in fear and anxiety behaviors, we wanted to further explore the potential differential involvement of BLPs in the modulation of learned fear. We investigated the effect of GRP and NMB receptor activation and blockade on the expression of learned fear using the conditioned emotional response paradigm, assessing their effect on the acquisition (drugs administered before training), expression (drugs administered before testing), and reconsolidation (recall test 24 hr after drug administration) of learned fear. Our results demonstrated attenuation of fear expression in both contextual and cued fear assay following central administration of GRP. Similarly, NMB reduced fear expression in the contextual portion of the test. Taken together our findings suggest that both the GRP and NMB system participate in the expression of learned fear.

In summary, this extensive research project provides new evidence suggesting that both endogenous BLP systems, GRP and NMB, are significantly involved in the modulation/facilitation of stress-, anxiety- and fear-related behaviors. There intrinsic role appear to be different, whereas NMB seems to mediate both anxiety and fear, GRP seems to selectively alter more robust stress-related behaviors, such as fear. Incidentally, this

thesis project provides important support for the involvement of this family of peptides in stress responses. It also provides interesting new evidence in different mechanism(s) of action that might underlie stress, anxiety and fear. This new evidence should help in the development of more specific, and therefore efficient, therapeutic agents in the prevention and treatment of stress-related disorders.

General Introduction

Exposure to stressors is considered an important precipitating factor for various psychiatric disorders, including depression, anxiety and post-traumatic stress disorders as well as schizophrenia. Over the course of a lifetime, one can be exposed to a variety of stressors ranging in degree of severity, anywhere from waiting in a long lineup or losing your keys to more serious stressors such as death of a loved one, war or natural disasters (e.g. tsunami or hurricane). Interestingly, not everyone submitted to stressors will develop a stress-related disorder(s). In fact, it is thought that 25% of the population will at some point in their life develop a stress-related illness, sometimes leading to short or even long-term hospitalization. Albeit, the incidence of stress-related illnesses in our fast pace society is reaching startling magnitude.

Currently available pharmacological treatments alleviate the symptoms, as opposed to eliminating or even preventing the development of such debilitating conditions. The drugs of choice, benzodiazepines, are effective at treating stress-related disorders, primarily those associated with anxiety. Unfortunately, the beneficial effects of benzodiazepines, are often obsfucated by incapacitating adverse effects, such as sedation, memory impairments, increasing the effect of alcohol, and most importantly physical dependence.[385] This underscores the pressing need for the development of effective and selective anxiolytic compounds that do not carry all the undesirable side effects of the currently available drugs. Thus, it is crucial to focus on delineating mechanisms underlying the response to various types of stressors, including those mitigating anxiety and fear-type responses. In this context, investigations focused on

identifying the diverse mediators and neural circuits involved in modulating fear and/or anxiety-like responses are needed.

This introduction is thus devoted to discussing the different systems thought to play a role in the mediation of anxiety, fear and stress responses; specifically, how certain neurotransmitter and neuropeptidergic systems respond to and govern such responses. Special attention will be paid to the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system and behavioral alterations as they constitute the key components of the stress response. The initial portion of the introduction will attempt to define and differentiate anxiety- and fear-responses, followed by the neurochemical regulators that affect one or both of these responses. Research in our lab has focused on determining the role of corticotrophin-releasing hormone (CRH) and bombesin-like peptides (BLPs), including bombesin (BB), neuromedin B (NMB) and gastrin-releasing peptide (GRP) in aversive and appetitive events. These neuropeptides and their role in the general stress response will be discussed in detail. Overall, this thesis investigates the specific involvement of NMB and GRP in anxiety and fear-related responses.

Stress, Fear and Anxiety

Although the terms fear, stress and anxiety are often used interchangeably, there is a substantial amount of evidence suggesting that these responses are distinct [94, 292, 293, 435] and can be modulated independently. For instance, patients diagnosed with posttraumatic stress disorder commonly show a normal fear response, but have abnormal anxiety states.[94]

Theoretically, fear responses are short lived and are typically associated with explicit cues or stimuli, thus involving conditioning. However, it is important to note that fear can also be unconditioned, occurring without previous association with a fear-eliciting stimulus.[500] Certain stimuli have characteristics that elicit an innate fear reaction in animals and humans. Conversely, anxiety is not necessarily associated with specific cues, does not involve conditioning and the response is long-lasting.[94, 158, 280] McNaughton and Corr [292] differentiate fear and anxiety by the resulting behavior emitted by the individual. Fear involves a more active type of behavior where the goal is avoiding the threat or danger, for example fleeing or freezing (defensive avoidance). Behaviors that are aimed at approaching the threat or stimulus (defensive approach) as opposed to avoiding it are viewed as an indication of a state of anxiety. For example, risk assessment in an elevated plus maze would be indicative of an anxiety response. Additionally, anxiety-related responses tend to be less robust than fear responses [158] and are attributable to a future *potential* threat rather than an immediate and identifiable threat. Unlike fear, anxiety responses usually do not cease when a specific cue disappears, they are thought to be more chronic in nature and may last well beyond the initial exposure to a threatful stimulus.

Fear, stress and anxiety can also be differentiated functionally by considering the neural systems and brain circuits mediating these stress-related responses.[94, 158, 292] Physiological and tract tracing studies have revealed a high degree of overlap between the neural pathways involved in fear and anxiety, however there are some clear distinctions between the neuroarchitecture mediating fear and anxiety type responses. According to

McNaughton and Corr (2004) fear responses involve the hierarchical activation of the periaqueductal gray (PAG), the medial hypothalamus, the lateral, basolateral and central nuclei of the amygdala (CeA), which will in turn stimulate the anterior cingulate area to finally terminate at the ventral stream of the prefrontal cortex. In support of this pathway, lesioning the anterior cingulate gyrus prevented the acquisition of fear learning and memory in various fear conditioning paradigms.[58, 221] Another study revealed that the regional cerebral blood flow increased at the level of the cingulate gyrus following acquisition of fear conditioning as compared to before the conditioning session.[109]

Similarly, McNaughton and colleagues (2004) suggest that anxiety responses first produce activation of the PAG followed by the medial hypothalamus, the lateral and basolateral nuclei of the amygdala, the septo-hippocampal system, the posterior cingulate area and finally terminate at the dorsal stream of the prefrontal cortex. Several studies have implicated the differential involvement of these brain structures in anxiety. For instance, microinjection of a benzodiazepine into the PAG alters conditioned sensitivity to pain, suggesting a role for the PAG in mediating anxiolytic action.[167] Previous studies have also revealed that hippocampal lesions have a similar effect on behavior as compared to the administration of anxiolytic compounds.[292] Furthermore, human studies using neural imaging have demonstrated increased activity in the left anterior cingulate area combined with decreased activity in the right anterior cingulate, right prefrontal cortex and right parietal lobe during self-generated anxiety.[217]

Davis and Shi (1999) proposed that the bed nucleus of the stria terminalis (BNST) and the CeA are the central brain sites differentiating fear from anxiety.[94] The CeA has many projections to the nucleus reticularis pontis caudalis, the ventral cochlear root neurons and the motorneurons in the spinal chord, which have been identified as the pathway mediating the fear-potentiated startle response, a widely used animal model of fear.[256] In this paradigm when a light (conditioned stimulus), previously paired with a footshock (unconditioned stimulus), is presented on its own, it causes an increase in the startle amplitude of animals. This fear-potentiated startle phenomenon is cue-bound and thus the effect on startle disappears immediately after the light is turned off.[94, 216] Studies have revealed that the fear-potentiated startle response was greatly attenuated in animals with amygdalar lesions.[60, 183] In the same vein, microinfusion of a non-NMDA receptor antagonist into the CeA attenuated the fear-potentiated startle response.[216, 244] In contrast, administration of a glutamate antagonist into the bed nucleus of the stria terminalis (BNST) did not alter the fear-potentiated startle response in these animals.[94, 500] suggesting that this brain structure and/or neurotransmitter system is not involved in the mediation of fear-related responses. Furthermore, fear-potentiated startle was extinguished following neurochemical lesions of either the lateral or the basolateral nucleus of the amygdala, regardless of whether the lesion was applied before or after training.[430] Davis and Shi (1999) propose that the basolateral amygdala is responsible for learning the association between the conditioned stimulus and the footshock, relaying the information to the CeA which ultimately results in altered behavior. Studies, using retrograde and anterograde tracers, suggest that projections from the basolateral amygdala to the CeA also receive projections from pain centers, supporting their involvement in

fear conditioning; whereas projections from the basolateral amygdala to the BNST do not receive input from pain centers.[444] Evidence further demonstrates that the lateral nucleus of the amygdala is involved in fear conditioning involving auditory cues and in turn relays the neuronal message to the CeA to affect behavior.[250, 445] Moreover, the lateral amygdala, like the basolateral amygdala, has been proposed as an essential component involved in the formation of the association between the unconditioned stimulus (US) and the conditioned stimulus (CS). An US can be defined as a stimulus with inherent characteristics that commonly elicit fear. In contrast, a CS does not possess intrinsic fear-eliciting characteristics on its own, but can evoke fear when paired with an US. Lesions to the lateral amygdala blocked the acquisition of fear memory.[30] Together, these studies support the involvement of the aforementioned neuronal pathways in the mediation of fear responses.

Investigations into the role of the BNST have demonstrated that fear-potentiated startle responses or conditioned freezing (indicative of a state of fear) using explicit cues were not affected by lesions to the BNST [251], suggesting that this structure is not involved in conditioned fear responses. The light-enhanced startle paradigm has been proposed as an animal model to study anxiety.[500] This paradigm is based on the knowledge that the startle response to a white noise is also enhanced by exposure to a bright light relative to baseline and this is produced without prior pairing that stimulus with an aversive event (e.g. footshock). This effect is innate and continues to be present for an extended period of time even in the absence of the light, characteristics indicative of a state of anxiety.[500] Whereas the light-enhanced startle response was attenuated by lesions to the basolateral

nucleus of the amygdala or the BNST, it was not affected by the inactivation of the CeA, supporting the contention that the BNST is involved in anxiety-related behaviors, whereas the CeA is not.[500, 501] The light-enhanced response has been shown to be sensitive to anxiolytic drugs, such as benzodiazepines, buspirone and propanolol.[102, 500] Moreover, microinfusion of a GABA synthesis inhibitor into the BNST resulted in a significant increase in the time spent in the open arms of the elevated-plus maze (EPM), indicating an anxiolytic action.[501]

Taken together, these results demonstrate a clear distinction between fear and anxiety responses. These studies suggest a primary role of the CeA in the mediation of certain fear responses, whereas anxiety-related responses are dependant on the activation of the BNST. Both these structures have efferent projections to various hypothalamic and brain stem areas intimately implicated in fear and anxiety [92], to regulate behavioral responses to threatening stimuli. Efferent projections from the basolateral amygdala to both these structures are believed to be the anatomical substrates impelling the animal to respond to emotionally significant stimuli. It is noteworthy that although fear- and anxiety-related responses are distinct, they are often activated simultaneously and are intimately related. For example, anxiety can result from modulation of a pre-existing fear. [292]

The Stress Response

When exposed to potentially harmful stimuli, stress-related responses, including anxiety and fear, bring into play many systems in an attempt to mold and shape the most

adaptive response(s). The behavioral responses are preceded or accompanied by internal physiological changes, preparing the organism for the overt flight or fight response. Indeed, the behavioral responses occur in concert with neuroendocrine, autonomic and immune responses to stressors. Furthermore, emotional and cognitive processes are considered to be amongst the determinants of these responses.[358, 439, 473] For this thesis, only the neuroendocrine, autonomic and behavioral determinants of the stress response will be reviewed.

It is worthy of mention that the neurochemical (neuroendocrine and autonomic) and behavioral responses involved in the stress response can be influenced by several different factors. For instance, an individual's response to a potential stressor will be determined first by organismic characteristics, such as species, genetic makeup, age and gender. Secondly, the previous stress history and early life experiences will also play an important role in mitigating the reaction to threatening stimuli and the action(s) taken by the organism. Finally, the response can also be influenced by characteristics inherent to the stressor, for example, if the stressor is psychological or physical in nature, if the stressor is mild or severe, acute or chronic. These aspects of the stressor or event will influence the final action taken by the individual (see Figure 1).

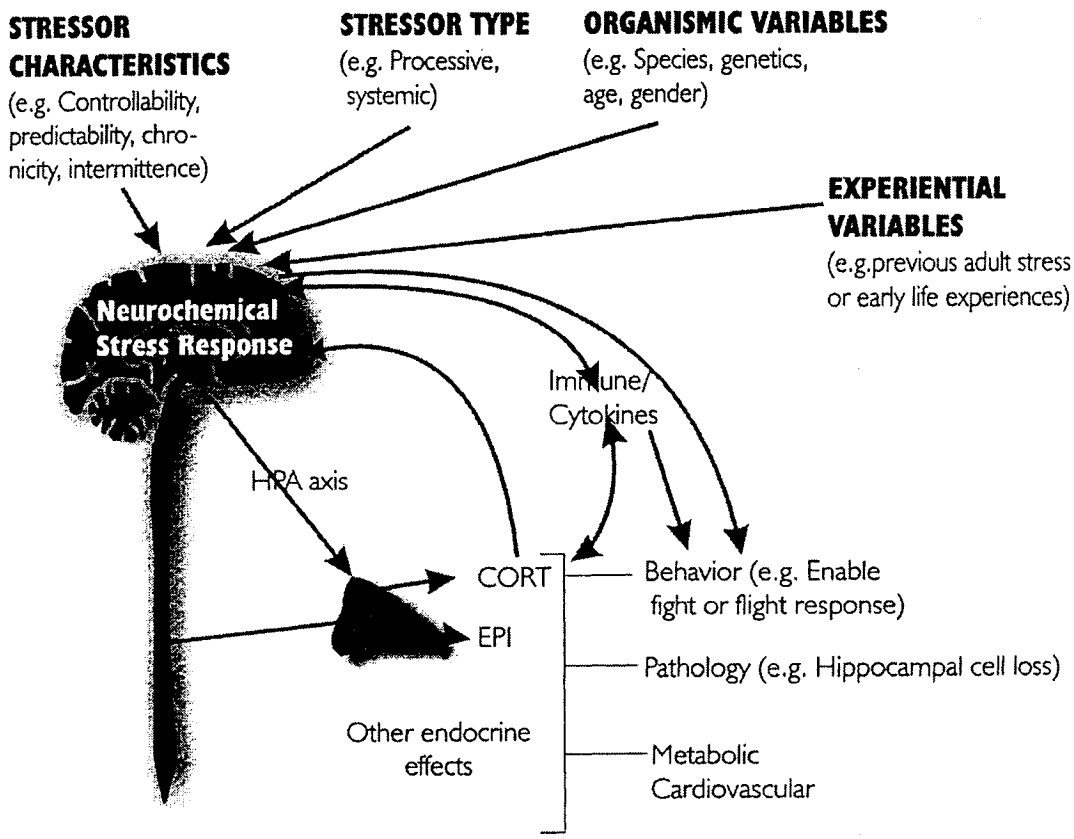


Figure 1 Factors influencing the neurochemical and behavioral response to stressor exposure, adapted from.[297]

The neuroendocrine response: the hypothalamic-pituitary-adrenal (HPA) axis

Exposure of an organism to a potentially harmful stimulus or event triggers a complex cascade of physiological and behavioral alterations that facilitates an adaptive response to the threat at hand. The response to stressor exposure ultimately results in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. This activation implicates the complex participation of many different neurotransmitters or hormones, the most important of which include corticotropin-releasing hormone (CRH), arginine-vasopressin (AVP), adrenocorticotropic hormone (ACTH) and the glucocorticoids.

The genesis of HPA axis activation is thought to involve the activation of the neurons emanating from the parvocellular layer of the paraventricular nucleus of the hypothalamus (PVN), specifically those producing CRH and/or AVP. These two peptides are transported to the median eminence (ME) where they are released into the portal blood system through which they reach the pituitary gland.[179, 197, 306, 464] At this site, they act on the anterior portion of the pituitary binding to adenohypophysal corticotroph cells causing the synthesis and release of pro-opiomelanocortin (POMC). This peptide is eventually converted to ACTH and released into the systemic blood system, reaching the cortex of the adrenal gland. The stimulation of the adrenal cortex by ACTH then triggers the synthesis and release of glucocorticoids, (cortisol in humans and corticosterone in non-human animals).[178, 197, 306, 464] The termination of HPA activation is, in part, mediated by a negative feedback loop, involving the inhibition by glucocorticoids at the PVN and the anterior pituitary, thus blocking the further synthesis and release of CRH and ACTH.[306] Consequently, it is the glucocorticoids that will act to

limit the duration and/or magnitude of the stress response, ensuring that the adverse consequences of HPA axis activation are not prolonged above what is necessary.

The exact mechanisms mediating this feedback inhibition have not been fully elucidated. Thus far, two distinct types of receptors for the glucocorticoids have been identified in the mammalian brain, known as mineralocorticoid receptors (type I) and glucocorticoid receptors (type II). The mineralocorticoid receptors, have a high affinity for the glucocorticoids, and are activated when levels of glucocorticoids are at their lowest, in order to maintain the basal activity of the HPA axis. In contrast, the type II receptors are thought to be involved in the mediation of the stress response. Thus, the type II receptors take on greater importance only when the glucocorticoids levels are high, for example when a stress response is activated.[306, 434] Consequently, as the glucocorticoid levels increase, mineralocorticoid receptors become saturated, and the type II receptors (particularly at the hypothalamic level) take on a more prominent role as they progressively become activated, which sets in motion the negative feedback loop, shutting down the HPA axis and the stress response. When the glucocorticoid type II receptors become activated (at the hypothalamic and/or anterior pituitary level), they are internalized and transported to the nucleus of the cell. There, they act on gene expression, controlling (in this case inhibiting) the synthesis of key peptidergic (i.e. CRH and AVP) as well as hormonal systems (ACTH).[482]

Considering the prominent role of the HPA axis in stress-related responses, it is not surprising that this system has been implicated in the genesis of stress-related

disorders such as depression and anxiety disorders (i.e. post-traumatic stress disorder). It is thought that although HPA activation has a survival value in mounting the stress response on a short-term basis, protracted activation can be detrimental or maladaptive to the organism.

Neurotransmitter and neuropeptide systems influencing the HPA axis

The control of the HPA axis in stress and anxiety-related responses are known to be influenced by various neurotransmitter and/or neuropeptidergic systems. In the following section, we will focus on the key neurotransmitters and neuropeptides implicated in the stress response. Specifically, we will review the role of neural circuits utilizing dopamine (DA), serotonin (5-HT), epinephrine and norepinephrine (NE), as well as cholecystokinin (CCK), CRH and arginin-vasopressin (AVP), in this context.

There is evidence suggesting that the dopaminergic system and the HPA axis may functionally interact. Specifically, it is thought that stimulation of the HPA axis during stressor exposure facilitates DA neurotransmission. Indeed, increases in homovanillic acid, a DA metabolite, have been reported following HPA axis stimulation (with CRH or ACTH administration) in humans.[383, 384] Moreover, administration of glucocorticoids enhances DA release in various stress-related brain regions, including the nucleus accumbens, hypothalamus and the caudate nucleus.[419, 511] Studies investigating the effect of modulation of the DA system on HPA activity have demonstrated that both D1 and D2 receptor subtypes mediate the stimulatory effect of cocaine on the HPA axis at the level of the hypothalamus by acting on CRH release; although blockade of these two receptor

subtypes did not alter basal CRH or HPA activity at the hypothalamus or the pituitary gland.[529] The medial prefrontal cortex (mPFC), rich in DA, has been implicated in the behavioral, autonomic and endocrine modulation of the stress response. Results from previous studies have revealed a reduction in the expression of the immediate early gene, c-fos, in CRH neurons of the PVN following infusion of both a D1 antagonist, SCH23390, and a D2 antagonist, sulpiride, in response to IL-1 β administration.[454] Interestingly, blockade of D2 receptors attenuated c-fos expression following exposure to a milder stressor (air puff stress), whereas administration of D1 receptor antagonist failed to alter the stressor-induced c-fos expression.[454] These results suggest that the D1 and the D2 receptor subtypes might be differentially involved in the stress response.

Daftary and colleagues (2000) proposed that the increased release of ACTH following stressor exposure might be regulated, in part, by NE action at the PVN. This contention is supported by both the high levels of NE at this site and the elevated density of NE positive neurons following exposure to a threat/stressor.[85, 224] In addition, more direct studies using *in vivo* techniques have reported increased NE release at the PVN during stressor exposure, which is thought to elicit the release of ACTH via activation of adrenergic receptors present on CRH neurons.[96, 224, 351, 479] The NE system is thought to modulate stressor-elicited release of pituitary hormones (ACTH, thyroid-stimulating hormone, and prolactin).[87] Additionally, experiments using *in vivo* techniques have shown that increased release of NE at the PVN may, at least indirectly, increase plasma ACTH by stimulating adrenergic receptors located on CRH neurons.[96, 224]

Serotonergic activity is also implicated in HPA axis functioning. Central 5-HT is generally synthesized and released from the raphé nuclei which have multiple projections throughout the brain affecting many systems including the HPA axis. The 5-HT cells within the raphé nuclei, mainly the dorsal and medial portions, show early gene expression following stressor exposure.[68, 226, 380] Similarly, CRH administration at the dorsal raphé nucleus, increased the firing rate in these neurons.[218] Furthermore, the release of 5-HT at the PVN, originating from the dorsal raphé nucleus, stimulated the release of CRH from that same brain site, supporting the involvement of 5-HT in HPA axis activation.[218] This synaptic communication between the raphé nuclei and the CRH neurons in the PVN represent a direct pathway through which 5-HT can participate in the regulation HPA axis activity during stressor exposure.[228]

As mentioned previously, HPA axis functioning is also influenced by several neuropeptide systems. Some of the major systems include those using CCK, CRH and AVP. Specifically, Porter and colleagues reported increases in ACTH [431] and corticosterone release in animals administered either CCK-8 or CCK-33 [382], two CCK receptor agonists. Moreover, when animals that have previously been subjected to a chronic cold stress regiment were pretreated with a CCK-B receptor antagonist, they displayed an enhanced ACTH response following exposure to a single session of restraint stress.[27] Interestingly, these chronically stressed animals do not show increases in CCK-B receptor mRNA in the paraventricular thalamic nucleus (a stress relevant site), suggesting the involvement of CCK-B receptors in acute, but not chronic stress.[27] The two CCK receptor sybtypes (CCK-AR and CCK-BR) are involved in the genesis of panic

attacks and are thought to ultimately participate in the development of panic disorders.[318, 531] In effect, administration of CCK in humans elicits panic attacks in both panic disorder and healthy individuals.[35, 36] The underlying mechanism(s) mediating the panicogenic effect of CCK seem to necessitate HPA axis activation. Indeed, CCK-4 agonist administration evoked the release of both ACTH and cortisol in healthy subjects.[33, 34, 36, 104, 531] Taken together, there is ample evidence supporting the contention of an interaction between the CCK system and HPA axis activity in stress-related responses and disorders.

In addition to CCK, several other peptides have been implicated in the stressor-induced activation of the HPA axis. As previously discussed, CRH plays an important role in HPA axis activity. Stimulation of the HPA axis elicits the release of CRH from the PVN into the portal blood system triggering the release of ACTH from the pituitary [112]. Tract-tracing studies have shown that CRH-containing neurons of the parvocellular division of the PVN project to the external zone of the ME, where released CRH enters the portal blood system, eventually reaching the anterior pituitary, where it potentially regulates ACTH synthesis and/or release.[7] Indeed, pharmacological administration of CRH provokes the release of ACTH.[28, 48, 493] In contrast, infusion of CRH antiserum or CRH antagonists blocked the stressor-induced elevation in ACTH in rats whether it is administered centrally or peripherally.[338, 349, 401, 402] Interestingly, although this blockade of CRH receptors markedly affected the stressor-elicited activation of the HPA axis, it did not affect the basal activity of the axis [342], further supporting its involvement in stress-related physiology and behavior. These findings are further supported by results

from studies using CRH and CRH receptor mutant animals. Investigation of HPA axis activity in CRH deficient mice revealed lower levels of circulating ACTH and CRH in these animals compared to the wild-type group.[333] This blunted ACTH response to stressor exposure has also been observed in CRH binding protein overexpressing and CRH 1 receptor deficient animals, providing further evidence for the importance of CRH in HPA activity.[264, 451, 478] Deletion of the CRH 2 receptors appears to exert the opposite effect, an elevation in ACTH release in response to stressor exposure.[81] One explanation for this difference is that CRH 2 receptor subtype might act as a buffer during stressor exposure to prevent over activation of the stress response via CRH 1 receptors.[81] Whilst, CRH overexpressing mice exhibit increased levels of both ACTH and corticosterone [461], CRH knockout animals show evidence of blunted ACTH and corticosterone under basal and stressful conditions.[113, 332] An abundance of evidence has been proposed supporting the involvement of CRH in the regulation of the HPA axis, as seen by changes in ACTH release.

Although CRH is thought to be the major regulator of ACTH release, it acts concurrently with AVP to influence the HPA axis.[15, 505, 506] 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. Moreover, pretreatment with an AVP receptor antagonist blocked the AVP-induced increase of plasma ACTH, as well as the ability of AVP to potentiate CRH induced ACTH release.[26, 400] Van Dijken and Colleagues (1994) demonstrated that under chronic stress conditions, AVP becomes the dominant peptidergic signal mediating ACTH release.[489] Moreover,

Bartanusz et al [23] have also shown that AVP takes on a more prominent role than CRH as an ACTH secretagogue following exposure to an acute stressor followed by the passage of time (14 days). Finally, stressor exposure increased AVP mRNA and peptide (AVP) content in CRH neurons at the PVN and median eminence respectively following acute stressor exposure.[23] Therefore, it would appear that CRH does not act by itself to trigger a stress response and HPA axis activation, but works concurrently with AVP to produce an adaptive response when faced with a potential threat.

In conclusion, these results provide evidence that activation of the HPA axis engages the collaborative action of several different systems and supports a role for major neurotransmitters systems including DA, epinephrine NE, 5-HT, and major neuropeptidergic systems including CCK, AVP as well as CRH in the mediation of the stress response.

The autonomic response: The autonomic nervous system (ANS)

Although activation of the HPA axis is known as the prototypical stress response, other systems also appear important for an adaptive response to a homeostatic threat. The ANS acts synergistically with the HPA axis to prepare the animal to deal with threatening situations at hand.[225] This system plays a role in the behavioral and physiological processes by acting on a variety of systems and organs in the body. The behavioral and cognitive changes that occur include an increased arousal and state of vigilance, heightened attention, selective memory enhancement, alert cognition and attention span as well as analgesia.[197, 306] In addition, the physiological changes observed are generated

in an effort to redirect the energy stores within the body to where they are most urgently needed. These alterations include the production and release of glucose, stimulation of amino acids and free fatty acids as a source of energy, as well as cardio-vascular changes, such as an increase in heart rate, blood pressure and respiration.[476] Activation of the ANS also suppresses vegetative processes, such as digestion, growth, reproductive and immune functions, as well as a concomitant increase of catabolic processes.[197, 306] These behavioral and physiological changes are due, in part, to the release of NE and epinephrine from the adrenal medulla. Adrenal functioning is under the control of the sympathetic nervous system through postganglionic neurons, in response to stressor exposure. The activation of a stress response, in eliciting ANS activation, also implicates the simultaneous release of NE in the brain.[111] The role of epinephrine in preparing the animal to respond to the stimulus or stressor is relatively minor in comparison to the role played by NE, although it does serve necessary functions.

Neurotransmitter and neuropeptide systems influencing the ANS

The activation of the sympathetic nervous system (SNS) is under the control of the central nervous system (CNS). The majority of the inputs are noradrenergic, and originate at the locus coeruleus (LC). This is known as the LC-NE/sympathetic system (LC/NE system). These noradrenergic pathways project to various brain regions, including those intimately involved in the stress and anxiety-related responses. The LC projections are quite wide-spread, however, in the context of the stress response, the projections of particular interest include those to the PVN, central nucleus of the amygdale (CeA), nucleus of the bed n ucleus of the solitary tract (BNTS), periaqueductal

grey, hippocampus, parabrachial nuclei as well as many regions of the cortex, particularly the prefrontal cortex.[18, 307, 473, 487, 488] Anatomical studies demonstrate that areas responsible for coordinating the autonomic response to stressor exposure, namely the periaqueductal grey and the nucleus of the solitary tract, have functional synaptic projection to the ventral portion of the LC; whereas, the CeA and the BNTS project to the dorsal portion of the LC.[86] Lesions of the CeA blocked the stress-induced activation of the LC by cardiovascular stress, supporting its involvement in autonomic stressors.

Like the noradrenergic system, DA also exerts an influence on the ANS. First, the anatomical distribution of DA and its receptors suggest its involvement in autonomic functions. Different DA receptor subtypes have been identified on systemic arteries (D1 and D5 subtypes) as well as on sympathetic neuron junctions (D2 and D4 subtypes).[398] Moreover, all the different DA receptor subtypes have been localized on the kidneys.[398] Many other organs are reported to express different DA receptor subtypes, including the lungs, lymphocytes, on vagal ganglionic neurons, providing further evidence of the role of DA in the regulation of the autonomic nervous system.[398] Secondly, using pharmacological studies, Amenta and colleagues (2002) have demonstrated that DA and/or DA agonist administration was sufficient to trigger hypotension, cardiac contraction, diuresis and natriuresis in treated subjects. It has also been reported that DA release is increased in brain regions known to modulate autonomic activity following exposure to a stressor, resulting in increased heart rate and enhanced blood pressure.[137] Together, this evidence demonstrates a likely influence of the dopaminergic system in autonomic regulation.

Groenink and colleagues (2003) have extensively studied the effect of different 5-HT receptor subtype knockout models on autonomic activity in order to gain insight into the role played by 5-HT on this system in the context of the stress response. Evidence suggests that the different receptor subtypes serve differing roles in autonomic functioning. For example, basal autonomic activity is undisturbed in 5-HT 1_A receptor knockout mice (1_A-KO); however, the stressor-induced autonomic response is altered in these animals.[159] More specifically, basal heart rate and circadian rythmicity of 1_A-KO do not differ from the wild-type control animals, but when exposed to a novel environment (novelty-induced stress), these knockout animals display an enhanced heart rate.[160, 360] Furthermore, blockade of 5-HT 1A receptors using a specific receptor antagonist resulted in a reduction of the sympathetic activity as well as reduction in heart rate, thought to be due to an alteration of the 5-HT 1A autoreceptor function.[286] These results confirm that 5-HT receptors and the serotonergic system act as a modulatory factor in autonomic functioning.

As with the HPA axis, the activity of the ANS is under the influence of several peptidergic systems as well. It has been proposed that the release of NE influences the activity of the CRH system(s), and likewise, stimulation of CRH has been shown to reciprocally induce activation of NE systems.[73, 481] For instance, central administration of CRH activates the release of NE, epinephrine, glucose and glucagon.[47, 84] On the other hand, CRH receptor antagonist administration (for example αh-CRH) blocked the CRH-induced increase in epinephrine and NE levels without impinging on basal activity of these transmitters in the CNS.[49] Animals with a functional deletion of CRH receptors

exhibit altered epinephrine levels as compared to their wild-type counterparts.[333] In addition to its effects on regulating epinephrine and NE levels during autonomic activation, CRH also influences other dimensions of the ANS. For instance, exogenous CRH increased mean arterial blood pressure, elevated heart rate, as well as oxygen levels.[47, 48, 131, 161]

Autonomic activation following stressor exposure also appears to involve the participation of the AVP system(s). Central administration of AVP in animals increased heart rate, blood pressure and plasma levels of epinephrine and NE, effects falling under the control of the ANS.[411, 528] Moreover, AVP receptor antagonist administration blocked the glutamate-induced activation of the SNS.[278, 283] Alterations in cardiac functioning were hampered by pretreatment with the same AVP antagonist infused in the NTS or the vagal complex.[84] Although the mechanisms underlying AVP action on autonomic activity are not well understood, it is thought that these effects may be mediated via the LC. Alterations in blood pressure and heart rate were observed following microinjection of AVP at the LC; and this effect was prevented by treatment with a mixed $AVP_{1A/B}$ receptor antagonist.[25]

CCK immunoreactivity has been identified in autonomic regions of the rodent spinal cord, suggesting the involvement of this peptide in the control of the ANS.[484] Most of the studies looking at the effects on CCK on the ANS have focused on gastrointestinal functions.[136, 427] Although, one study using a CCK receptor antagonist,

loxiglumide, revealed that there were no effects on pancreatic secretion, gastric secretion or gastric emptying in rats or mice.[427]

Based on these findings, it would appear that the activity of the ANS in the context of the stress response is also greatly influenced by several different neurotransmitter and/or peptidergic systems known to be implicated in mediation of stress-related responses. In studying the stress response, the role of the ANS is often overlooked. The evidence that the same neurotransmitter/neuropeptide systems that modulate the HPA axis concurrently influence the activity of the ANS to produce a complete and adaptive response to stress highlights the importance of this system.

The behavioral response: behavior and stress-related paradigms

The endogenous neurochemical, neurophysiological and neuroendocrine changes evoked by stressors, also impact on the overt behavioral responses evoked by the stressors. Behaviors commonly associated with a state of stress and/or anxiety include reduction in food intake, increased grooming and scratching, decreased exploration of a novel environment but increased exploration of a familiar milieu.[125, 194, 227, 369, 440, 459] Furthermore, stressors impact the performance in a wide range of behavioral paradigms thought to reflect anxiety-like or depressive behaviors. The subsequent section will focus on some of the more relevant behavioral paradigms, including the open field, elevated-plus maze (EPM), Vogel test (or punished drinking test), fear-potentiated startle and conditioned emotional response paradigms. Several neurotransmitter/neuropeptide

systems differentially influence stressor-induced behaviors in the various paradigms and thus play an important role in the behavioral expression of the stress response.

Dopamine (DA)

As stated earlier, DA plays an important role in the physiology of stress and anxiety, but also acts on the behavioral expression of the response to a stressor.[117] Research shows that alterations of the dopaminergic system impact the behavioral output of animals in different stress and anxiety paradigms. The effect of DA on three anxiety-related tests will be reviewed: the open field (open field), the EPM and the vogel test; and two fear-related tests: the fear-potentiated startle and the conditioned emotional response or fear conditioning paradigms. Studies using different DA receptor antagonists have revealed that blockade of dopamine D2 receptors increased locomotion in the open field at high doses, but decreased the same behavior at low doses.[311] This effect can be explained by activation of the D2 autoreceptors at low doses of the antagonist, whereas, at higher doses, the antagonist reaches the D2 postsynaptic receptors. In contrast, D3 receptor antagonism failed to alter locomotor activity.[311] Quinpirole (specific D2 receptor antagonist) enhanced locomotor activity in the open field, a response described as being a continuous, consistent locomotion in which the center of the field was not entered.[220] These results were further supported by experiments using genetically manipulated animals. Indeed, several studies have reported a marked hyperactivity in DA transporter deficient mice.[123, 405, 455, 480] These animals also exhibited low motor coordination and reduced rearing behavior.[123] Further evidence implicating dopamine and its receptors in anxiety and fear type behaviors are demonstrated in studies using D2

and D3 receptor agonists. Generally, various dopamine agonists tested thus far for anxiolytic properties have failed to influence animals' behavior in the EPM.[309] However results using this paradigm are somewhat conflicting. For example, Ropinirole caused an increase in the time spent in open arm, although this was true only when administered intraperitoneally.[309, 413] Another DA agonist, S32504, increased the number of open arm entries, however, had no effect on the time spent in the open arms.[309] While results presented using the EPM are inconclusive, using a different paradigm, the conflict drinking test (Vogel), an anxiolytic effect following DA receptor agonist administration has consistently been observed. Indeed, administration of three different D3 receptor agonists, S32504, 7-OH-DPAT and BP 897, resulted in inceases in the number of accepted shocks in the Vogel conflict drinking test at high doses, a useful paradigm for the screening of anxiolytic properties of drugs.[413] It is noteworthy that many DA receptor agonists are known to also have some effects on both 5-HT and/or noradrenergic receptors. Therefore, it is possible that the anxiolytic effect of DA might be mediated, at least in part, through other receptors.[310] The dopaminergic receptors D1 and D2 are thought to play a role in fear-potentiated startle.[154] Impairment of both acquisition and expression of the fear-potentiated startle response were observed following intraamygdalar D2 receptor antagonist (raclopride) treatment.[154, 155] Similarly, administration of a D1 receptor antagonist (SCH 23390) prevented the acquisition of fear-potentiated startle in animals.[155] Studies conducted using the conditioned emotional response paradigm (CER) have proposed a role for dopaminergic systems in fear expression and extinction, but DA does not appear to be implicated in the acquisition phase of fear memory.[124, 327, 328, 393] Lesions of the medial prefrontal cortex, resulting in

DA loss, produced longer extinction periods in both contextual and cued fear conditioning, without affecting the acquisition of fear conditioning.[124, 327, 328, 393] Further evidence for the involvement of dopaminergic neurotransmission selectively in expression of conditioned fear is supported by pharmacological studies using dopaminergic agonist and antagonists. Infusion of either a D1/D2 receptor antagonist, a D2 receptor antagonist or a DA agonist attenuated expression of conditioned fear, as seen by decreased freezing behavior of animals, but failed to alter acquisition of fear memory. [162, 370] Based on these results, it would appear that an equilibrium in DA concentrations is required for expression of conditioned fear. This equilibrium is achieved by the simultaneous action of the DA agonist and antagonist in keeping the DA homeostasis or optimal levels facilitating fear expression.[370] However, when a D1/5 receptor antagonist was infused, animals showed an inability to learn fear conditioning, but did not show any differences in the expression of conditioned fear.[192] The D1/5 receptor agonist did not affect either acquisition or expression of learned fear conditioning. These findings are further supported by results of investigations using DA receptor deficient mice. Mice lacking the D1 receptor showed no differences in their capacity to learn fear conditioning, but displayed delays in the extinction of conditioned fear.[114] Moreover, deletion of the D4 receptors failed to alter both acquisition and expression of learned fear.[117]

These findings presented above help confirm the role of DA and its different receptor subtypes in the mediation of behavioral responses related to stress and anxiety. DA has been shown to be involved in many aspects of stressor-dependant behaviors by

acting on locomotor activity (open field), anxiety-like behaviors (EPM and Vogel), as well as fear acquisition and expression (FPS and CER paradigms).

Serotonin (5-HT)

In addition to its effect on endocrine and autonomic functioning under stressful conditions, 5-HT also plays an important role as a modulator of anxiety and fear-related behaviors. Regulation of locomotor activity, a behavior known to be associated with a state of stress or anxiety, is thought to be one of the main functions of the serotonergic system.[195] Systemic or central administration of a 5-HT 5-HT_{1A} receptor agonist (full or partial agonist) decreased the stressor-induced inhibition of locomotor activity in rodents as measured in the open field test.[13, 61, 63, 266, 447, 460, 512] In contrast, administration of several 5-HT reuptake inhibitors failed to increase locomotor activity (an anxiolytic-like effect) in the open field.[166, 196, 403] Similarly, studies have found no effect of the 5-HT_2 receptor antagonists on anxiety, with the exception of ritanserin, which increased locomotion in animals tested in the open field, indicative of an anxiolytic action of the compound.[294] Finally, non-specific 5-HT agonists exerted anxiogenic-like effects, as seen by decreases in locomotor activity in the open field.[391] Studies using the EPM as an animal model to assess anxiety have revealed that 5-HT_{1A} receptor deficient animals display anxiety-like behaviors, as shown by increased time spent in the closed arms (anxiolytic portion) of the maze and/or decreased time spent in the open arms (anxiogenic portion).[352] Evidence from two different types of genetic animal models suggests that overexpression or deletion of the 5-HT_{1A} receptor causes anxiety-like behavior. Although, additional studies using various tests of anxiety are needed to confirm this

intriguing relationship. In contrast, results obtained from pharmacological studies using the Vogel test appear to support previous reports of an anxiolytic effect of 5-HT_{1A} receptor antagonism. Indeed, administration of various 5-HT_{1A} receptor antagonists increased punished drinking behavior (Vogel test), indicative of an anticonflict effect of the compounds.[156, 504] Paradoxically, two studies reported that administration of a 5-HT_{1A} receptor agonist also increased the number of shocks accepted in the Vogel test [261, 308], suggestive of an anxiolytic-like effect. These seemingly conflicting results might be due to the presence of 5-HT1A receptors both pre and post synaptically. Certainly, the different location of these receptors might mediate different functions. Akin to the results seen on anxiety-related behaviors, 5-HT also exerts effects on fear responses, although the effects seem to be dependant on the regime of administration. For instance, a 5-HT reuptake inhibitor, citalopram, administered acutely facilitated fear conditioning in the CER test, while *chronic* infusion of the same compound produced impairments in learned fear.[56] Moreover, alterations in the serotonergic system, achieved by lesions to the median raphé nucleus, have consistently been shown to impair contextual and cued acquisition and expression of fear conditioning, as demonstrated by decreases in freezing behavior occurrences in lesioned animals compared to sham animals.[21, 295, 448, 449]

Epinephrine and Norepinephrine (NE)

Like DA and 5-HT, both epinephrine and NE regulate the behavioral manifestations of stress and/or anxious states in various animal models. For example, mice lacking the α_{2A} adrenergic receptor demonstrated a reduction in locomotor activity in the open field accompanied by a similar reduction in the time spent in the open arms of

the EPM as compared to their wild-type counterparts.[243] Loss of adrenergic function significantly increased locomotor activity and exploration in the open field, indicative of an anxiolytic effect.[246] Furthermore, it would appear that the LC-NE system is involved in mediating the behavioral response to subsequent stressor exposure, as locomotor activity of animals in an open field was correlated with NE release in the hippocampus following exposure to a mild stressor.[414] Studies looking at genetic predispositions to stress susceptibility revealed that animals genetically hyper-responsive to stress, the Wistar-Kyoto strain, despite their exaggerated endocrine response to stress, show a blunted behavioral response to stressor-induced reduction in locomotor activity following acute immobilization stress.[358] In addition, the Wistar-Kyoto rats failed to show a fear-potentiated startle response.[358] These effects are thought to be a consequence of a deficiency in the noradrenergic system given that the behavioral responsivity is accompanied by a diminution in NE release.[358] In contrast, blockade of α_1 adrenergic receptors in the CeA failed to alter stressor-induced behavior in the EPM.[65] However, administration of both α_1 adrenergic receptors and β-adrenergic receptors in the BNST prevented the stressor-induced decrease in open arm exploration.[64] Based on these results, it would appear that the role of the noradrenergic system is both paradigm and site-specific. Loss of noradrenergic neurotransmission induced anxiogenic-like behaviors in the Vogel test, as shown by decreases in the number of shocks accepted by toxin-treated animals.[135] Research has shown a role of epinephrine and NE in fear-related responses as well.[90] In this vein, α_2 adrenergic receptor agonist treatment blocked acquisition of fear memory, but failed to alter contextual fear conditioning.[90] Conversely, administration of the α_2 adrenergic receptor antagonist blocked contextual fear

expression. These results are further supported by findings using α_2 receptor deficient animals.[90] Animals with a functional deletion of the α_2 receptor displayed elevations in the occurrence of freezing behavior in response to a cue, but no alterations in freezing to the context.

Cholecystokinin (CCK)

In addition to catecholamines, neuropeptides also play an important role in mediating behaviors associate to stress and anxiety. Infusion of a CCK-B receptor agonist increased locomotor activity in the open field, an effect akin to stressor exposure.[89] Likewise, activity in the open field of one strain of mice (Balb/c) was enhanced by administration of CCK antagonists.[223] Animals naturally lacking CCK-A receptors (due to genetic abnormality) exhibited reduced locomotor activity in an open field test.[521] whereas animals lacking CCK-2 receptors display hyperactivity.[2] Consolidation of the data presented on the open field test requires additional studies to further investigate the role of CCK and its receptor subtypes in stressor-induced inhibition of locomotor activity in a novel environment. Administration of CCK-8 in the dorsal periaqueductal gray evoked anxiogenic behavior, as seen by a reduction in the percentage of time spent in the open arms of the EPM.[341] Furthermore, CCK-A receptor deficiency reduced time spent in the anxiogenic portion and number of open arm entries of the EPM. [521] One study has suggested a primary role of the CCK-1 receptor subtype in anxiety-like behavior in the EPM.[119] Specifically, when rats were administered with a CCK-1 receptor antagonist, they displayed increased time spent in the open arms, whereas treatment with either CCK-4 or CCK-2 receptor antagonists failed to alter the

behavior of animals in this paradigm, with the exception of a slight decrease in the time spent in the closed arms. Additionally, in the FPS test, animals infused with CCK-8 displayed heightened fear potentiation to the tone, whereas administration of a CCK-4 agonist did not exert any effect on the FPS response.[173] Animals with high anxiety levels, as evidenced by increased fear-potentiated startle responses, display a reduction in CCK-B receptor density in both the basolateral and central amygdaloid nuclei, but showed no alterations in CCK-B receptor binding in the nucleus accumbens.[515] Results of this study further demonstrate a down-regulation of amygdalar CCK-B receptor density in animals exhibiting increased anxiety-like responses in the FPS paradigm.

Corticotropin-releasing hormone (CRH)

As mentioned earlier, CRH is one of the key peptides involved in the mediation of stress responses. Therefore, one would expect stressor-related behaviors to also arise, in part, from altered CRH system(s). The influence of CRH on stressor-induced behavior can be opposite depending if the animal is in a familiar or novel environment.[41, 112, 466, 471] Whereas CRH increased locomotor activity in a familiar environment [112, 466], it reduced locomotion in a novel environment.[41, 466, 471] Several studies have demonstrated enhanced locomotor activity in an open field arena following administration of CRH at low doses. [252, 253, 417, 466] Conversely, high doses of CRH were shown to have the opposite effect.[40, 41, 190, 252, 466] More recent evidence supporting the anxiogenic effect of CRH on locomotion in the open field is provided by studies using genetic manipulation, by knockout or overexpression of CRH, CRH receptors or CRH binding protein. Indeed, animals overexpressing CRH exhibit reduced locomotor activity in the open field, but

showed no differences in that particular behavior when placed in a familiar environment.[490] Moreover, CRH binding protein knockout mice showed trends towards elevated locomotor activity.[206] Whereas the results of some studies support previous findings, others seem to yield evidence that is contradictory. Overexpression of CRH binding protein (CRH-BP-OE) in the pituitary of mice was reported to elicit enhanced locomotion in a familiar environment (open field).[206] These contradictory findings might be explained by the detection of excessively high levels of CRH in stress-relevant brain regions like the PVN in these CRH-BP-OE mice, which is thought to be a compensatory mechanism.[57] Results from the EPM have also served to further confirm the anxiogenic properties of CRH. For instance, when administered centrally, CRH reduced time spent in the open arms of the EPM.[457] CRH overexpressing mice showed a similar decrease in the time spent in the open arms or open arm entries.[177, 462, 490] These effects were blocked by pretreatment with CRH antagonists. Indeed, antagonism of CRH receptors, by several antagonists, evoked increases in the time spent in the open arm area of the EPM and the stress potentiated EPM test.[67, 257, 267, 271, 530] Studies corroborate previous findings by means of knockdown strategies where functional deletion of the CHR1 receptors significantly increased time spent in the open arms and the number of entries therein.[262] In the same vein, CRH-binding protein over expressing mice also demonstrated increased time spent in the open arms as well as increased open arm entries [57], while CRH-binding protein knockout animals showed a decrease in both of these measures.[206] Although CRH appears to play an important role in anxiety-related behaviors, its role in fear-related behavioral responses seems less clear. The majority of investigators agree that CRH plays at least a minimal role in fear.[103, 113, 262, 503] Results show a normal fear

potentiated startle response in animals administered centrally with αh-CRH, a CRH receptor antagonist.[103] Likewise, CRH1 receptor deficient mice present no alterations in the fear potentiation of the startle amplitude.[503] However, Yilmazer et al (2002) showed that animals displaying a more robust fear potentiated startle response following presentation of a cue have elevated levels or CRH immunoreativity in the CeA, but not in the basolateral amygdala.[524] Other studies report that CRH-induced increases in fear-potentiated startle in rats were blocked by administration of a novel CRH antagonist, CP-154,526 [441] or following injection of αh-CRH.[467] The use of the CER paradigm to investigate the role of CRH and its receptors in fear has yielded more positive results. Indeed, centrally administered CRH has been shown to induce freezing immediately following infusion.[59, 77, 175, 443] Several studies have demonstrated an attenuating effect on freezing behavior following administration of various CRH1 receptor antagonists, including αh-CRH, CP-154 526, Antalarmin, and DPC904.[78, 105, 113, 184, 201, 503] In conclusion, results of these studies suggest a role of CRH in stress-related behavior, yet this peptidergic system appears to play a more prominent role in mediating anxiety-related behaviors rather than those associated with a state of fear.

Arginine-Vasopressin (AVP)

As seen earlier, AVP activity is closely related and dependant on CRH function, therefore one might also expect a possible role for AVP in stress and anxiety-related behaviors. Although most studies have focused on CRH as a stress and anxiety-related peptide, a few studies have looked at the role of AVP in these behaviors. Previous findings revealed higher levels of AVP release following exposure to the open arm of the

EPM.[508] Conversely, treatment with different AVP V1 receptor antagonists elicited a increase in both the percent of time spent in the open arms as well as open arm entries in the EPM.[157, 508] These same anxiolytic effects were observed in the Vogel test, where administration of SSR149415, an AVP V1 receptor antagonist, increased the amount of punished drinking.[157] To our knowledge, data expressing a potential effect of AVP on anxiety and fear responses is scarce. Nonetheless, one study proposes the V1b receptor antagonist, SSR149415, as a potential anxiolytic compound. This antagonist is effective if taken orally and does not exhibit the side effects seen with the typical anxiolytic treatment benzodiazepines (e.g. diazepam) on motor activity, sedation, memory or cognitive functions. The anxiolytic effects of SSR149415 have been demonstrated in various animal models of Generalized Anxiety Disorder, including punished drinking, elevated plus-maze, light dark, and social interaction as well as fear-related paradigms such as fear-potentiated startle. It has also been shown to be effective in reducing depressive-like symptoms in rodents and its efficacy is similar to the antidepressant drug, fluoxetine, in various animal models including the forced-swim test, exposure to chronic mild stress and subordination stress.[442]

Bombesin-like peptides (BLPs)

Bombesin, a tetradecapeptide initially isolated from the skin of the European frog Bombina bombina [9] was shown to affect several biological or physiological functions. Indeed, the initial studies revealed that administration of bombesin (BB) to mammals altered various functions such as thermoregulation [50] and stimulation of gastric motility [116, 470]. This amphibian peptide was also found to have important behavioral effects in

rodents such as increased grooming and scratching behavior [230, 231, 330], appetite suppression [230, 231, 238], inhibition of sodium intake [97] as well as increased locomotor activity in familiar settings and decreases locomotor activity in a novel environment. The capacity of BB to alter physiological functioning in most mammals tested, including humans, suggested the presence of endogenous BLPs and their receptors in mammals. To date, two major families of mammalian counterparts of BB have been identified, namely the 27 amino acid gastrin-releasing peptide (GRP) with its truncated (decapeptide) form neuromedin C , and neuromedin B (NMB), with 3 forms differing in their number of amino acid makeup.

The NMB-10, composed of 10 amino acids, was first isolated from porcine spinal cord.[312] Minamino and colleagues (1985) isolated two larger forms of NMB, identified as NMB-30 and NMB-32 comprised of 30 and 32 amino acids respectively. It has been suggested that the larger forms of NMB may be precursors for the decapeptide NMB-10. [316, 339, 340, 428] On the other hand, GRP was isolated from porcine non-antral gastric tissue. [289] The examination of the amino acid chain of these BLPs reveal a striking structural and molecular homology with BB (see Table 1), more specifically at their carboxyl terminal portion, thought to be the more biologically active fraction of the peptide.[235, 312, 513] Although these peptides are widely distributed, their presence within various stress-relevant regions suggests their potential involvement in the mediation of stress-related responses.

Table 1: Amino acid sequence comparison of the c-terminal portion of each of the BLP subfamily members [312-315]

Bombesin subfamily	
BB	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}	***G ly-Asn***-His-***Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$***
Alytesin	pGlu-Gly-Arg-Leu-***Gly***-Thr-***Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$***
Ranatensin subfamily	
NMB_{1-32}	x-Val-His-Ser-Arg-***Gly-Asn***-Leu-***Trp-Ala***-Thr-***Gly-His***-Phe-***Met-NH$_2$***
NMB_{23-32}	***Gly-Asn***-Leu-***Trp-Ala***-Thr-***Gly-His***-Phe-***Met-NH$_2$***
Ranatensin	pGlu-Val-Pro-***Gln-Trp-Ala-Val-Gly-His***-Phe-***Met-NH$_2$***
Ranatensin R	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$_2$***
Litorin	pGlu-***Gln-Trp-Ala-Val-Gly-His***-Phe-***Met- NH$_2$***
Rhodei-litorin	pGlu-Leu-***Trp-Ala***-Thr-***Gly-His***-Phe-***Met- NH$_2$***
Phyllolitorin subfamily	
Leu^8-phyllolitorin	pGlu-Leu-***Trp-Ala-Val-Gly***-Ser-***Leu-Met- NH$_2$***
Phe^8-phyllolitorin	pGlu-Leu-***Trp-Ala-Val-Gly***-Ser-Phe-***Met- NH$_2$***
Thr^5-Leu^8-phyllolitorin	pGlu-Leu-***Trp-Ala***-Thr-***Gly***-Ser-***Leu-Met- NH$_2$***

Note: Structural homology of BLPs at the c-terminal region (in italic bold). "x" represents the amino acid chain preceding the sequence indicated.

Peptide distribution

Peripheral distribution of BLPs

Both NMB and GRP have been found to be widely distributed peripherally throughout the gastrointestinal tract as well as the central and peripheral nervous systems, and show little to no interspecies heterogeneity.[339] In the peripheral nervous system, NMB immunoreactivity has been observed in the trigeminal and dorsal root ganglia.[497] It has also been observed in the esophagus and the rectum.[339] Conversely, GRP immunoreactivity has been observed in nerve fibers of the gut, more specifically in

nerves of the muscular layer [126] and in nerves of the smooth muscle and myenteric ganglia.[107, 320]

Colocalization of BLPs in the CNS

There is evidence of colocalization of NMB and GRP in a limited number of regions in the central nervous system. Immunohistochemical examination of the distribution of these two BLPs in mammalian brain has revealed immunoreactive signals for both NMB and GRP in the pyramidal layer of the CA3 region of the hippocampus, the medial preoptic nucleus of the hypothalamus (lateral, medial and central), the parabrachial nucleus (central lateral, dorsal lateral and superior lateral) and finally the LC.[497] However, the distribution of NMB, GRP and their respective receptors differ greatly in most regions of the CNS.

Distribution of NMB in the CNS

High levels of immunoreactive (ir)-NMB have been localized in the forebrain areas. Specifically, in situ hybridization studies have revealed the presence of NMB in the external plexiform layer and the mitral cell layer of the olfactory bulb, in the polymorph layer of the dentate gyrus of the hippocampal formation.[497] More moderate hybridization signals of prepro NMB mRNA were identified in the mitral cell layer of the accessory olfactory bulb, the pyramidal layer of the CA3 region of the hippocampus, the CeA, the substancia nigra, the ventral tegmental area as well as different brain stem regions, including the principal sensory nucleus, the dorsal motor nucleus of the vagus nerve, the peridorsal tegmental nucleus, the nucleus incertus and more importantly, the

hindbrain raphé. Finally, the weakest signals for NMB mRNA were found in the lateral habenula and the reticular nucleus of the thalamus, the BNST, the supraoptic, arcuate and medial preoptic nuclei of the hypothalamus. NMB was also found in low concentrations in various brain stem areas such as the auditory (nucleus trapezoid body, ventral nucleus lateral lemniscus), visceral (area posterna, parabrachial nucleus), motor (facial nerve), reticular core (LC, median and hindbrain raphé nuclei, pontine reticular nucleus, gigantocellular reticular field, lateral reticular nucleus) and the spinal cord (substancia gelatinosa).[497]

Distribution of GRP in the CNS

The expression pattern of GRP is quite distinct from that of NMB. Whereas NMB was present in very few regions of the hippocampal formation (strictly in the CA3 region) and the amygdala (exclusively in the CeA), GRP expression in these regions is much more pronounced. The initial studies on the distribution of GRP in the brain, using immunohistochemical techniques, identified ir-GRP neurons in the hypothalamus, interpeduncular nucleus, central grey area, dorsolateral tegmental nucleus, dorsal parabrachial nucleus, NTS and the trigeminal complex.[356] These preliminary results were confirmed and complemented with the use of *in situ* hybridization techniques. GRP mRNA signals were present in high to moderate levels in the medial and lateral entorhinal areas, presubiculum, parasubiculum, the pyramidal layer of the subiculum, the pyramidal layer of the CA1, CA2, CA3 areas of the hippocampus, the granular layer of the dentate gyrus, as well as the amygdalohippocampal area, the anterior amygdaloid area, the posterior part of the cortical nucleus, the medial, central, lateral, basolateral, and

basomedial nuclei of the amygdala.[325] Moderate to weak concentrations of hybridization signals for GRP mRNA were detected in the isocortex (II, III, V and VI), the tenia tecta, the anterior olfactory nucleus, the medial nucleus and the nucleus diagonal band of the septum, the magnocellular preoptic nucleus and the substancia innominata (basal ganglia), as well as several nuclei of the thalamus (anteroventral, medial geniculate, posterior intralaminar) and the hypothalamus (median preoptic, anteroventral, suprachiasmatic, paraventricular, medial preoptic area and the posterior hypothalamic area). Lastly, GRP was also observed in the somatosensory area, the auditory area, the NTS, the parabrachial nucleus, the central grey, the LC, the interfascicular nucleus, the paranigral nucleus, the rostroventrolateral reticular nucleus as well as the substancia gelatinosa of the spinal cord.[497, 509]

Receptor distribution

Four different receptor subtypes have been identified with affinity for BB-like peptides.[150, 336, 387, 458, 498] These receptors are commonly referred to as BB_1, BB_2, BB_3, and BB_4. The first three receptor subtypes are known to exist in amphibians and mammals, although the BB_4 receptor subtype has only been identified in the amphibian brain.[336] The ligands for the BB_1 and the BB_2 receptor subtypes are the only endogenous compounds known to bind with very high affinity with the BLP receptors.[24, 237] The BB_1 receptors have higher affinity for NMB and are therefore known as NMB-preferring receptors; whereas the BB_2 receptors have higher affinity for GRP and are labeled as GRP-preferring receptors.

Distribution of BB_3 receptor subtype

Most studies assessing the role of BLPs have primarily focused on the BB_1 and BB_2 receptors, partly due to the fact that their endogenous ligands are well characterized. More recently attention has also been focused on the role of the BB_3 receptor subtype, which has a 47% homology with the BB_1 and a 51% homology with the BB_2 receptors.[425, 426] Southern blot analyses have revealed the presence of these receptors largely in the uterus, testis and lung carcinoma cells in humans and rats, but not in mice.[120, 346] In the CNS, the receptor gene for BB_3 was shown to be expressed mostly in the hypothalamic regions, more specifically in the PVN, arcuate and dorsomedial nuclei as well as the spinal cord.[346] Additionally, *in situ* hybridization studies have demonstrated that the BB_3 gene is also expressed in the parabrachial nucleus and the medial and central nuclei of the amygdala.[516] Although the role of these receptors have not yet been determined, functional deletion of the BB_3 receptor subtypes induced a variety of physiological deficiencies in animals. In particular, BB_3 knockout mice exhibit high levels of obesity, metabolic defects [347], and hyperresponsiveness to taste stimuli.[516] These BB_3 deficient mice have also been found to respond better to social isolation compared to the wild-type group.[516] These data suggest a possible involvement of the BB_3 receptor subtype in the modulation of taste preference, the regulation of neural mechanisms involved in isolation effects as well as the development of obesity.

Peripheral distribution of BLP receptors

Similar to the distinct distribution pattern for NMB and GRP, the expression patterns of BB_1 and BB_2 receptors are also differ markedly. However, in contrast with

peptide distribution, the BLP receptors expression is much more widespread throughout the brain and nonneural tissues. Peripherally, BB_1 receptors are found in high numbers in a subpopulation of the pituitary gland, the thyrotrophs [188], the esophagus, the stomach, the larger intestine, the kidney as well as the bladder.[229, 498] In contrast, the tissue distribution of BB_2 receptors in the periphery is not as localized as that of the BB_1 receptors. These receptors are found in relatively large quantities throughout the digestive system, urogenital system, the respiratory system, the heart and skin [229] as well as in lower levels in the lactotrophs and the somatotrophs of the pituitary gland.[188, 189]

Distribution of BB_1 receptors in the CNS

Autoradiography and in situ hybridization studies have identified strong signals for BB_1 receptors in all nuclei of the olfactory bulb, the hippocampus and the thalamus. Moderate levels of BB_1 receptors have been found in the medial nucleus of the amygdala, along with low levels in the amygdalohippocampal area, the nucleus lateral olfactory tract, the bed nucleus accessory olfactory tract and the cortical and intercalated nuclei of the amygdala; whereas NMB is exclusively found in the CeA. Furthermore, moderate to weak signals were detected in the isocortex (layer V and VI), the BNST, substantia nigra, the PVN, periventricular and ventromedial nuclei of the amygdala. Several brain stem areas along with the nucleus ambiguous, the peridorsal tegmental nucleus, the supragenual nucleus, the dorsal, medial and hindbrain raphé nuclei, the interpeduncular nucleus, the gigantocellular reticular field, the lateral paragigantocellular reticular field, the parvocellular, pontine and lateral reticular nuclei and finally throughout the spinal cord also express BB_1 receptors.[343, 497]

Distribution of BB_2 receptors in the CNS

Like BB_1 receptor subtype, the BB_2 receptors are widely distributed throughout the brain. Immunoreactive signals were localized in high concentrations in the olfactory bulb, nucleus accumbens, olfactory tubercule, basal caudate putamen, CeA, CA3 region of Ammon's horn of the hippocampus, paraventricular thalamic, central medial and paracentral thalamic nuclei, as well as the substancia gelatinosa of the spinal cord. Moderate levels of BB_2 receptors were found in the granule cell layer, the layer V and VI of the cortex, temporal cortex, occipital cortex, BNST, anterior amygdaloid area, cingulated, frontal, parietal and insular cortex, throughout the hypothalamus, the rhomboid thalamic nucleus, arcuate nucleus, medial amygdaloid nucleus, primary olfactory cortex, subiculum, interpeduncular nucleus, entorhinal cortex, LC, parabrachial nucleus as well as the floor of the 4th ventricle.[236, 323, 510, 527] Moreover, *in situ* hybridization studies detected signals for the BB_2 receptors in the isocortex, the dentate gyrus, nucleus of the olfactory tract, magnocellular preoptic nucleus, basal ganglia, nucleus ambiguous, throughout the hypothalamus, nucleus accumbens, central grey and finally in the NTS.[24, 325, 498] The raphé nuclei express both BB_1 and BB_2 receptor subtypes.[297, 325]

In summary, neurons expressing ir-BLPs and BLP receptors are extensively distributed throughout the CNS, and are present within various stress-relevant regions, including the PVN, arcuate nucleus, ME, medial preoptic area, amygdaloid nuclei, BNST, hippocampus, LC, NTS, olfactory bulbs and pituitary gland. These data support the contention that this family of peptides might be involved in the mediation or

expression of the stress response. The distinct distribution pattern of the BLPs and their receptors further suggests that NMB and GRP might be differentially implicated in the regulation of stress and anxiety-related responses.

The role of BLPs in stress and anxiety

In addition to the localization of BLP and their receptors in stress-relevant regions, another indication that this family of peptides is involved in the mediation of stress-related responses comes from the observation that stressor exposure is associated with changes in BLP 1) receptor densities, 2) tissue concentrations, and 3) release patterns. Indeed, site-specific increases in tissue concentration of BLPs and an increase in the density of their receptors at stress-sensitive brain regions have been observed following exposure to a variety of stressors.[297] For example, exposure to acute immobilization stress is associated with increased ir-BLP levels at the hypothalamus and medulla, as well as increased density of related receptors within the PVN and NTS.[220] In keeping with these observations, the release of BLPs is also affected by stressor exposure. Indeed, exposure to an acute stressor increased the interstitial levels of both NMB and GRP at the anterior pituitary [297] and the CeA.[302] Based on these results, it would appear that BLPs are involved in the integration and/or modulation of the stress response.

Effect of BLPs on the HPA axis

One way of elucidating the processes or mechanism(s) by which BLPs affect stress responses is to assess their influence on the HPA axis/endocrine system in relation to stressor exposure. Specifically, it would be of interest to determine whether BLPs

influence blood concentrations of ACTH and corticosterone. Evidence from our laboratory and those of others has shown that centrally administered BLPs stimulated the release of ACTH from the anterior pituitary gland and of corticosterone from the adrenal cortex.[140, 212, 213] No studies to date have investigated the effects of centrally administered NMB on ACTH or corticosterone release. However, systemic administration of NMB is known to increase circulating levels of ACTH and corticosterone.[274, 277] Unlike NMB, effects of peripherally administered GRP on the release of these hormones have been less consistent. Sander LD and Thomas RM (1991) found that GRP significantly altered both ACTH and corticosterone levels whether it was delivered intravenously or intraperitoneally. One *in vitro* study also showed increased ACTH and corticosterone concentrations following administration of high doses of GRP.[140] However, two studies failed to see a stimulatory effect of GRP on plasma levels of ACTH or corticosterone.[140, 348] One explanation for these discrepant results might be the considerable difference in dosage used in the experiments. Thus, methodological and variations in types of subjects utilized may also have affected the outcomes obtained. Irrespective of the existing inconsistency in the literature, there is sufficient evidence that BLPs do influence HPA activity as demonstrated by their effect on ACTH and corticosterone.

Effect of BLPs on the ANS

In addition to their effect on HPA activity, there is ample evidence suggesting that BLPs are also involved in the regulation of ANS responses. Studies have identified high densities of ir-BLPs in sympathetic ganglia and splenic nerves, supporting a possible role in ANS activity. Interestingly, ir-BLPs are present primarily in noradrenergic cells.

Furthermore, central administration of BLPs dose-dependently increased systemic and central epinephrine, NE and plasma glucose levels.[43, 45] Epinephrine and GRP are concurrently released from the adrenal gland and the nerves of the stomach respectively, to influence gastric secretion by binding to the beta 2-adrenergic receptors, suggesting a possible interaction of these compounds in the regulation of gastric outflow.[53] It is thought that GRP exerts its effect on the SNS and the release of epinephrine from the adrenal gland through central mechanisms.[43, 44, 258, 259] Indeed, effects of GRP on the SNS were hampered by ganglionic blockade, vagotomy or adrenalectomy [258, 259], supporting the contention that GRP regulates the SNS through central but not systemic mechanisms. [31, 258, 259] In addition, central administration of BLPs elevated plasma glucose concentrations.[288, 376, 379] Studies have demonstrated that insulin-induced hypoglycemia in animals significantly increased GRP concentrations [472], which was followed by a marked increase in plasma glucose levels. Results from more recent studies investigating the effect of genetic manipulation on glucose regulation have found altered glucose tolerance as well as an attenuation of the insulin response in BB_2 receptor deficient mice.[368] In addition to its effect on glucose regulation, GRP has also been shown to be involved in insulin and glucagon activity. It is thought that GRP serves to increase both insulin and glucagon levels in rats.[204, 205] This effect is mediated through a calcium dependant process where GRP facilitates either calcium uptake, inhibits the calcium-dependant potassium channels or reduces the repolarization of the cells.[204, 205, 499] Moreover, these effects appear to be species dependant as studies conducted with dogs showed opposite effects. In these animals, GRP seemed to act by eliciting a decrease in glucose levels, whereas it was previously shown to increase glucose levels in rats, mice

and sheep.[288] Although the results using different species seem contradictory, they do strengthen and confirm a role for GRP in gastric hormone regulation. Although fewer studies have examined the role of NMB in glucose regulation, data show a similar effect of NMB on glucose, insulin and glucagon homeostasis. Like GRP, NMB also dose-dependently elevated plasma glucose and glucagon levels in rats.[209, 376, 379] Administration of NMB also caused an increased release of insulin from pancreatic cells, an effect facilitated by elevated glucose levels.[208, 337]

Thermoregulation and arterial blood pressure, two additional functions controlled by the ANS, are also influenced by BLPs. Central administration of either NMB or GRP dose-dependently decreased body temperature in animals, with GRP having a greater potency than NMB.[185] Interestingly, pretreatment with a specific BB_2 receptor antagonist completely blocked the BB-induced decrease in body temperature, whereas a relatively specific BB_1 receptor antagonist had no effect, suggesting a differential role for these BLPs in hypothermia [483], with the BB_2 receptor subtype primarily mediating these thermoregulatory effects. However, one study has shown a mild effect of NMB on thermoregulation.[343, 344] Intracerebroventricular administration of a NMB agonist slightly decreased body temperature in BB_1 receptor deficient mice; whereas GRP agonist administration resulted in a robust reduction in temperature in both wild-type and BB_1 receptor deficient mice.[343, 344] These results indicate a primary role for BB_2 receptor suptype in thermoregulation and a more secondary role for the BB_1 receptor in this function.[343, 344] Further evidence suggesting autonomic modulation of BLPs is provided

by the finding that systemic GRP administration dose-dependently increased blood pressure in animals.[185]

Effect of BLPs on behavior

In addition to their effect on the endocrine and autonomic systems, BLPs have also been implicated in the control of stress related-behaviors. Indeed, BLPs appear to promote behaviors that are commonly associated with a state of stress or anxiety, including suppression of food intake, intense grooming and scratching behavior, and decreased locomotor activity in a novel environment as well as increased locomotion in a familiar environment.[194, 198, 231] Evidence supporting the involvement of BLPs in the mediation of the aforementioned stress-related behaviors (feeding, grooming, and locomotor activity) will be presented.

In vivo studies looking at the release of BLPs reported increased interstitial levels of GRP bilaterally at the medial prefrontal cortex upon exposure to a cue previously paired with a palatable snack.[301] Increased GRP release was also noted before (anticipation), during (ingestion) and after (digestion) presentation of a meal.[165] Conversely, previous studies have shown that at the PVN, GRP release was decreased during the ingestion of a meal, as compared to levels prior to and after ingestion [377], an effect not detected at other brain sites studied. These studies suggest that the role of BLPs on feeding behavior might differ in a site-specific manner. Further support for the involvement of BLPs in satiety is provided by studies investigating the effect of exogenously administered peptides. For instance, bilateral microinjection of GRP at the

CeA (at doses ranging from 50 and 100 ng) significantly inhibited food intake in rats [121], whereas administration of higher or lower doses of the peptide were without effect. This suppression of food intake was reversed by pretreatment with a BB_2 receptor antagonist, suggesting involvement of this receptor subtype in satiety. Additionally, the effect of GRP appears to be transient in nature, decreasing the size and duration of the first meal, increasing the length of the first postprandial intermeal interval exclusively, without altering the size or the intermeal interval of subsequent meals.[121 421-423, 474] Thus it would appear that GRP acts through mechanisms occurring shortly after ingestion of a meal (within 5 min postprandial) to alter feeding and intermeal interval duration.[452] The suppressive effect of GRP on food intake is thought to be a consequence of a decrease in the reinforcing nature of the food present. In support of this contention, Thaw and Quinn (2003) and Rushing and Houpt (1999) report that infusion of GRP reduced ingestion of sucrose and milk by reducing the reinforcing characteristics of these compounds.[421, 475] These studies support an alteration of the appetitive or reinforcing nature of food in lieu of a direct effect on appetite. Further studies have attempted to elucidate the mechanisms underlying the effect of GRP and BLPs on satiety. Administration of a CCK-A receptor antagonist (devazepide) significantly increased gastric emptying, completely reversing the inhibitory effect of GRP on this function, and thus providing evidence of a CCK-dependant action of GRP.[240] Unlike GRP, the effects of NMB on feeding behavior have yielded inconsistent results. One study reported that systemic administration of NMB (5 nmol/kg) significantly decreased the size and the duration of spontaneous meals.[422] Another study also showed a reduction on food intake in animals administered with NMB.[239] However, in a more recent study, systemic infusion of NMB failed to alter

feeding behavior at different doses (2.5, 5 or 10 nM/kg).[474] This lack of NMB effect on feeding was further supported by observations in NMB deficient mice; NMB knockout mice did not show differences in their feeding behavior or pattern as compared to their wild-type counterparts.[345]

Increased grooming behavior is believed to be another reliable indicator of the "stress" level of rodents. Central administration of both GRP and NMB induced excessive grooming and scratching behavior.[146, 491, 492] While the GRP-induced grooming is intense, the effect of NMB is not as robust. The induction of grooming and scratching following BLP administration is transient, appearing as soon as 1 min following central infusion and dissipating by 30 min. In this regard, NMB appears to have an exceedingly short half life, probably due to its quick degradation by endogenous peptidase after administration.[121, 376] The mechanisms underlying the BLP effects on grooming behavior are not well understood. There is some evidence in the literature suggesting that this effect might be mediated through the dopaminergic and/or opioid systems.[303, 491, 492] Indeed, blockade of both DA and opioid receptors attenuated BLP-induced grooming and scratching.[303, 491, 492]

Another behavioral consequence of stress and anxiety is alterations in exploratory activity. [297] The release of BLPs in response to stressor exposure is believed to cause an increase in exploratory activity in a familiar environment while decreasing the same behavior in a novel milieu.[297] In support of this contention, studies using mice lacking BB_2 receptors displayed higher levels of spontaneous locomotor activity in their home

cage (familiar environment) as compared to wild-type mice.[496] Furthermore, central administration of NMB or GRP reduced exploratory behavior in animals as measured in an open field test (novel environment), an indication of a state of stress or anxiety.[194]

Further evidence for a role for BLPs in stress-related behavior is provided by the finding that these peptides are involved in the mediation and/or modulation of fear or aversive learning and memory processes. Systemic administration of GRP immediately following training in a memory task, significantly improved performance in mice, an effect seen only when relatively low doses of scopolamine were administered. Consequently, these results suggest that GRP was able to counteract the impairing effects of scopolamine on memory, but only when the memory deficits were relatively mild.[433] The dose of GRP administered seems to be an important factor to take into account as previous research has shown that administration of high doses of GRP did not incur enhancing effects on memory, but rather induced amnesia in animals, nevertheless, these results do suggest a role for GRP in memory processes.[132] Further support for the involvement of a physiological role of BLPs in fear and aversive learning and memory is provided by pharmacological studies using BLP receptor antagonists. For instance, Roesler and colleagues (2003, 2004a, 2004b) demonstrated that treatment with a specific BB_2 receptor antagonist (RC-3095) impaired performance in a memory test involving emotional memory, whether the antagonist was administered systemically or centrally, as well as before or after the training session.[407-409] Furthermore, GRP and NMB appear to influence a particular form of memory. Specifically, BB_2 receptor blockade with RC-3095 did not produce memory deficits in a hippocampal-dependant task (Morris water

maze), supporting the contention that GRP and its receptors are selectively involved in aversive and fear-related memory, and not as much in non-aversive, non-spatial and/or non-emotionally based types of memory processes.[407-409, 445, 494] While the underlying mechanisms mediating BLPs effects on aversive learning and memory processes are currently not well understood, there is evidence for the involvement of the GABAergic system, as pretraining intrahippocampal or intra-BLA treatment with muscimol (direct GABA receptor agonist) blocked the memory deficit effect of RC-3095 administration in rats.[408] Based on these results, it would appear that the BB_2 receptors serve more of a secondary or modulatory role in aversive memory formation rather than a direct role in memory processes. This contention is supported by the reversal by muscimol of the memory inhibition effect of the BB_2 receptor antagonist administration, RC-3095.[408] Moreover, Shumyatsky and his colleagues (2002) have identified high levels of BB_2 receptor densities on GABA interneurons in the lateral nucleus of the amygdala (LA), a brain region known to be involved in fear memory and learning. The BB_2 receptor deficient mice exhibited higher levels of freezing behavior in a conditioned emotional response paradigm (conditioned fear) immediately upon presentation of the conditioned stimulus (CS; a tone) or to the context where they previously receive a footshock; whereas wild-type mice displayed delayed freezing (few seconds) to presentation of the CS and their freezing occurrences were lower than the mutant mice. These findings suggest that GRP knockout mice might benefit from faster memory processing and learning due to the absence of GABA inhibition, and further support a role for BLPs in the regulation of memory function through their action on GABA function.[445] The BB_2 receptors are also thought to form partnerships with other neurotransmitter systems in the

modulation of memory formatrion.[494] Venturella and colleagues (2005) demonstrated that, although blockade of GRP receptors was able to prevent cell proliferation triggered by dexamethasone, it did not attenuate the memory enhancing effect of dexamethasone given following training on a memory task. Based on these findings, it is possible that the BB_2 receptor subtype helps regulate or facilitate aversive memory consolidation, partly through interactions with the GABA and/or glucocorticoid systems, but is not necessarily a direct participant in these processes.

Although the role of NMB in memory has not been well investigated to date, one study has demonstrated the possible participation of the NMB/BB_1 receptor subtype system in a memory-related task.[519] When subjected to a 30 minute restraint stress prior to conditioning, BB_1 receptor deficient mice presented longer step-through latencies in the passive avoidance test compared to their wild-type counterparts. This impairment in aversive memory performace/conditioning in BB_1 receptor deficient mice suggests that this system may serve a function in aversive memory processes. However, further studies are needed in order to establish stronger evidence for the involvement of the NMB system in memory.

Interaction between BLPs and neuropeptide/neurotransmitter systems

While the exact neural mechanisms mediating the effects of BLPs are unknown, there is evidence of communication with CRH system(s). Interestingly, both BLPs and CRH, and their respective receptors, follow similar distribution patterns.[212] Additionally, central administration of CRH dose-dependently produces similar endocrine, autonomic

and behavioral changes as those elicited by stressor exposure or central BLP administration.[211, 212] Therefore, there is reason to believe that these two peptidergic systems might influence each other to exert their effects. Specifically, our laboratory has proposed that BLPs may mediate their effects, at least in part, by acting on the CRH system. Initially, animals exposed to a stressor (restraint stress) show simultaneous increases in the release of BLPs and CRH at the CeA.[302] Moreover, central administration of BB elicits the release of CRH from both the ME and the anterior pituitary.[212] Finally, our laboratory has further revealed that blockade of CRH receptors with αh-CRH attenuated the BB-induced behavioral (decreased food intake, increased grooming), endocrine (increase in plasma levels of ACTH and corticosterone), and autonomic (increase in plasma levels of NE, epinephrine and glucose) effects.[213, 378] Similarly, Garrido and colleagues (1998) found that pretreatment with αh-CRH completely blocked the GRP-induced elevations in plasma levels of ACTH and corticosterone. *In vitro* studies using isolated pituitary cells, along with pharmacological studies, have demonstrated that the CRH-induced release of ACTH was markedly potentiated by GRP administration and this effect was blocked by pretreatment with BB_2 receptor antagonist or with CRH antiserum.[19, 348] Taken together, these findings support the possibility of a communication between BLP and CRH systems in the mediation of stressor-related responses.

In addition to their effects on CRH activity, it would appear that BLPs might influence stress and anxiety responses through their interactions with 5-HT, a neurotransmitter known to play an important role in the response to stressor exposure.[71,

[502] Indeed, careful consideration of the serotonergic system in BB_1 receptor deficient mice revealed that these mice expressed higher levels of 5-HT as compared to their wild-type counterparts.[522] Further investigations in NMB-deficient mice revealed a diminution in anxiety levels as reflected by decreased marble burying in these animals, a behavior though to be modulated via serotonergic neurons.[520] Additionally, a significant downregulation of the expression of $5\text{-}HT_{1A}$ autoreceptors was observed in these mutant mice. Pharmacological administration of BB and related peptides caused a depolarization of a subpopulation of 5-HT neurons in the dorsal raphé nucleus.[374] This depolaroization seemed to occur by activation of postsynaptic receptor located on these serotonergic neurons. Increasing evidence points to a functional interplay between BLPs and 5-HT, however, very little information on the nature of this interaction is currently available. There is, however, one study demonstrating that blockade of BB_2 receptor subtype resulted in an attenuation of the stressor-induced activation of the serotonergic system and the HPA axis.[138] They also found increased 5-HT release following GRP administration. It is important to note that these effects were observed only at the PVN. Furthermore, levels of 5-HT were extrapolated from measures of 5-HIAA concentrations, a 5-HT metabolite, which may not necessarily reflect enhanced transmitter release.[66, 83, 200]

Overall Thesis Objectives:

As can be appreciated from the preceding review of the literature, evidence has accumulated that strongly supports involvement of the BB family of peptides in the mediation and/or modulation of the stress response. Gastrin-releasing peptide and NMB along with their respective receptor subtypes are localized in many stress-relevant brain regions including elements of the HPA axis. Moreover, exogenous administration of these mammalian BB counterparts elicits many endocrine, autonomic and behavioral effects, reminiscent of the stress response. However, despite many of their similarities, GRP and NMB, seem to have distinct distribution patterns, as well as subtle differences in their endocrine, autonomic and behavioral effects, suggesting that GRP and NMB may play differential roles in HPA activation and stress-related responses. Thus, the overall objective of this dissertation was to further characterize the differentiation between GRP and NMB in terms of their involvement in 1) the general stress response and 2) stress-related responses, namely anxiety and fear. Six specific objectives addressing particular questions have been proposed in an attempt to attain this overall objective.

1 - Does exogenous administration of BB-related peptides mimic the effects of stressor exposure? Specifically, does central administration of NMB or GRP activate the HPA axis as measured by increased levels of ACTH and corticosterone? Furthermore, is the increased release of plasma ACTH and corticosterone induced by stressor exposure attenuated by blockade of BB_1 and/or BB_2 receptors?

2 - If a peptide is physiologically relevant in the modulation of the activity of the HPA axis, then one might expect to see changes in its endogenous release in response to stressor exposure. Thus, in Chapter II we wanted to investigate 1) if acute stressor exposure (appetitive or aversive) altered the availability of GRP and NMB at a selected stress-relevant brain sites and 2) are such stressor-elicited changes in the release of peptides affected by prior stress history?

3 - Does central administration of NMB and GRP alter anxiety-like or fear-type responses as measured using animal models of anxiety (open field and elevated plus maze) and fear (fear potentiated startle paradigm)? Conversely, does the blockade of BB1 or BB2 receptors produce anti-anxiety or fear-reducing effects as assessed in these same paradigms?

4 - If NMB and/or GRP do play a role in anxiety and fear-related responses, what are the underlying mechanisms by which they mediate these effects? Specifically, do NMB (and/or GRP) mediate their effects via the serotonergic system?

5 - Does chronic infusion of antisense oligodeoxynucleotides directed against BB_1 or BB_2 receptors produce anti-anxiety effects using animal models to assess anxiety?

6 - Are GRP and NMB involved in the mediation of fear-related responses? Does infusion of GRP, NMB or their respective receptor antagonists affect the acquisition and expression of conditioned fear?

Preface to Chapter I

Most of the earlier research investigating the role of BLPs was done using the native amphibian peptide, namely BB itself. Subsequent studies demonstrated that administration of BB elicited endocrine, autonomic and behavioral effects, which resembled those elicited by stressor exposure. However, few, if any, studies have assessed the differential role(s) of the mammalian BB counterparts, NMB and GRP, in this context. The objective of this set of experiments was to assess the specific effects of GRP and NMB in HPA activation.

Chapter 1

The role of bombesin-like peptides in the regulation of the HPA axis

Abstract

Evidence suggests involvement of bombesin-like peptides (BLPs) in the mediation and/or modulation of the stress response; however the specific roles of the mammalian BB analogues, neuromedin B (NMB) and gastrin-releasing peptide (GRP) in hypothalamic-pituitary-adrenal (HPA) axis and sympathetic activation have not been well delineated. Thus, the objectives of the present investigation were to assess the effects of 1) central administration of NMB or GRP on stress hormone (ACTH, corticosterone) and glucose levels and 2) blockade of NMB (BB_1) and GRP (BB_2) receptors on the stressor-induced release of ACTH, corticosterone and glucose. Results showed that blood glucose and corticosterone levels were significantly elevated above baseline following all treatment conditions. Central administration of GRP attenuated the stressor-induced increases in blood corticosterone concentrations. However, NMB administration failed to alter glucose or corticosterone levels. We then assessed if stressor-induced elevation of glucose and corticosterone could be attenuated or blocked by administration of BLP receptor antagonists. Glucose and corticosterone levels increased above baseline levels following stressor exposure in all treatment groups (vehicle, GRP antagonist (GRPa) and NMB antagonist (NMBa)). Whereas central administration of GRPa significantly potentiated the stressor-induced rise in corticosterone levels, the NMBa was without effect. These findings support the notion that BLPs can affect the outcome of stressor-elicited HPA axis and sympathetic activation.

Introduction

When an event is perceived as a potential stressor (or a threat to homeostasis), a cascade of behavioral and physiological processes is triggered. One system known to play a primary role in the integration of the response is the hypothalamic-pituitary-adrenal (HPA) axis.[17, 176] The activation of the HPA axis is commonly measured by changes in levels of two hormones, namely adrenocorticotropin hormone (ACTH) and/or corticosterone.[176, 373] In addition to the activation of the HPA axis, stressors also cause a concomitant activation of the sympathetic division of the autonomic nervous system.[39, 197] One of the physiological changes associated with sympathetic activation is an increase in blood glucose concentrations.[45]

Bombesin (BB), a tetradecapeptide isolated from the skin of the amphibian *bombina bombina*, has been implicated in the regulation of the stress response.[297] In support of this contention, a number of studies measuring bombesin-like peptide (BLP) immunoreactivity (ir-BB) have identified high concentrations of these peptides in brain sites representing key nodes of the HPA axis, especially the hypothalamus and the pituitary gland.[147, 189, 326, 356] Using in situ hybridization technique, BLP mRNA and their receptors have been located in the paraventricular, suprachiasmatic, supra optic, preoptic, arcuate and mammilary hypothalamic nuclei, the median eminence and the pituitary gland.[24, 147, 189, 323, 497, 510] In addition, extra-HPA brain regions that are known to be involved in the stress response have also been found to express BLPs and their receptors. For instance, the nucleus of the solitary tract, the parabrachial nucleus, the bed nucleus of the stria terminalis, the cortical and central amygdaloid nuclei and the hippocampus

contain relatively high levels of ir-BB.[72, 323, 326, 356, 369, 510, 527] Moreover, BLP receptors have been identified in the locus coeruleus and the dorsal raphé nucleus, further supporting the involvement of this family of peptides in the mediation and/or modulation of the stress response.[254, 323]

Several laboratories, including ours have provided pharmacological and physiological evidence demonstrating the involvement of BLPs in the mediation of the stress response. For example, exogenously administered BB caused a dose-dependant increase in plasma levels of ACTH and corticosterone.[163, 213, 277] Moreover, BB administration also elicits behaviors typically associated with a state of stress or arousal, such as increased grooming, scratching, exploration in a familiar environment and decreased food consumption and exploration in a novel (presumably stressful) environment.[142, 194, 284, 296, 378, 440] We have further shown that stressor exposure is associated with site-specific alterations of 1) endogenous levels of BLPs [211, 298, 302] and 2) BLP-receptor densities.[211]

Although, BB per se is not present in mammals, its two mammalian counterparts namely, gastrin-releasing peptide (GRP) and neuromedin B (NMB), are, as discussed earlier, differentially expressed in mammals. Whereas NMB has a higher affinity for the BB receptor subtype 1 (BB_1), GRP has greater affinity for BB receptor subtype 2 (BB_2). Gastrin-releasing peptide has previously been shown to affect the release of ACTH and corticosterone, and thus the activity of the HPA axis.[138-140] Some of these effects are due to direct action of these peptides on specific endocrine cells, as in vitro exposure of

isolated pituitary and adrenal gland to high doses of GRP, provoked the release of ACTH and corticosterone.[139] This effect was completely blocked by infusion of a specific GRP antagonist. Furthermore, central administration of GRP increased the secretion of ACTH and corticosterone at all doses tested.[140] Again, the specific GRP antagonist completely blocked the GRP-induced increase of these stress hormones.[140]

While there is evidence demonstrating that exogenously administered BB or GRP causes elevations in plasma levels of ACTH and corticosterone *in vitro* and *in vivo* [139, 140, 213], experiments linking NMB to the activation of the HPA axis are sparse. For instance, one study by Malendowicz and Nussdorfer [277], demonstrated that a subcutaneous injection of NMB elicited a significant rise in plasma levels of ACTH and corticosterone. However, no studies to date have explored the endocrine effects of centrally administered NMB or have attempted to directly compare the effects of GRP and NMB on HPA axis activity. Thus, if NMB is involved in the regulation of the HPA axis, this should be reflected by increased plasma levels of corticosterone, ACTH and glucose in response to a central administration of this peptide. Furthermore, blockade of BB_1 receptors with a BB_1 receptor antagonist should attenuate the rise in corticosterone, ACTH and glucose caused by stressor exposure.

In view of these considerations, the goal of the present study was to explore the role of BLPs in the functioning of the HPA axis. In a first study, we aimed to assess whether central administration of GRP or NMB would differentially affect blood levels of corticosterone and glucose as well as plasma levels of ACTH. We also wanted to

determine if the blockade of BB_1 and/or BB_2 receptors would attenuate some of these stressor-induced changes.

Materials and Methods

Subjects

Male Sprague-Dawley rats obtained from Charles River (St-Constant, Quebec, Canada) were used in both experiments. Animals weighed approximately 300 g at the time of their arrival and were housed individually in a temperature (23°C) and humidity-controlled (60 %) room. They were maintained on a 12-hour light/dark cycle (lights on at 07:00). Rats had free access to food (Purina Rat Chow) and tap water. All experimental procedures were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the University Animal Care Committee.

Drugs

Gastrin-releasing peptide and Neuromedin B-30 (porcine) (Phoenix Pharmaceuticals, Belmont, CA, USA), GRP antagonist (Leu^{13}-ψ-CH_2NH-Leu^{14}) – bombesin and Neuromedin B antagonist, BIM 23127 (Bachem, King of Prussia, PA, USA) were dissolved in Kreb's Phosphate Ringer Buffer (KRB).

Surgical procedure

Rats were anesthetized (pentobarbital, 60 mg/kg, i.p.) and stereotaxically implanted with a permanent 22-gauge guide cannula (containing a removable obturator) aimed at the 3rd ventricle. The coordinates, obtained from Paxinos and Watson [361] were

from level skull AP: -4.4 mm; DV: -4.4 mm; L: 0. The cannula was anchored to the skull surface using 4 stainless steel screws and acrylic dental cement. Animals were allowed a 1-week recovery period before the experiment started. Animals were then acclimatized to handling and mock central injection procedures. All experiments were performed between 9:00 and 12:00 hrs.

Experimental protocol

Experiment 1: ***Effects of central NMB and GRP administration on plasma levels of corticosterone, and glucose.***

On the day of testing, rats were randomly divided into 3 groups as follows: 1) Vehicle (3 μl of KRB; i.c.v.; n = 7), 2) NMB (0.31 nmoles/3 μl; i.c.v.; n = 8), and 3) GRP (0.31 nmoles/3 μl; i.c.v.; n = 7). Both NMB and GRP were freshly dissolved in KRB prior to administration. Microinjections were delivered in a 3 μl volume infused over a 60 s period via an injection cannula (protruding 0.5 mm beyond the guide cannula) connected to an infusion pump (Harvard Apparatus, MA) via polyethylene tubing. The blood samples for glucose and corticosterone measurement were collected at 0, 15, 30 and 60 min following BB infusion (0.5 μg/3 μl/60 s) by lancing the rat's tail close to the tip with a sterile surgical scalpel blade (size 10) and then wiping the first droplet of blood away. Subsequent blood droplets were collected (2 for each time point). One droplet was collected with a glucose strip and immediately analyzed for glucose content using a portable glucose meter Elite^{TM} (Ames, Miles, Canada). A second droplet was blotted onto preprinted circles on S&S filter paper (Schleicher & Schuell, Mandel Scientific), allowed to dry at room temperature and were stored at –20°C until analysis for

corticosterone levels. Blood was eluted from the filter paper by placing one 2.5 mm punch of filter paper in a 12 x 75 culture tube containing 100 µl of Dulbeco's phosphate buffered saline (Sigma, USA) and then shaking the tubes on an automatic shaker (50 rpm) for 1 hr at room temperature. Tubes are stored overnight at 4°C. On the following day, tubes are again agitated on the automatic shaker for 1 hr at room temperature and corticosterone levels in the eluted samples were determined using a commercial RIA kit (ICN Pharmaceuticals, CA). Blood glucose levels were measured from whole blood using a portable Elite^{TM} glucometer (Ames, Miles, Canada). Before returning the rat to its home cage, an additional droplet was extracted and deposited on a glucometer test strip. The reaction was allowed to take place for 55 s and the glucose concentration was determined. At the 60 min time interval, animals were sacrificed by decapitation and trunk blood was collected for subsequent analysis of plasma ACTH levels. Trunk blood was collected in tubes containing EDTA (to prevent blood clotting), centrifuged and the plasma was stored in microcetrifuge eppendorf tubes at –80°C until analysis. Plasma samples were thawed and assayed for ACTH levels using a commercial RIA kit (ICN Pharmaceuticals, CA).

Experiment 2: ***Effects of blockade of BB_1 and/or BB_2 receptors on levels of corticosteron, and glucose following mild stressor exposure.***

The design of this experiment was identical to that of Experiment 1 with the exception that rats were randomly divided in to the following groups: 1) NMB antagonist (NMBa; 1 µg/3 µl; n = 8), 2) GRP antagonist (GRPa; 1 µg/3 µl; n = 7), and 3) Vehicle injection (3 µl of KRB; n = 7). Briefly, a baseline tail blood sample was taken as

described above, and animals were then injected with the respective drug. Fifteen minutes following drug infusion, an additional blood sample was taken, after which time rats were exposed to an air puff stressor. The stressor consisted of 5 puffs of air delivered in the facial region of a rat at a rate of 1 puff every 30 s for 2 min. Subsequently, a final blood sample was taken 30 min following drug administration for corticosterone content analysis (as described above).

Statistical Analysis

All results are expressed as means ± S.E.M. In both experiments, corticosterone values were analyzed separately using two factor repeated measure ANOVAs. The between-groups factor was Treatment condition (vehicle, GRP, NMB and vehicle, GRPa, NMBa) and the within-subjects factor was Time (0, 15 and 30 min). Post hoc comparisons were conducted using Newman-Keuls multiple comparison tests. A value of $p < 0.05$ was considered statistically significant.

Results

Experiment 1: *Effects of central NMB and GRP administration on plasma levels of corticosterone and glucose.*

Figure 1 shows blood corticosterone levels and Figure 2 depicts blood glucose concentrations, at 0, 15 and 30 min following central administration of either vehicle, NMB or GRP agonists. The overall two factor repeated measure analysis revealed that blood corticosterone concentrations varied as a function of Treatment x Time interaction ($F(4, 42) = 3.246$; $p < 0.05$). 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 by 15 min post drug injection and remained elevated for the remainder of the testing period. The follow up tests further revealed that prior to drug administration, levels of corticosterone were comparable for all groups. In rats treated with GRP, the rise in corticosterone was significantly attenuated in comparison to the vehicle control, at the 15 min interval. However, by the 30 min interval, there were no significant differences in corticosterone levels across the various treatment groups.

The overall two-factor repeated measure analysis revealed that blood glucose levels did not vary as a function of Treatment x Time interaction. However, the simple effect of Time revealed that there was a significant difference in blood glucose concentrations over time. Blood glucose levels were significantly elevated above baseline values by 30 min following drug injection for all treatment conditions until the end of the experiment.

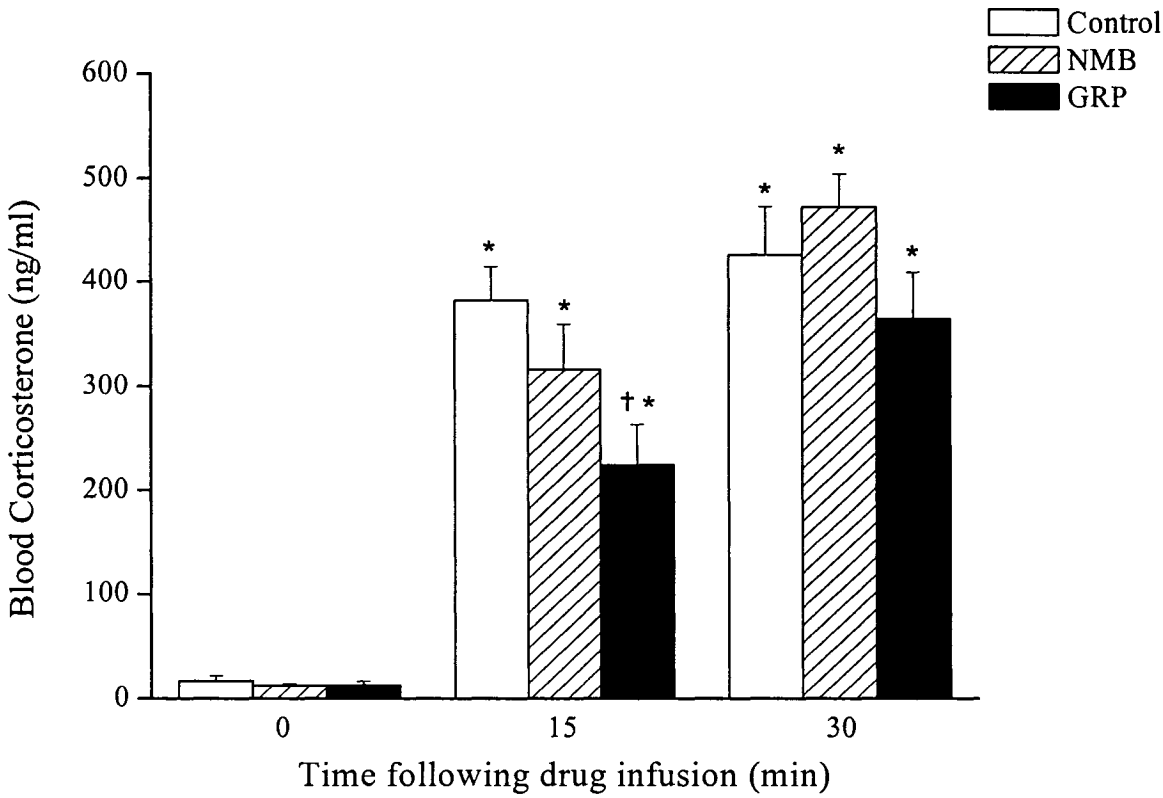


Figure 1 Blood concentrations of corticosterone (mean ± S.E.M.) at the 0, 15 and 30 min time intervals following drug administration in rats that received either vehicle (open columns), NMB-30 (hatched columns) or GRP (solid columns).
* significantly different from (within-treatment condition) baseline values at $p < 0.05$.
† significantly different from (between-treatment condition) time-matched control (vehicle) values at $p < 0.05$.

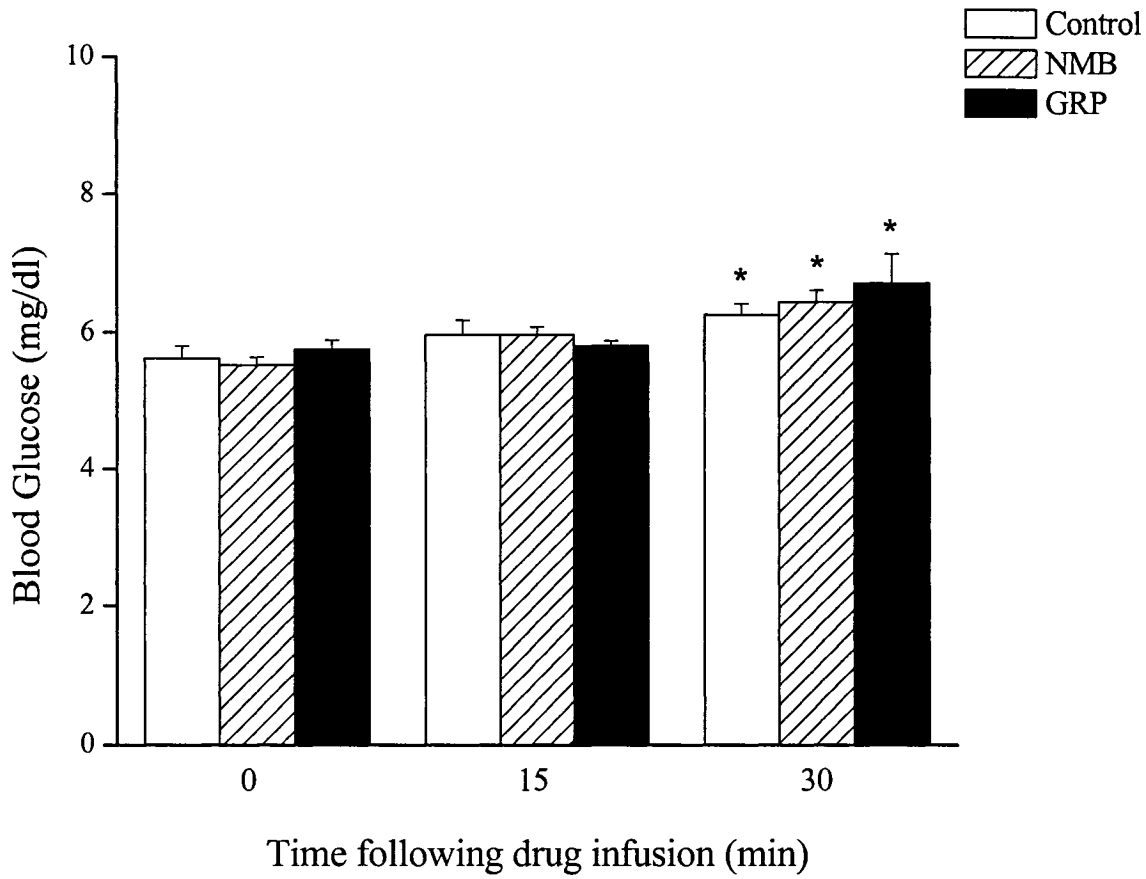


Figure 2 Blood concentrations of glucose (mean ± S.E.M.) at the 0, 15 and 30 min time intervals following drug administration in rats that received either vehicle (open columns), NMB-30 (hatched columns) or GRP (solid columns).
* Significantly different from (within-treatment condition) baseline values at $p < 0.05$.

Experiment 2: ***Effects of blockade of BB_1 and/or BB_2 receptors on levels of corticosterone and glucose following mild stressor exposure.***

Figures 3 and 4 show blood concentrations of corticosterone and glucose, respectively, at 0, 15 and 30 min following central administration of either vehicle, NMB antagonist or GRP antagonist. The overall two factor repeated measure analysis revealed that blood corticosterone concentrations varied as a function of Treatment x Time interaction ($F(4, 38) = 2.698$; $p < 0.05$). Newman-Keuls multiple comparisons of the simple effects for this interaction revealed that blood corticosterone levels were significantly elevated above baseline values in all treatment conditions by 15 min post drug injection and remained elevated for the entire 30 min testing period. Stressor exposure significantly increased corticosterone levels above baseline in all groups tested (vehicle, GRPa and NMBa). The follow up tests further revealed that prior to drug administration, levels of corticosterone were comparable for all treatment groups. At the 15 min interval, levels of corticosterone were significantly elevated above the vehicle condition following GRPa administration. Although the corticosterone levels remained elevated at the 30 min interval, there were no significant group differences.

The overall two-factor repeated measure analysis revealed that blood glucose levels did not vary as a function of Treatment x Time interaction. However, there was a significant difference in blood glucose concentrations with the passage of time. Blood glucose levels were found to be significantly elevated above baseline values by 30 min following drug injection for all treatment conditions.

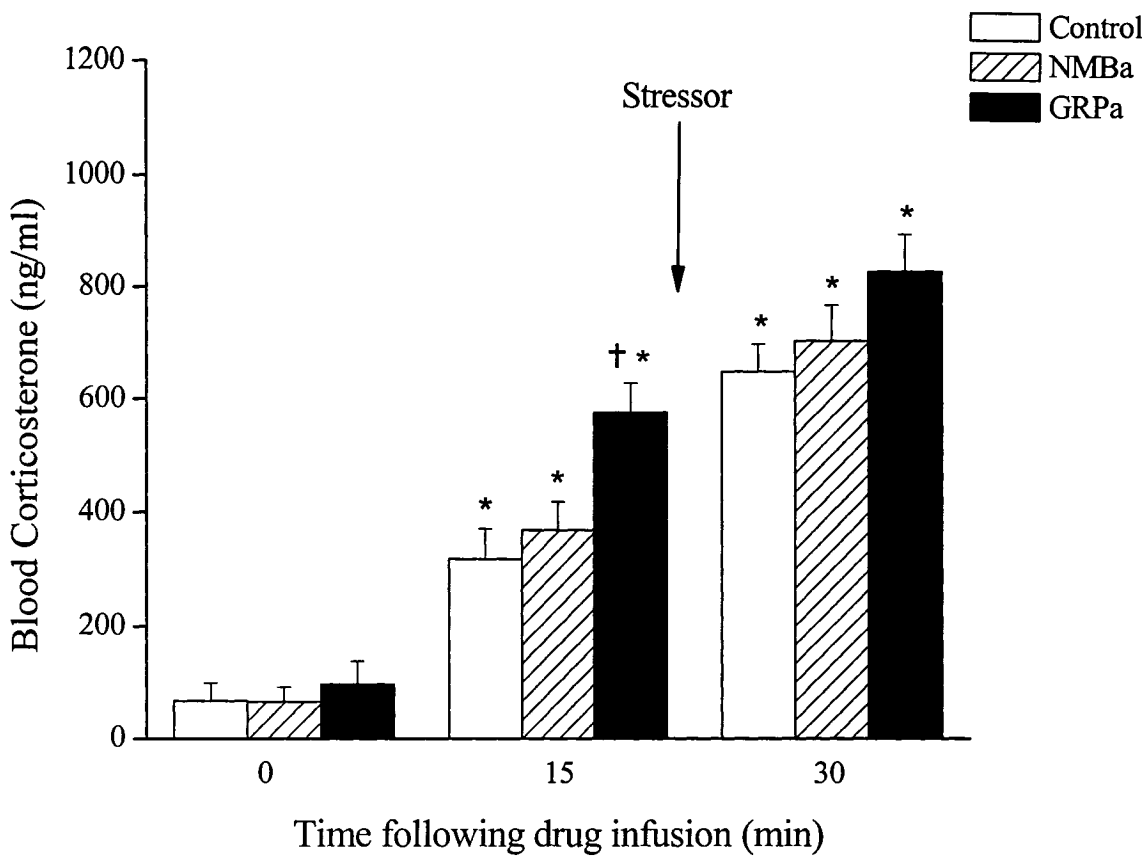


Figure 3 Blood concentrations of corticosterone (mean ± S.E.M.) at the 0, 15 and 30 min time intervals following drug administration in rats that received either vehicle (open columns), NMB receptor antagonist (NMBa; hatched columns) or GRP receptor antagonist (GRPa; solid columns).
* significantly different from (within-treatment condition) baseline values at $p < 0.05$.
† significantly different from (between-treatment condition) time-matched control (vehicle) values at $p < 0.05$.

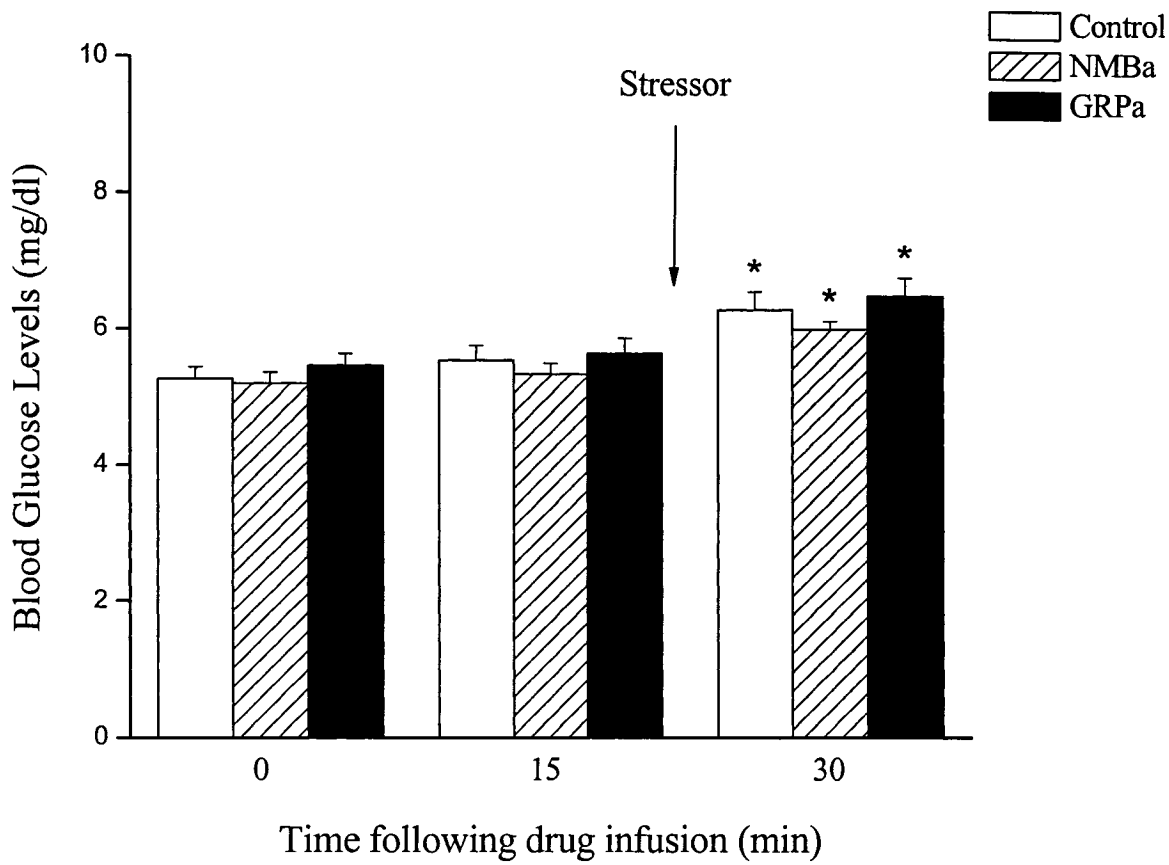


Figure 4 Blood concentrations of glucose (mean ± S.E.M.) at the 0, 15 and 30 min time intervals following drug administration in rats that received either vehicle (open columns), NMB receptor antagonist (NMBa; hatched columns) or GRP receptor antagonist (GRPa; solid columns).
* significantly different from (within-treatment condition) baseline values at $p < 0.05$.

Discussion

It has been demonstrated that BB does modify the stress response. Previous studies have shown that BB-like immunoreactive material is released in response to stressor exposure [298,302] and that BB dose-dependently increased the circulating levels of ACTH and corticosterone.[213] In addition, there is evidence indicating that centrally administered BB elicits behaviors akin to stressor exposure.[142, 194, 284, 296, 378, 440] More recent studies have implicated endogenous mammalian BLPs, NMB and GRP, in stress-related responses. Low doses of GRP activate the HPA axis as indicated by increased plasma levels of both ACTH and corticosterone.[140] However, to date, the effects of centrally administered NMB on the HPA axis activity are not known. Consequently, the present study aimed to determine whether NMB and GRP administered centrally influenced HPA axis activity. In order to determine whether endogenous peptides of this family, play a physiological role in the mediation or modulation of the stressor-induced elevation in corticosterone, we investigated the impact of peptide antagonists on stress-induced HPA activation. Results from the present study demonstrated that 1) central GRP administration attenuated the stressor-elicited rise of blood corticosterone levels and 2) that pretreatment with a GRP antagonist significantly enhanced the stressor-induced rise in corticosterone levels. In contrast, NMB administration had little or no effect on stressor-induced changes in glucose or corticosterone levels.

Results revealed that corticosterone and glucose levels were significantly elevated above baseline values following mild stressor presentation, in all treatment groups (GRP, GRPa, NMB-30, NMBa and vehicle). The rise observed in corticosterone and glucose

concentrations following vehicle injection are likely attributable to the stress associated with the experimental procedures, tail lancing and handling. It is noteworthy that the changes in corticosterone levels observed following GRP and GRP antagonist administration were significantly different from the control condition at 15 min post-injection. Corticosterone levels were significantly attenuated following GRP injections; whereas administration of GRP antagonist further exacerbated corticosterone elevations induced by the stressful experimental procedures.

The present results on the effects of GRP agonist and antagonist administration on corticosterone release appear to be somewhat inconsistent with past findings. For example, Garrido et al. reported that central administration of GRP significantly increased plasma levels of corticosterone at all doses tested.[140] Additionally, Olsen and colleagues demonstrated that central administration of GRP at low doses (7-700 pmoles) caused an increase in ACTH levels.[348] Conversely, our results show a significant attenuation in corticosterone concentrations following GRP injection relative to the control condition. A possible explanation for these differences may have to do with the fact that our test condition included the presence of a mild stressor. Furthermore, Garrido and colleagues, for instance, used much lower doses of GRP (0.001-0.1 μg) as compared to the dose utilized by us (0.84 μg or 0.31 nmoles). It has been well documented that the effects of some drugs follow an inverted U-type curve eliciting expected results at median or optimal doses, but having the opposite effect at lower or higher doses.[115, 290, 354, 408] In keeping with this contention, are the findings from Tache and colleagues [469] where high doses of BB failed to affect corticosterone levels in rats. It is also interesting to consider

the possibility that whereas GRP may stimulate corticosterone release under basal (non-stressed) conditions, it may have the opposite effect in conditions where an animal is exposed to a stressor challenge. Together, these results suggest that the effect of GRP on corticosterone is likely contingent on the dose of the peptide administered and the type of concomitant stressor the animal is exposed to.

Interestingly, Shumyatsky et al. [445] recently found that mice lacking the BB_2 receptors exhibited more freezing behavior as compared to wild-type animals, suggesting an anxiolytic effect of GRP on stress and/or fear. They found that BB_2 receptors are located on GABA interneurons of the lateral nucleus of the amygdala. Consequently, when these interneurons are activated, they promote the release of GABA from the interneurons, which in turn inhibit the primary neurons involved in fear conditioning and stress-response. Thus our findings are consistent with notion that GRP may, through the activation of GABAeric interneurons, attenuate stressor perception and/or expression. It would be of interest to carry out microinjection studies aimed at assessing the effects of GRP administration directly into such stress-relevant regions (like the lateral amygdala). It would also be of interest to verify this contention by using GRP concomitantly with drugs that modulate GABA receptors.

No significant differences were found in corticosterone or glucose concentrations following treatment with NMB at doses used. To our knowledge, this is the only study to date investigating the central effect of NMB on HPA axis activity. One previous study reported that subcutaneous injection of NMB increased corticosterone and ACTH

levels.[274, 277] Based on these past findings as well as our own, it would appear that while NMB is capable of eliciting the release of ACTH and corticosterone, it does not do so via a central mechanism(s). It is possible that the effect of centrally available NMB on stress-related responses is not mediated directly through the HPA axis, but rather through other systems. The pituitary gland, a key element in the HPA axis, is rich in BB_1 receptors supporting its involvement in HPA axis functioning.[189] Furthermore, it has been suggested that the role of NMB in the regulation of the anterior pituitary functioning might be of a paracrine nature.[172, 199, 276, 363] Therefore, it is likely that NMB acts on the HPA axis through its paracrine effect on the pituitary gland [189] bypassing the CNS due to the fact that the pituitary is located just outside the blood brain barrier. However, the effects of NMB on HPA axis activity via central mechanisms should not be ruled out completely until a wider array of doses and different analogues of NMB are tested.

In an earlier study conducted in our laboratory, central injection of NMB was found to elevate blood glucose levels.[376] However, in the current study, NMB (at the doses used) failed to further increase stressor-elevated glucose levels. Once again, it is possible that whereas NMB may affect sympathetic tone under non-stressful conditions, it may have little or no effect under more threatening or stressful conditions. It should also be noted that different NMB analogues may have differential effects. For instance, in earlier experiments conducted in our laboratory (see Plamondon and Merali (1993)) effects of the NMB-32 and NMB-10 analogs were assessed, whereas in the current study we utilized the NMB-30 analog. The NMB-10 analog was only able to induce hyperglycemia at very high doses (3.1 nm), whereas NMB-32 was able to yield its effect

at lower doses (0.31nm). Higher doses of NMB-30 might also need to be tested (0.31–3.1 nm), as only one dose was tested (0.31 nm) in the current experiment. It is possible that being a shorter sequence of amino acids, NMB-30 might be more readily available to metabolism/catabolism by peptidases in the rat. Like NMB, GRP did not alter stressor-elicited rise in blood glucose concentrations. Since only a single dose of GRP was assessed in the current study, it would be of interest to test additional doses of GRP.

Taken together, these results provide further support for the contention that BLPs play a role, at least in part, in the regulation of the HPA axis, as shown by the effects of GRP and its antagonist on stressor-elicited changes in corticosterone levels. Based on our results, it can be hypothesized that NMB may play a paracrine role on HPA activity locally at the anterior pituitary but not through its effects within the brain. There is evidence to suggest that these peptides may serve a physiological function in the mediation of the stress response through their effects on the HPA axis.

Preface to Chapter II

Although the first set of experiments provided evidence suggesting a linkage between BLPs and the mediation of the stress response, the evidence was pharmacological in nature. In order to obtain more direct evidence of the physiological involvement of these peptides in the mediation and/or modulation of the stress response, we sought to assess more *dynamic* peptidergic fluctuations, associated with stressor exposure. We also wanted to compare the release profile of these BLPs with those of other peptides implicated in the stress response. Thus, in the next set of experiments we assessed (using push-pull perfusion) the dynamics of stressor (air-puff)-induced *in vivo* release (interstitial levels) of BLPs, as well as other stress relevant peptides, namely CRH and AVP, at the anterior pituitary. Reports suggesting that stressor-exposure may induce long-term changes in peptide expression, prompted us to examine the potential role of BB-related peptides in this phenomenon. Specifically, we examined whether the subject's prior stress history (previous exposure to acute or chronic restraint) influenced the subsequent stressor-induced release of the BLPs, CRH and AVP.

Chapter II

Influence of previous stressor experiences on the in vivo release of CRH, AVP and BB-related peptides at the anterior pituitary

Abstract

Repeated exposure to stressors or with passage of time following an acute stressor exposure, has been reported to increase the expression of arginine vasopressin (AVP), especially in corticotropin releasing hormone (CRH) neurons co-expressing AVP, within the hypothalamus. This may increase the potential for subsequent stressor-elicited enhancement of hypothalamic-pituitary-adrenal (HPA) functioning as these peptides synergistically stimulate pituitary ACTH secretion. In addition, members of the bombesin (BB) family of peptides (including its mammalian analogues gastrin-releasing peptide (GRP) and neuromedin B (NMB)) may be co-localized with CRH in the median eminence and may play a role in the mediation and/or modulation of the CRH stress response. In the present investigation, chronic stressor exposure (daily restraint over 14 days) was associated with increased co-expression of CRH and AVP at the median eminence. However, such enhancement of CRH and AVP co-expression, as evidenced by immunohistochemical techniques was not apparent 14 days after acute stressor episode exposure. In contrast, *in vivo* interstitial levels of anterior pituitary CRH, AVP, GRP and NMB were elevated 14 days after a single restraint episode, and the latter three peptides were elevated following chronic stressor exposure as well. Basal corticosterone levels, in contrast, were unaffected by acute or chronic stressor exposure history. Upon exposure to an acute novel (heterotypic) stressor (air-puffs), and to a lesser extent following consumption of a highly palatable food (metabolic stressor), a more rapid and pronounced enhancement of pituitary peptide availability was apparent in naïve controls compared to rats previously subjected to acute or chronic restraint. Corticosterone was equally increased by the air-puff and food ingestion across the stressor conditions. It

seems that several peptidergic systems, including those of the typical ACTH secretagogues (CRH and AVP), as well as satiety peptides (GRP and NMB) undergo long lasting changes not only when these systems have been primed by chronic stressor exposure, but also with the passage of time following a single stressor episode.

Introduction

Corticotropin releasing hormone (CRH), is a primary regulator of the hypothalamic-pituitary-adrenal (HPA) system.[178, 306, 353] In addition to being released in response to stressor exposure, this peptide is also released in response to spontaneous food ingestion, at certain hypothalamic and amygdaloid sites.[302, 379] This suggests that either food ingestion is perceived as a metabolic stressor, or that appetitive or rewarding stimuli can also activate the HPA axis.[88, 372] Predictably, central CRH administration induces anxiogenic-like behaviors, including the suppression of food intake [176, 186, 187, 227, 333, 450, 456, 468], while CRH antagonist (alpha-helical CRH) pretreatment attenuated restraint-induced anorexia, enhanced the hyperphagia induced by neuropeptide Y, reduced the latency to begin eating and increased the duration of feeding induced by tail pinch.[176] Given that defensive behaviors are incompatible with those associated with feeding, it follows that those systems associated with stressor reactivity should be linked, directly or indirectly, with those subserving reductions of consummatory behavior.

In addition to CRH, bombesin-like peptides (BLPs), best recognized for their ability to suppress food intake (satiety peptide) [133, 142, 164, 300] act to moderate the stress response.[297] In this respect, gastrin releasing peptide (GRP) and neuromedin B (NMB), mammalian analogues of bombesin (BB), are present in all major components of the HPA axis, including the hypothalamus, pituitary and adrenal gland.[108, 147, 189, 199, 313, 324, 325] These peptides are elevated in response to stressors [211, 302] and promote HPA activation.[140, 213, 277, 304, 348, 383, 439, 501]

It has been reported that exposure to chronic stressors, or acute stressor episode followed by the passage of time, provokes increased peptide expression in CRH neurons co-expressing arginin-vasopressin (AVP) within PVN neurons terminating at the external zone of median eminence.[22, 98-101, 268, 437, 477] It is thought that increased peptide availability would translate to increased peptide release upon subsequent challenge, and would synergistically stimulate (or exacerbate) ACTH secretion.[15, 145, 400, 486] However, this supposition remains to be directly assessed. The present investigation determined whether the effects of chronic stressors and/or the passage of time following an acute stressor exposure would be accompanied by altered CRH, AVP, GRP and NMB availability at the anterior pituitary, and whether peptidergic and corticosterone changes would be further affected upon presentation of a novel stressor challenge or in response to appetitive stimuli.

Materials and Methods

Animals

Individually housed male Sprague-Dawley rats (weighing between 325 and 400 g), obtained from Charles River (St. Constant, Quebec) 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. All experimental procedures were approved by the Animal Care Committee of the University of Ottawa, and met the guidelines provided by the Canadian Council on Animal Care.

Experimental Protocol

All rats were acclimated to a Graham wafer snack (~3.5 g of Christie's Honeymaid®) over a 14 day period, so that upon its introduction rats immediately approached and consumed this palatable snack. Thereafter, rats (n = 10/group) were randomly assigned to one of three groups and exposed to either 1) no stress, 2) chronic restraint (20 min daily restraint for 14 days; restraint comprised an experimenter restraining the rat manually in a gloved hand while the rat rested on a flat counter) or 3) acute restraint (a single 20-min restraint session followed by 14 days of no stressor exposure). Following the chronic restraint group's last stressor exposure, rats from all three groups were anaesthetized (60 mg/kg pentobarbital; i.p.) and 20 gauge guide cannulae (plugged with removable stainless steel stylettes) stereotaxically implanted 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.[361] 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 to acclimate them to the test environment for a 48 hr period.

On the test day (i.e., following the 48 hr acclimation period), the stylet within the guide cannula was replaced with a push-pull probe aimed at the anterior pituitary. Three baseline dialysate samples were collected followed by the presentation of the Graham wafer snack (~3.5 g of Christie's Honeymaid®) to which animals had previously been acclimated. Commencing immediately after snack ingestion 5 further dialysate samples

were collected and used for determination of peptides and hormones. Thereafter, 3 baseline samples were again collected, after which rats received stressor exposure comprising a series of 5 air-puffs (a 5 sec puff/min) from a compressed air source directed at the rat's face. This was followed by the collection of 5 further dialysate samples. The samples (150 μl) were collected at 10 min intervals, immediately frozen on dry ice and stored at –80°C until radioimmunoassay (RIA) analysis.

Pull-pull perfusion and the effects of stressor exposure on test day

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.

A separate experiment assessed the effects of the stressor procedures (chronic restraint, acute restraint or no treatment) on CRH and AVP expression in the external zone of the median eminence. Rats were exposed to the stressor or control treatments as in the initial experiment. However, instead of rats having cannulae implanted, they were perfused intracardially with 0.9 % saline followed by a solution of 4% paraformaldehyde

and 0.4 % -saturated picric acid in 0.16 M phosphate buffer (pH 6.9). Brains were removed and placed in the fixative for 90 min at 4°C and subsequently transferred to a solution of 10% sucrose in 0.1 M phosphate buffer (pH 7.2), containing 0.01% sodium azide, until sectioning.

Histology

Following completion of the dialysis 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, BLPs (GRP and NMB) [272] Corticosterone was also measured in the perfusates using a micro-adaptation [32] of the commercial RIA kit (ICN pharmaceuticals, Canada). Briefly (for CRH, AVP, GRP 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, GRP 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}]BB (iodinated in our laboratory according to Salacinski et al.[429]), 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.[486] 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 BB antibody (α-BB kindly provided by T. Moody, NCI, Rockville, MD) recognizes the C-terminal of BB and cross reacts strongly with GRP_{1-27} (110%), BB (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, BB, or substance P.

Immunohistochemistry

Brains were cut on a cryostat (Reichert-Jung Frigocut, model 2800) and stored at -40°C until processing. Coronal 5 µm sections through median eminence were reacted for

AVP-ir and CRH-ir to determine the extent of AVP/CRH-ir co-localization within terminals of the external zone of the median eminence. Slides were rehydrated with PBS (normal saline in 10 mM sodium phosphate) for 2 min and then further washed for 20 min in the buffer. For double labeling, sections were incubated, overnight at 4°C in a humid chamber, with anti-CRH antiserum (1:400 dilution; polyclonal rabbit, Peninsula Laboratories, CA) combined with anti-AVP antiserum (1:800; polyclonal guinea pig, Peninsula Laboratories, CA). All antibodies were diluted with 10 mM PBS containing 0.3% Triton-X. After further rinsing with PBS, slides were incubated at 37°C for 45-60 min with secondary, biotinylated goat anti-guinea pig (Vector laboratories, CA; 1:50 dilution) and fluorescein conjugated donkey anti-rabbit (Amersham, UK; 1:20) immunoglobulins. Finally, sections were incubated a further 1 h at 37°C with Texas Red conjugated streptavidin (Amersham, UK; 1:100 dilution). Slides were cover-slipped with an anti-fade medium containing 0.1 % p-phenylenediamine in phosphate buffered glycerol.

Immunohistochemical quantification

As AVP and CRH immunoreactive varicosities are particularly dense within the external zone of the median eminence, thin cryostat sections (5 μm) were cut at levels corresponding to plates 44-49 in Paxinos and Watson[361], and were separated by an interval of 50 μm. Digital images were acquired using Northern Eclipse (Empix Inc.) imaging software and a Sony XC75 PAL CCD camera in conjunction with a Matrox frame grabber with integration chip. A standard number of integrations were used for each image depicted within a given figure and no individual image manipulations were

performed. After image acquisition, gray level and density measurements were made using Image Tool software (UTHSCSA Image Tool for Windows, version 2.00).

Integrated density (mean gray level x pixel area) was determined for AVP and CRH fluorescence within the median eminence of digitized images. Objects within a section were defined by manual thresholding, such that background fluorescence and dimly immunofluorescent terminals were excluded from the analysis. Quantification of AVP and CRH within the median eminence was made at rostral (-1.7 mm), medial (-1.94 mm) and caudal (-2.06 mm) levels relative to bregma. These measures were then pooled for each animal in order to obtain a relative measure of peptide immunoreactivity across the entire extent of the brain region.

Statistical Analysis

Basal differences of GRP, NMB, CRH, AVP (pmol/10 μl) and corticosterone concentrations, as a function of the stressor treatments, were determined through a mixed measures ANOVA wherein stressor treatment was considered the between-group variable, and samples was the within-group variable. Specific differences between groups were assessed by Newman-Keuls multiple comparisons of significant main effects or means comprising the simple effects of significant interactions ($p = .05$).

To determine the effects of the ingestion of a submaximal quantity of Graham Wafer and that of acute stressor challenge (air-puffs) on peptide and corticosterone levels, two types of analyses were used. The choice of conducting two separate analyses was

predicated on the fact that appreciable within-group variability frequently exists with respect to basal peptide levels, and thus it is difficult to determine the meaningfulness of absolute changes of peptide levels in response to a challenge (food ingestion or air-puff) among rats with high versus low basal peptide levels. In the present investigation this difficulty was compounded by the fact that the animal's stressor history influenced basal peptide levels. Specifically, in those animals where peptide levels were ordinarily low, "relative" changes induced by the challenge may be obfuscated by the disproportionate contribution attributable to animals with relatively high basal levels of a given peptide. By converting the peptide levels to a percent of baseline values, the relative contribution of every animal is equal to one another. Of course, the possibility remains that peptide changes among rats with high basal levels may actually be undervalued. Nevertheless, because neither approach adequately represents the potential impact of a treatment (such as food ingestion or an acute air-puff stressor) analyses were also undertaken assessing the proportionate change of peptide levels following snack ingestion or administration of the air puff stressor.

The first set of analyses was conducted using the absolute peptide levels, while in the second the proportionate change of each peptide was analyzed. In the latter analysis the peptide levels (3 samples prior to presentation of the snack) were averaged individually over the baseline samples and defined as 100%. Values were then expressed as a percentage of that baseline. In analyzing the effects of the air-puff stressor, the 3 samples taken prior to the air puffs were averaged and the resulting value defined as 100%. The post-stressor levels were then expressed as a function of the pre-stress

baseline. The proportionate peptidergic and hormonal changes associated with the various treatments were analyzed independently using a mixed measures ANOVA (Stressor Treatment as the between group variable x Sample as the within group variable). In all cases, the Huynh-Feldt correction was used. Post hoc comparisons were conducted using Newman-Keuls multiple comparisons ($\alpha = .05$) of the main effects or simple effects of significant interactions.

Results

Immunohistochemical variations of CRH and AVP within the median eminence

Table 1 shows the CRH and AVP density, as well as the co-localization of these peptides at the external zone of the median eminence. Analyses of the CRH and AVP densities indicated that while CRH immunoreactivity was not affected by the stressor treatments ($F < 1$), the AVP immunoreactivity was moderately increased, particularly in response to the chronic stressor, although this outcome was not statistically significant, $F(2, 12)$ 2.91, $p < 0.09$. However, it was observed that the percentage of CRH terminals that co-localized AVP varied significantly with the treatment, $F(2, 12) = 5.64$, $p < 0.01$. The multiple comparisons confirmed that relative to control animals (53.6 ± 4.10 %), a greater number of CRH terminals were co-reactive for AVP following the chronic stressor regimen (68.2 + 2.10 %), whereas the acute treatment did not provoke a significant effect in this respect (59.2 + 2.7 %). Figure 1 depicts the co-expression of AVP and CRH within CRH terminals located within the external zone of the median eminence.

Table 1 Effects of acute and chronic stressor history on CRH and AVP density and the co-localization of these peptides at the external zone of the median eminence

Stressor history	CRH density	AVP density	% colocalization
Control	28132.8 ± 2418.3	22309.2 ± 3683.9	53.6 ± 4.1
Acute	31890.4 ± 3121.4	27834.2 ± 1339.1	59.2 ± 2.7
Chronic	29938.6 ± 3364.4	31323.2 ± 2433.7	68.2 ± 2.1*

Note. Data for CRH and AVP density represents arbitrary numbers (mean gray level x number of pixels in the image ± SEM).

* significantly different from non-stressed control condition at $p < 0.05$.

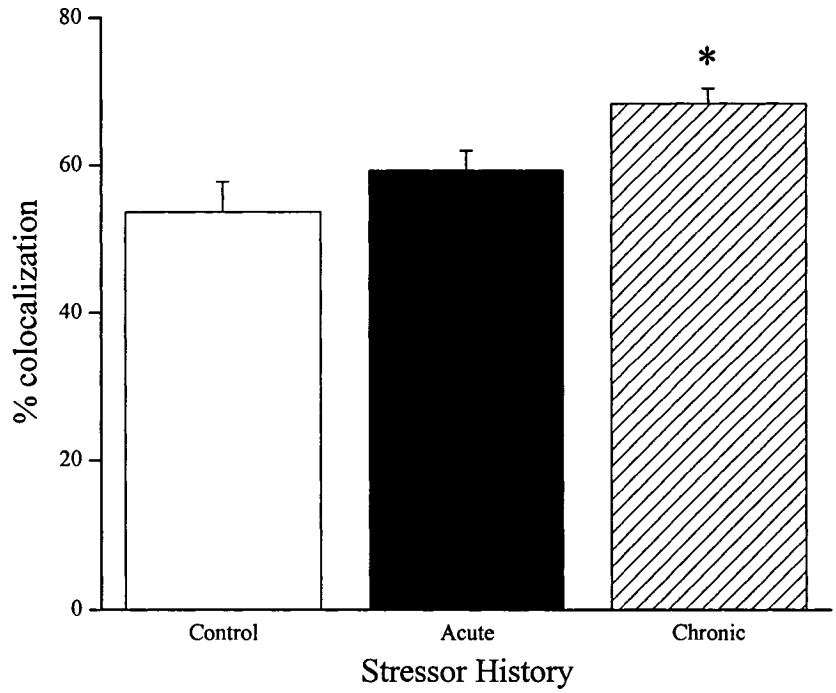


Figure 1 Co-expression of AVP and CRH within CRH terminals located within the external zone of the median eminence in previously non-stressed control rats (open columns) , those previously exposed to 1 session of restraint (acute; solid columns) followed by a 14 day stressor-free period, or rats previously exposed to 14 sessions of restraint (chronic; hatched columns). * $p < 0.05$ relative to control rats.

Effects of acute and chronic stressor history on basal interstitial levels of CRH, AVP, GRP, NMB, and corticosterone at the anterior pituitary

Interstitial levels of GRP, NMB, CRH, AVP (expressed as pmol/10 μl) and corticosterone (expressed as pmol/15 μl) as a function of the prior acute and chronic restraint treatments are depicted in Figures 2-6, respectively. These figures also show the change of peptide and hormone levels following consumption of the palatable snack, as well as the changes induced by the air-puff stressor. The upper panel of the figures shows the absolute concentrations of each neurochemical, while the lower panels depict the percentage change of the peptides as a function of the palatable snack consumption and exposure to the air puff.

It is clear that the initial treatment rats received (chronic stressor exposure, acute stressor exposure followed by a 2 week stressor-free period and no stressor) had marked effects on basal peptide release, i.e., prior to the presentation of the snack (CRH: $F(2,23) = 8.41$, $p<0.001$; AVP: $F(2,27) = 10.87$, $p<0.001$; BB/GRP: $F(2,24) = 4.106$; $p<0.05$; and NMB: $F(2,26) = 31.91$, $p<0.0001$). The follow-up comparisons indicated that while CRH levels were elevated in acutely stressed rats, such an effect was not evident among rats that had been exposed to the chronic stressor regimen. In the case of AVP both the chronic restraint and the acute stressor increased the interstitial levels of this peptide, but in this instance the AVP level in chronically restrained rats was significantly higher than in acutely restrained animals. In the case of GRP it was again observed that acute restraint increased interstitial levels of these peptides, whereas the effect of the chronic stressor was relatively modest, such that the elevated GRP was not significantly different

from that of non-stressed rats. With respect to NMB, the acute and chronic stressors were equipotent in increasing interstitial levels of this peptide in comparison to that of non-stressed rats. Finally, in contrast to these peptides, corticosterone measured at the pituitary did not vary as a function of the stressor experience that rats had received ($F < 1$).

Effects of Graham Wafer ingestion and the air puff stressor on interstitial levels of GRP, NMB, CRH, AVP and corticosterone at the anterior pituitary

Analyses of the *absolute* peptide levels revealed that the ingestion of Graham Wafers and the air-puff stressor provoked only modest effects on interstitial peptide levels, irrespective of stressor history (see Figures 2 –6). However, the effects of the treatment were more dramatic when considered from the perspective of the change from baseline peptide values. Analysis of the percent scores indicated that the exposure to a palatable snack significantly influenced peptide concentrations. Specifically, interstitial CRH levels varied as a function of the Previous stressor condition x Sample (time) interaction, $F(10,105) = 2.56$, $p < 0.01$. The follow-up tests indicated that among rats that had not previously been exposed to a stressor, the snack provoked a rise of CRH that reached significance by the second sampling period and remained elevated thereafter. While CRH did not increase in response to the snack in acutely stressed rats, among animals previously exposed to the chronic stressor regimen, CRH levels were increased, reaching statistical significance at the 3rd sampling period.

During the ensuing three samples, which served as the basal levels to assess the effects of the air-puff stressor, CRH concentrations remained stable in control rats. However, among rats in the two stressor conditions the CRH concentration continued to rise. In the chronic stressor condition CRH content increased from <2 to approximately 4 pmol/ 10 µl while in the acute stressor condition the CRH levels increased from 8 to 12 pmol/10 µl. As a result the interstitial CRH levels differed from that of previously non-stressed rats, $F(2, 25) = 70.27$; $p < 0.0001$. The follow-up tests confirmed that CRH in the acute stressor condition significantly exceeded the levels among chronically stressed rats, which in turn, exceeded that in the non-stressed animals.

Analyses of the absolute CRH concentrations before versus after the air-puff stressor indicated that this manipulation was without effect. However, analysis of the percentage change of CRH indicated that relative to the second baseline period, interstitial levels of this peptide varied as a function of the interaction between the Previous stressor condition x Samples following the air puffs, $F(10,120) = 2.59$; $p < 0.01$. Follow-up comparisons of the simple effects revealed that exposure to the air-puff stressor elicited an increase of CRH that was more pronounced and occurred more readily in previously unstressed rats than in the previously stressed groups. Although the absolute increase of CRH was modest in the previously non-stressed rats, relative to their own baseline the rise of CRH was actually quite pronounced (>100%). The rise was evident as soon as at the 1st sampling period following the air-puff, peaked at the second sample, and declined somewhat thereafter, although levels still exceeded baseline. In the chronically stressed rats the increase was significantly less pronounced, and was only

apparent at the 3rd and 4th samples following the air-puff stressor. In those rats that had previously received the acute stressor treatment, later exposure to the air-puff stressor did not significantly increase CRH relative to baseline.

Unlike the effects evident with respect to CRH, proportionate changes of interstitial AVP levels were unaffected by the snack consumption. During the three samples taken prior to air-puff exposure, levels of AVP differed as a function of the stressor history, $F(2, 29) = 9.05$; $p < 0.001$. Levels of the peptide were greater in the chronically stressed rats than in those that had received a single restraint session, which in turn exceeded that of non-stressed animals. Following exposure to the air-puff the proportionate change of AVP, depicted in the lower portion of Figure 3, varied as a function of the Treatment condition, $F(2,27) = 4.67$, $p < 0.05$ and Samples following the air-puff stressor, $F(5,135) = 4.72$, $p < 0.01$. Although the interaction between these variables was not significant, it is clear from Figure 3b and confirmed by the follow-up comparisons that in the previously non-stressed rats subsequent exposure to the air puff stressor increased interstitial AVP levels at the 2nd sample relative to their own baseline and relative to that of rats that had previously received either the acute or chronic stressor treatments. The increase relative to baseline declined thereafter, but still exceeded baseline at the 3rd and 4th samples. In the previously stressed animals the increase over samples following the air puff was less pronounced, and with the exception of the second to last sample in the chronic stressor condition, AVP did not exceed baseline.

Interstitial GRP (in relation to baseline levels) indicated that the peptide accumulation varied as a function of the Previous stressor exposure x Samples taken

following ingestion of the snack, $F(5,105)=4.73$, $p < 0.01$. While the proportionate levels of GRP among control rats increased during the 4th and 5th post-ingestion sampling periods, among rats that had previously been exposed to restraint, snack ingestion was not associated with changes of this peptide (see Figure 4b).

During the period preceding the air puff stressor, the absolute levels of GRP varied as a function of the previous stressor treatment rats received, $F(2,24) = 4.11$, $p < 0.05$. Rats in the acute and chronic stressor groups exhibit increased levels of GRP relative to control condition, while not differing significantly from one another. Exposure to the air puff stressor did not affect the absolute levels of GRP. However, analysis of the proportionate changes of this peptide revealed that the change of GRP levels varied as a function of the Previous stressor treatment rats had received, $F(2,23) = 4.21$, $p < 0.05$ and over Samples following the air-puff stressor, $F(5,115) = 9.21$, $p < 0.01$. The follow-up comparisons indicated that relative to baseline the air-puff stressor increased GRP levels in each of the groups. Moreover, this effect was greater in the rats that had previously not been exposed to the stressor than in rats that had received the acute stressor, particularly during the first two sampling periods. In the two groups that had previously been stressed, the increased GRP relative to baseline was significant only at the 4th and 5th samples.

Snack consumption appeared to have more pronounced effects on NMB than on GRP. The proportionate change of NMB (relative to baseline) varied as a function of the Previous Stressor condition, $F(2, 20) = 3.50$, $p < 0.05$ and the Samples following snack

consumption, $F(5, 100) = 5.58$, $p < 0.01$. The follow-up comparisons indicated that, collapsed across treatment groups, NMB increased by the 3rd sampling period and remained elevated thereafter. Although the interaction between previous stressor treatment and Samples did not reach statistical significance, post-hoc tests indicated that the change of NMB was greater among previously non-stressed rats than those that had received either acute or chronic restraint. In fact, as depicted in Figure 5, in the control rats the NMB increase was evident as early as the 1st post ingestion sample, while moderate increases of NMB were only evident after the 3rd sample in the chronically stressed rats, while in the acutely stressed rats the increase of NMB was highly variable and non-significant.

Analyses of the absolute interstitial neuropeptide levels indicated that prior to the air-puff being administered, the absolute levels of NMB varied as a function of the previous stressor condition, $F(2,28) = 10.15$; $p < 0.001$, with the acute and chronic restraint conditions provoking an elevation of NMB levels relative to non-stressed animals. The effects of the air-puff stressor on absolute levels of NMB were modest and non-significant. In contrast, analyses of the percent change scores indicated that the air-puff stressor, in fact, influenced the peptide concentrations relative to baseline, $F(5,125) = 4.72$, $p < 0.01$, being elevated over the 3rd – 5th post air-puff samples relative to baseline. As in the case of the effects of food ingestion, the Treatment x Samples interaction was shy of significance ($p = .09$); however, it is clear from Figure 5 and confirmed by post-hoc tests, that a significant rise of NMB was evident in control rats as

soon as the first sample following the air puff, whereas among the previously stressed groups the increase was not statistically significant until the 4th sample.

Finally, analysis of the corticosterone changes indicated that corticosterone levels increased over time following food ingestion, $F(5, 105) = 5.01$, $p < 0.01$. Follow-up comparisons indicated that this increase was progressive over the 5 sampling periods, reaching statistical significance by the 3rd sample, and remaining stable over the ensuing samples. The extent of the corticosterone changes associated with food ingestion were, however, relatively modest, and did not differ as a function of the animal's stressor history. It will be noted that in chronically stressed animals, the increase of corticosterone following food ingestion, while variable, approached 50% whereas in the remaining two groups this increase was somewhat less pronounced, and in the main did not exceed 25%.

The effects of the air-puff on corticosterone concentrations at the pituitary were more pronounced than that elicited by food ingestion. Regardless of whether absolute or proportionate changes were assessed, the outcomes were the same. The ANOVA of the percentage change scores yielded a significant effect of Samples relative to the air-puff stressor, $F(5, 120) = 2.59$, $p < 0.01$. The air-puff stressor significantly increased corticosterone levels at the pituitary in each of the groups, irrespective of stressor history. Within each group this effect peaked at the 3rd sample and declined thereafter.

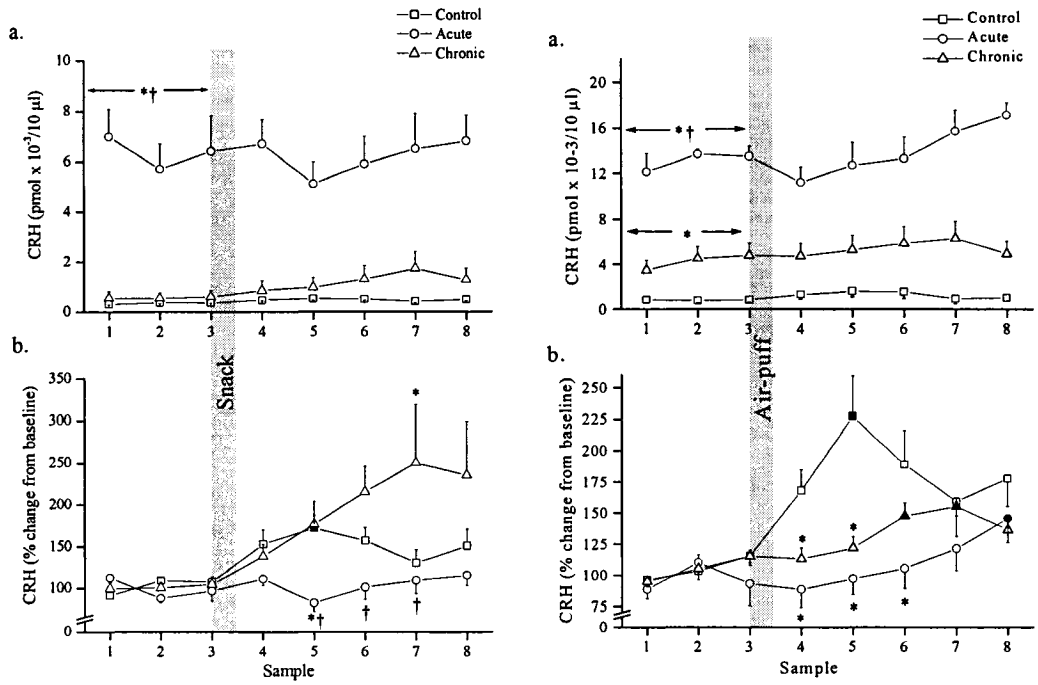


Figure 2a Interstitial levels of CRH (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following exposure to a palatable snack or air-puff stressor (5 min) in previously non-stressed control rats, those exposed to 14 sessions of restraint (chronic), or rats previously exposed to 1 session of restraint (acute) followed by a 14 day stressor-free period. * $p < 0.05$ relative to baseline values of control rats. † $p < 0.05$ relative to baseline values of chronically stressed rats.

Figure 2b shows the same data expressed as a percentage of the baseline scores. 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 $p < 0.05$.

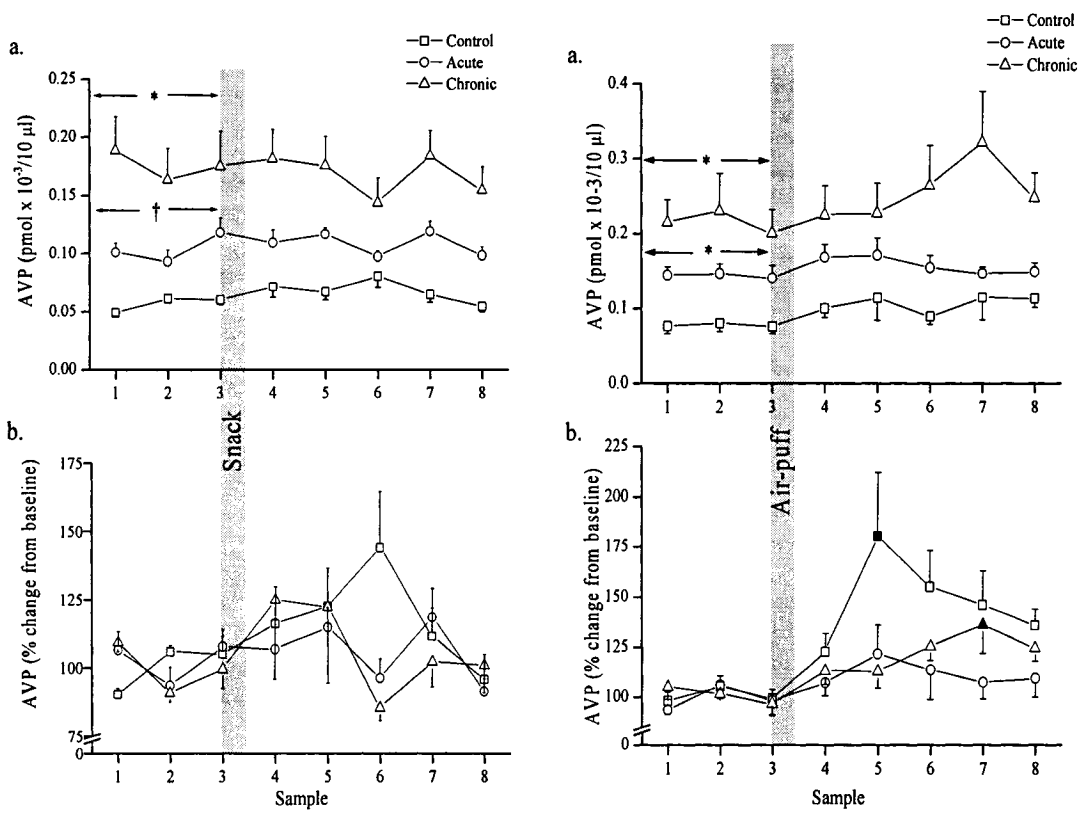


Figure 3a Interstitial levels of AVP (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following exposure to a palatable snack or airpuff stressor (5 min) in previously non-stressed control rats, those exposed to 14 sessions of restraint (chronic), or rats previously exposed to 1 session of restraint (acute) followed by a 14 day stressor-free period. * $p < 0.05$ relative to baseline values of control rats. † $p < 0.05$ relative to baseline values of chronically stressed rats.

Figure 3b shows the same data expressed as a percentage of the baseline scores. Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions.

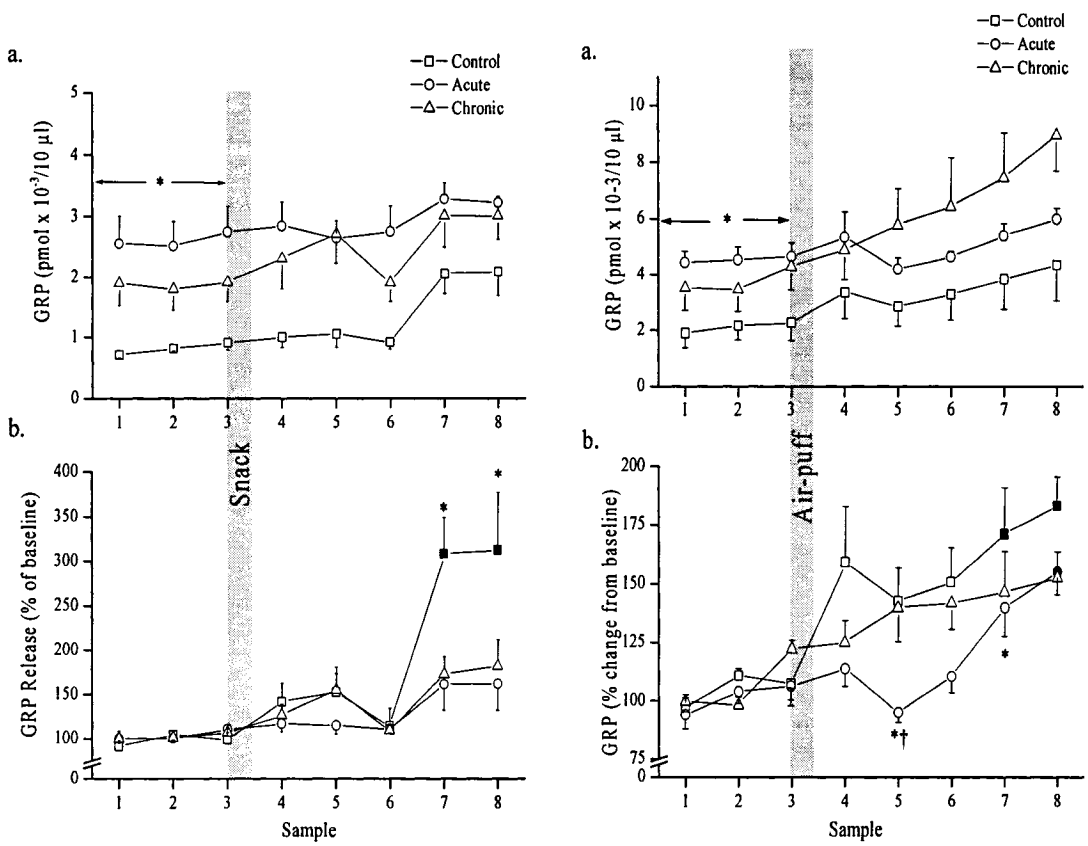


Figure 4a Interstitial levels of GRP (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following exposure to a palatable snack or air-puff stressor (5 min) in previously non-stressed control rats, those exposed to 14 sessions of restraint (chronic), or rats previously exposed to 1 session of restraint (acute) followed by a 14 day stressor-free period. * $p < 0.05$ relative to baseline values of control rats.

Figure 4b shows the same data expressed as a percentage of the baseline scores. 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 $p < 0.05$.

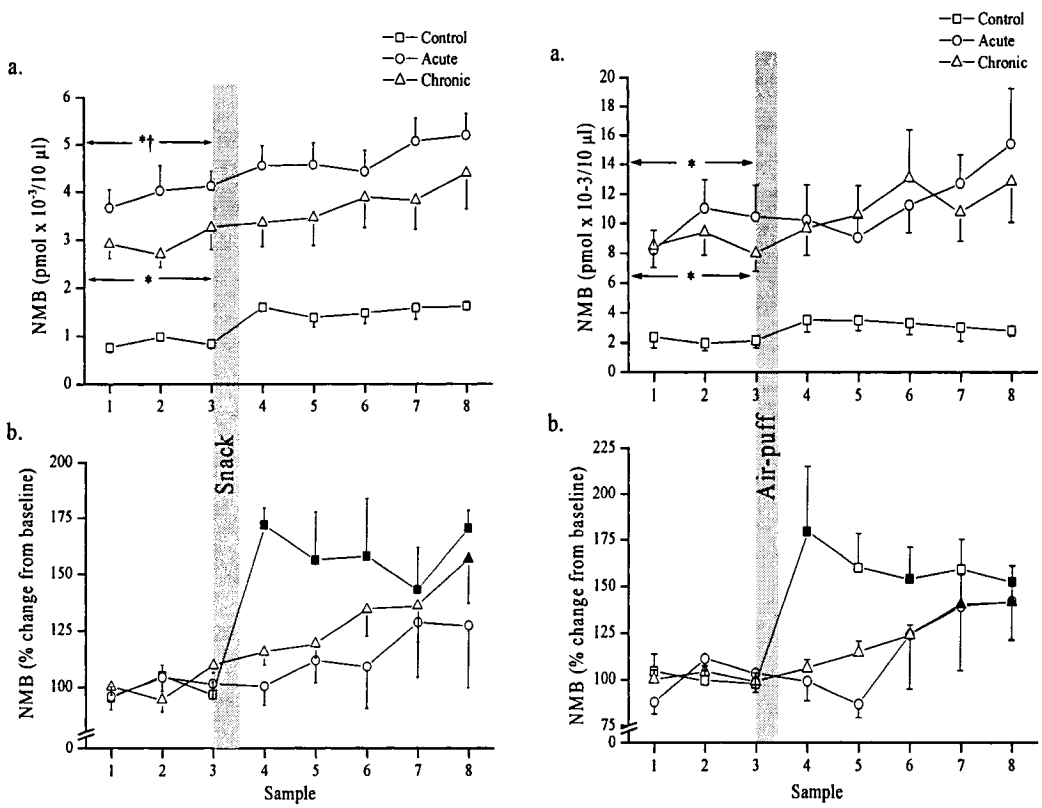


Figure 5a Interstitial levels of NMB (expressed as pmol/10 µl) at the anterior pituitary under basal conditions and following exposure to a palatable snack or air-puff stressor (5 min) in previously non-stressed control rats, those exposed to 14 sessions of restraint (chronic), or rats previously exposed to 1 session of restraint (acute) followed by a 14 day stressor-free period. * $p < 0.05$ relative to baseline values of control rats. † $p < 0.05$ relative to baseline values of chronically stressed rats

Figure 5b shows the same data expressed as a percentage of the baseline scores. Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions.

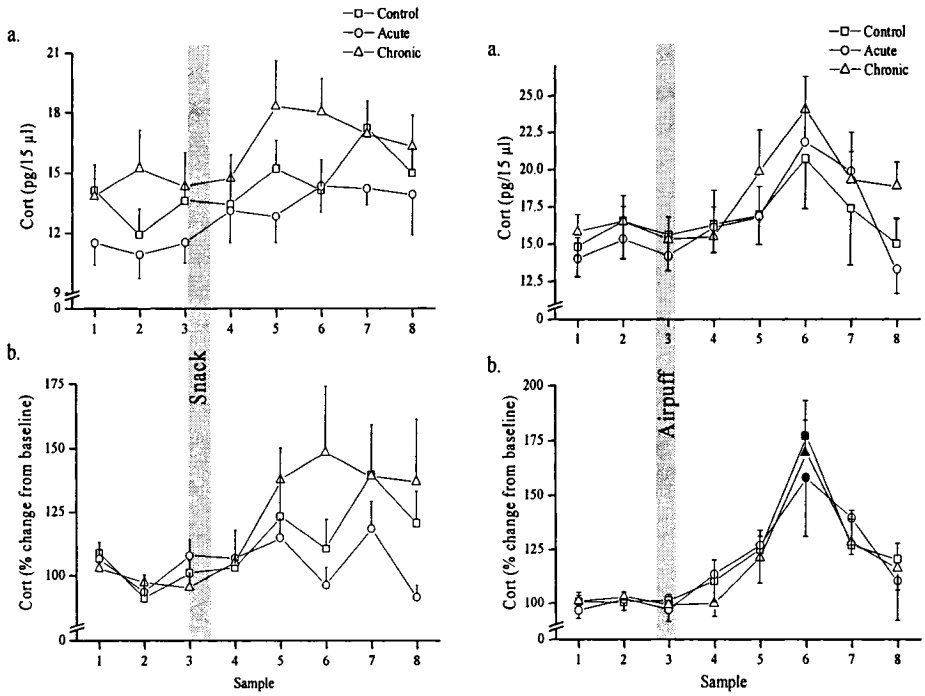


Figure 6a Interstitial levels of corticosterone (Cort) (expressed as pg/15 µl) at the anterior pituitary under basal conditions and following an air-puff stressor (5 min) in previously non-stressed control rats, those exposed to 14 sessions of restraint (chronic), or rats previously exposed to 1 session of restraint (acute) followed by a 14 day stressor-free period.

Figure 6b shows the same data expressed as a percentage of the baseline scores. Solid symbols represent points that are significantly different from the last baseline sample of respective treatment conditions.

Discussion

Indirect evidence derived from analyses of peptide mRNA expression, *c-fos* activation and post-mortem analyses indicated that stressors increase the turnover of CRH and/or AVP.[3, 22, 69, 99-101, 191, 268, 269, 273] Furthermore, enduring phenotypic changes occurred in the co-expression of these secretagogue at the external zone of the median eminence following chronic stressor exposure and with the passage of time following an acute stressor. Thus, these treatments may have engendered the potential for greater pituitary activation in response to subsequent challenges.[100, 99, 437, 477]

Consistent with these reports, it was shown in the present investigation that AVP increased in CRH terminals within the external zone of the median eminence. However, this outcome was evident in chronically stressed rats, but not with the passage of time following an acute stressor as previously reported.[437, 438, 489] Whether this represented differences in the stressors used across these studies or with respect to the specific timing following the acute insult is uncertain. Nevertheless, it is clear that given appropriate conditions, stressors may engender long-lasting phenotypic changes of CRH and AVP expression at the external zone of the median eminence.

In addition to the altered CRH/AVP co-expression, basal levels of interstitial pituitary CRH were significantly elevated in rats previously exposed to a stressor. Specifically, when assessed two weeks after an acute restraint stressor, ir-CRH levels were markedly increased relative to non-stressed rats. A similar increase, however, was not apparent in response to a chronic stressor. While acute exposure to restraint was

previously found to dramatically increases CRH mRNA in parvocellular neurons of the PVN [269, 375], such changes are typically observed within hours after stressor termination. The present data extend such previous observations, indicating that a single stressor episode can have long lasting effects on CRH availability at the pituitary. It is equally clear, however, that the *in vivo* basal CRH alterations did not parallel the immunohistochemical data, as neither acute nor chronic restraint produced changes in the density of CRH neurons at the external zone of the median eminence, and only the chronic stressor regimen was associated with increased AVP expression within CRH terminals.

Although it is difficult to reconcile the effects of the stressor treatments on CRH expression within the median eminence, and the increased availability of CRH at the pituitary, it ought to be considered that alterations (or lack thereof) of peptidergic densities measured by immunofluorescence do not necessarily reflect functional peptidergic release. Indeed, the "snap shot" assessment of post-mortem tissue peptide levels may have failed to detect changes in turnover, if for instance, peptide utilization were offset by compensatory changes in peptide synthesis or degradation. Interestingly, Aubry et al. [20] demonstrated that while an acute episode of immobilization provoked a persistent increase of AVP and CRH mRNA in the parvocellular division of the PVN, this was not accompanied by concomitant changes of AVP immunostaining in CRH terminals at the external zone of the median eminence. In this particular study, as in the present investigation, repeated stressor exposure was required to provoke an increase in the number of CRH terminals containing AVP.

In addition to CRH and AVP, it appears that the BB family of peptides may be involved in the regulation of HPA functioning.[139, 140, 277, 297, 348] Indeed, the stressor treatments in the present investigation increased the accumulation of these peptides at the anterior pituitary. This was the case in both chronically stressed rats and in those that were assessed two weeks after an acute stressor.

Although our results demonstrate enhanced interstitial levels of CRH, AVP, GRP and NMB at the anterior pituitary in rats with previous stressor experience, the neural mechanism(s) underlying such actions, as well as their significance, remains speculative, 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 either 1) a general decline in corticosterone levels, or 2) reduced glucocorticoid sensitivity of CRH neurons.[273, 375] It was reported that the characteristic increase of AVP mRNA observed after repeated restraint was only seen in adrenalectomized rats receiving low dose corticosterone treatments, but prevented by the higher dose.[375] Moreover, it has been demonstrated that exposure of rats to repeated immobilization elicits a corticosterone surge, which serves to down-regulate glucocorticoid receptors in the hippocampus and PVN.[273, 281] This may, in turn, temporarily impair glucocorticoid negative feedback, hence facilitating 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).[170, 219] Yet, repeated immobilization

increased AVP-binding affinity and AVP V1b receptor mRNA expression at the pituitary, suggesting that a previous stressor experience may result in a more dominant role for the AVP receptor in determining corticotroph responsiveness.[394]

In response to an acute stressor, in this instance, a series of air-puffs, a proportionate increase of peptide levels was provoked relative to baseline. However, although basal peptide levels were generally lowest in the naïve control group (relative to the acute or chronic stressor conditions), they were proportionately most responsive to the acute air puff challenge. Typically, air puff exposure rapidly increased peptide levels of control rats, whereas the increase in previously stressed animals was blunted and delayed. The functional significance of these findings, however, is uncertain as the marked corticosterone increase provoked by the air-puff was comparable irrespective of the animal's stressor history. Yet, these systems have been implicated in both anxiety and depressive illness, and hence the protracted elevations observed following acute and chronic stressors, may have psychopathological implications.

The mismatch between CRH and AVP with corticosterone levels have also been noted under other stressful conditions, suggesting that increased CRH or AVP availability does not uniformly produce a corresponding elevation in the end hormones.[5, 149, 174, 268, 489] It was reported that repeated stressor exposure, including restraint, had little effect on basal corticosterone or ACTH levels, despite significant changes of CRH and/or AVP mRNA expression.[106, 269, 282, 375] In this respect, 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 these secretagogues.[4, 10, 169, 170, 203]

In addition to being affected by stressors, ingestion of a palatable snack was associated with altered peptidergic availability at the anterior pituitary, comprising BLPs (GRP and NMB) and CRH, but not AVP or corticosterone. These findings are consistent with our previous observations that food intake was associated with the release of CRH and BLPs at the CeA [302], and increased tissue levels of BLPs in the median eminence/arcuate region.[379] While the interpretation of altered tissue levels is tenuous, it is possible that the increased BLPs at the median eminence/arcuate represents increased release and/or synthesis at this region, which in turn could translate into an increased availability of these peptides at the anterior pituitary.

Although interstitial levels of both NMB and GRP were significantly increased following snack consumption, their kinetics was distinct from one another. The increased NMB availability was immediate in onset and large in magnitude, while that of GRP was relatively delayed and less pronounced. This differing temporal profile makes sense in light of what is known regarding the distribution of these peptides. While the mRNA expression of NMB and BB_1 receptors are concentrated in the anterior pituitary [189], GRP mRNA expression is more prominent at hypothalamic structures, such as the PVN.[497] Thus, the immediate increase of interstitial NMB at the pituitary could represent local release, while the more delayed increase of GRP may be attributable to upstream release, most likely from hypothalamic structures. The functional significance of the increased

availability of these peptides at the pituitary remains uncertain. Exogenous administration of BLPs (including GRP and NMB) suppresses food intake [141-144, 231, 232, 234, 287, 300, 465], and this effect is antagonized by the blockade of BB_1 (using a selective BB_1 receptor antagonist, BIM-23127) and/or BB_2 receptors (using high affinity BB_2 receptor antagonists)[241, 242] or central administration of BB antiserum.[303]

As indicated earlier, BLPs have been shown to influence the effects of stressors on HPA functioning.[211, 212, 302] Likewise, while CRH is generally associated with the mediation of the stress response, it seems that this peptide is also involved in the regulation of ingestive behavior.[79, 245, 305, 399] Thus, it is not altogether surprising that CRH, like BLPs, are influenced by appetitive events. The functional significance of the release of CRH in response to appetitive events is uncertain. We have suggested previously that in several respects feeding would be appropriately considered as a stressor, given that in the wild feeding is, in fact, a potentially perilous exercise. Alternatively, some expenditure of internally stored energy supply may be necessary for preparing the organism to consume, metabolize, transport and store newly acquired raw materials. Finally, these peptidergic responses may be activated to draw attention to external events or cues of biological significance, such as those associated with food availability or a threat to survival.[302]

Consistent with reports that food intake increases plasma glucocorticoid levels [222, 302, 416], in the current investigation the ingestion of a palatable snack likewise induced an increase of interstitial corticosterone levels at the pituitary. Although the animals'

stressor history influenced the basal levels of CRH and AVP, as well as GRP and NMB which also may affect corticosterone levels, the corticosterone response associated with food ingestion was comparable irrespective of stressor history.

In conclusion, there is mounting evidence that hypersecretion of hypothalamic CRH and/or AVP may be involved in the pathogenesis of certain stressor-related psychiatric disorders, such as major depression and posttraumatic stress disorder.[174, 392] It also appears that the influence of stressors on the provocation of such pathological states is influenced by previous stressors experienced by the individual. In the present study, we demonstrated that rats chronically exposed to restraint, or with time following an acute stressor, basal interstitial levels of GRP, NMB, CRH and AVP were increased at the anterior pituitary. Although changes in the CRH and AVP systems following previous stressor experiences have been postulated using indirect markers, our results are the first to *directly* show that the availability of these secretagogues is altered by the organism's stressor history. Moreover, in response to a subsequent acute challenge, albeit positive (ingestion of a palatable snack) or negative (air-puff exposure), rats with previous stressor experience (and high basal peptide levels) showed a somewhat delayed and less pronounced increase of 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, increasingly more evidence has supported the view that the BB family of peptides function in a similar manner to that of other stressor relevant peptides in the mediation/and or modulation of the response to stressors and potentially might be in involved in the pathogenesis of stress-related disorders.

Preface to Chapter III

Since NMB and GRP appear to 1) modulate the HPA axis, and consequently the stress response, 2) be released within certain brain sites in response to stressor exposure, and 3) be influenced by the stressor history of the animals, we wanted to explore the behavioral involvement of these peptides in specific stress-related responses, namely anxiety- and fear-related responses. Thus, in chapter III, we assessed whether NMB and/or GRP play a role in anxiety and fear-related responses using several animal models of anxiety and fear, including performance in the open field, elevated plus maze, and fear-potentiated startle paradigm. We also evaluated the impact of BB_1 and BB_2 receptor blockade (using available antagonists) on these same anxiety- and/or fear-paradigms.

Chapter III

Role of gastrin-releasing peptide and neuromedin B in anxiety and fear-related behavior(s)

Abstract

Several lines of evidence have implicated bombesin (BB)-like peptides in the mediation and/or modulation of the stress response. However, the contribution of this family of peptides specifically to fear and anxiety-related responses has not been well characterized. The objective of the present investigation was to assess the effects of gastrin releasing peptide (GRP) and neuromedin B (NMB), two mammalian counterparts of BB, on fear and anxiety-related behaviors in animal models. To this end, the effects of i.c.v administration of GRP (0.30 nmoles), GRP (BB_2) receptor antagonist, [Leu^{13}-(CH_2NH)Leu^{14}]-BN (1.26 nmoles), NMB-30 (0.29 nmoles), NMB (BB_1) receptor antagonist, BIM 23127 (1.70 nmoles), and a mixed BB_1/BB_2 receptor antagonist, PD 176252 (0.621 nmoles), were assessed in several tests of anxiety, namely the open field, elevated plus maze (EPM) and in a fear potentiated startle paradigm (a model thought to reflect conditioned fear). The BB_1 receptor antagonist and the mixed BB_1/BB_2 receptor antagonist elicited anxiolytic effects in the open field and/or EPM, whereas the BB_2 receptor antagonist was without effect. In the fear potentiated startle paradigm, pretreatment with either the BB_1 receptor antagonist or the BB_2 receptor agonist attenuated the fear potentiated startle response, without affecting basal startle amplitude. These data suggest that both mammalian analogues of BB, NMB and GRP, are involved in stress responses. However, whereas NMB appeared to play a role in both anxiety and fear responses, GRP seemed to be selectively involved in fear responses.

Introduction

Bombesin (BB) is a fourteen amino acid peptide that was originally isolated from the skin of the frog, *bombina bombina*. Subsequently, two mammalian counterparts of BB have been identified, namely neuromedin B (NMB) and gastrin-releasing peptide (GRP). These peptides may serve as endogenous ligands for the four different receptor subtypes, designated as BB_1, BB_2, BB_3, and BB_4.[24, 150, 336, 387, 458, 498] It appears that NMB has greater affinity for the BB_1 receptor subtype, whereas GRP has a greater affinity for the BB_2 receptor subtype.[24, 237] In contrast, however, NMB and GRP appear to have only a weak affinity for the BB_3 and BB_4 receptor subtypes, suggesting that there may be other yet to be discovered peptides, that are endogenous ligands for these receptors.[343] Although BB_1 and BB_2 receptors are located throughout the mammalian central nervous system, their distribution patterns are distinct.[236, 325, 497] For example, there is a preponderance of BB_2 receptors and its endogenous ligand (GRP) in forebrain structures, various hypothalamic and amygdaloid nuclei and in the hippocampal formation.[24, 325, 497, 498] In contrast, NMB and BB_1 receptors are more pervasive in the lateral septum, olfactory regions, several thalamic nuclei, and the dorsal raphé nucleus.[24, 325, 343, 497, 498] The heterogeneous distribution patterns of GRP, NMB, BB_1 and BB_2 receptors raises the likelihood that these two peptidergic systems have distinct physiological and/or behavioral functions.

Earlier findings had identified BB and related peptides as potent suppressants of food intake and consequently, these peptides gained propriety as being satiety peptides.[134, 287, 300] However, subsequent studies have implicated these peptides in other

functions, including the stress response.[297] Indeed, central BB administration like traditional stressors, provokes the release of ACTH and corticosterone and evokes behaviors associated with fear and/or distress, including increased grooming and locomotor activity in a familiar environment, and decreased food intake and reduced locomotor activity in a novel (presumably stressful) environment.[142, 198, 231] In addition to stressor-like pharmacological actions of these peptides, stressors also provoke the release of endogenous BB-like peptides (BLPs) from stress-relevant brain regions such as the central nucleus of the amygdala and anterior pituitary gland.[297,302]

Although evidence supports the view that GRP and NMB are involved in the mediation of the stress response, few specifics are available regarding the differential roles of these peptides. Central GRP administration activates the hypothalamic pituitary adrenal (HPA) axis as reflected by the increased release of ACTH and corticosterone, an effect completely blocked by pretreatment with a competitive and specific BB_2 receptor antagonist.[140, 213] Although subcutaneous administration of NMB also elicits the release of ACTH and corticosterone, it is not clear if these effects are centrally mediated, or through effects of peripheral NMB at the pituitary level. The effects of centrally administered NMB on HPA axis function are currently not known.[275, 277]

Paralleling the neuroendocrine findings, BB has been implicated in the mediation of anxiety-related behaviors; however, the available data are not particularly compelling. In this regard, both BB_1 and BB_2 receptor deficient mice do not appear to differ from their wild type counterparts in the light dark box and elevated plus maze tests.[517, 519, 522]

However, decreased emotionality in other anxiety-related paradigms was observed in mice with BB_1 receptor deletion.[520] Furthermore, evidence indicates that GRP and related BB_2 receptors are involved in fear-related responses. Shumyatsky et al [445] demonstrated that BB_2 receptor deficient mice displayed greater and more persistent long-term memory of fear. In contrast, however, Roesler et al.[408] reported that microinjection of a selective BB_2 receptor antagonist directly into the basolateral amygdala, impaired memory retention on an inhibitory avoidance task suggesting that blockade of BB_2 receptors impairs aversive memory. Although the source for these inconsistencies is not clear, it is believed that multiple stress pathways exist which may be differentially activated by distinct stressor types (e.g., neurogenic versus psychogenic; innate vs. learned) and may contribute to anxiety in different types of paradigms.[171, 331] Moreover, there is evidence indicating that fear and anxiety may involve peptidergic actions (CRH) in different portions of the extended amygdala, namely the central amygdala and the bed nucleus of the stria terminalis.[92, 94] In light of these considerations, the present investigation assessed the influence of central administration of NMB and GRP agonists and antagonists in different behavioral paradigms, including those involving innate (unlearned) anxiety responses (open-field test and elevated plus maze) and conditioned responses (fear potentiated startle). This study was predicated on our working hypothesis that GRP and NMB may be differentially involved in anxiety and fear responses.

Materials and methods

Subjects

Male Sprague-Dawley rats (Charles River, St-Constant, Canada), weighing between 275-300 g at the time of surgeries were used. Animals were housed individually and maintained on a 12 hour light/dark cycle (lights on at 07:00 h). Temperature and humidity were kept constant at 23°C and 60%, respectively. Throughout the study, animals had free access to food and water. All experimental procedures were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the University Animal Care Committee.

Surgery

Animals were anesthetized with halothane (2.5%) and stereotaxically implanted with 5.5 mm stainless steel guide canulae (22 gauge) (Plastics One, Roanoke, VA) aimed at the third ventricle. The placement coordinates [362] used were 4.4 mm posterior to bregma, 0 mm lateral, and 4.4 mm below the skull surface. The cannula was anchored to the skull with 3 stainless steel screws and dental cement. Removable stylets (Plastics One, Roanoke, VA) were inserted into the guide cannulae until the experimental day. Animals were allowed one week of recovery time before the beginning of behavioral testing. During the recovery period animals were acclimated to handling as well as mock central injections.

Drugs and Injections

GRP (Phoenix; 0.30 nmoles), NMB-30 (Phoenix; 0.29 nmoles), the BB_2 receptor antagonist, [Leu^{13}-(CH_2NH)Leu^{14}]-BN (Bachem; 1.26 nmoles) and the BB_1 receptor antagonist, BIM 23127(Bachem; 1.70 nmoles), were dissolved in Krebs Ringer Buffered saline solution (KRB) consisting of (in nmoles): 2.7 K^+, 145 Na^+, 1.35 Ca^{2+}, 1.0 Mg^{2+}, 150 Cl^-, 0.05 ascorbate, pH 7.4 (Moghadden & Bunney, 1989). The non-peptide BB_1/BB_2 receptor antagonist (which only has modest effects on BB_2 receptors), PD 176252 (Parke Davis, UK; 0.621 nmoles) was dissolved in a 30% dimethyl sulfoxide (DMSO) and dH_20 solution.

Each drug solution was microinjected into the 3rd ventricle in a 3 µl volume infused over 60 s via an injection cannula (0.5 mm longer than the guide cannula) connected to an infusion pump through polyethylene tubing (Harvard Apparatus, MA). Following drug infusion, the injector was left in place for an additional 60 s to ensure complete diffusion.

Experiment 1a: ***Effects of central administration of BB_1 receptor agonist and antagonist on anxiety and/or fear responses in the open field, elevated plus maze and fear-potentiated startle paradigm.***

Open Field

The open field testing arena consisted of a light gray plastic chamber (57 x 57 cm; 30 cm high walls) equally divided into 36 squares. A black curtain surrounded the chamber to prevent spatial cues and influence of other extraneous stimuli. A video

camera mounted above the arena permitted the transmission of a video signal to a TV monitor located on the other side of the curtain.

Rats (n = 9/group) were were acclimatized to the testing room for 1 hour and then injected centrally (icv) with either vehicle (KRB solution), NMB-30 (0.29 nmoles) or the BB_1 receptor antagonist, BIM 23127 (1.70 nmoles) 15 min prior to testing. Each rat was then placed in the center of the arena and its behavior was monitored for 5 min and scored by an experimenter blind to the drug conditions. The total distance (number of squares crossed by the animals), number of entries and time spent in the center of the arena were recorded. Before introducing the next animal, the arena was thoroughly cleaned using a 70% ethanol solution to prevent detection of any residual odor from the previous animal.

Elevated-plus maze (EPM)

The EPM consisted of four arms (50 cm long and 10 cm wide) positioned in the shape of a plus (+), elevated from the floor (50 cm). Two of the opposing arms were surrounded by a 40 cm high wooden wall, whereas the other two opposing arms comprised open planks surrounded by a 0.5 cm ledge on all sides to prevent rats from falling off. The four arms connected to a central 10 x 10 cm square platform. As in the case of the open field, a black curtain surrounded the EPM, and behavior was recorded through a video camera mounted above the maze.

Immediately following the open field test, each rat was placed onto the open central platform of the EPM, such that he was facing one of the closed arms. The rats' behavior was monitored for 5 min and scored by a blind experimenter as follows: 1) frequency of entries in open arms (entry was counted only if all four paws were placed on an open arm), 2) time spent on the open arms, 3) frequency of entries in the closed arms, 4) time spent in closed arms, 5) number of unprotected head dips/risk assessments (head protruding over the edge of an open arm). Between tests, the EPM was cleaned with 70% ethanol.

Fear-Potentiated Startle

The startle chamber (Coulbourn Instruments) consisted of a sound attenuated chamber containing 2 calibrated platforms (18 x 10 cm) designed to measure the animal's startle response. Animals were placed in a teflon cage (18.5 x 11 cm) with a floor made up of stainless steel bars spaced 1.8 cm apart, and connected to a shock generator (Coulbourn instruments; H13-16). A high frequency speaker mounted (24 cm) above the platforms generated white noise, while tones were generated by a sonalert model tone generator (75kHz – Coulbourn Instruments), which was adapted to work with the acoustic startle apparatus.

Fear potentiated startle training and testing spanned 5 days. On the first day, naïve rats (n=8-10/group) were placed inside the startle chamber and were exposed to random bursts of white noise (95, 110, and 115 db) designed to acclimatize the rodents to the startle chamber and establish baseline startle amplitudes for each animal. On the

second day, animals received their first conditioning session (CS-US pairing). A 0.6 mA, 0.5 s footshock was administered during the last 500 ms of a 4 s tone (75 KHz). There were 7 trials in total with an average of 1 min (randomized) intertrial interval (ITI). On the third day, animals were tested for fear potentiation in the absence of drug to distinguish potentiators from non-potentiators. Twenty trials consisting of 110 db white noise bursts (random 1 min ITI) were followed by 5 trials where the tones were paired with noise, and finally, 5 noise alone trials. At the end of testing, animals were separated into drug conditions based on the combination of their baseline startle amplitude values (Day 1) and their fear potentiation values (Day 3). Rats that did not exhibit fear-induced potentiation on Day 3 were not included in the study. This procedure ensured no pre-existing group differences prior to testing. On Day 4, animals were reconditioned using the same procedure used on Day 2, and were retested on the 5th day, but following the administration (15 min prior to test) of vehicle, NMB-30 (0.29 nmoles) or BIM 23127 (1.70 nmoles). Cages were cleaned with 70% ethanol between testing of each animal.

Experiment 2: ***Effects of central administration of BB_2 receptor agonist and antagonist on anxiety and/or fear responses in the open field, elevated plus maze and fear potentiated startle paradigm***

The design of this experiment was identical to that of Experiment 1 with the exception that rats (n=8/group) were injected icv with either vehicle, GRP (0.30 nmoles) or a BB_2 receptor antagonist, [Leu^{13}-(CH_2NH)Leu^{14}]-BN (1.26 nmoles) 15 min prior to testing.

Experiment 3: ***Effects of BB_1/BB_2 receptor blockade on anxiety as assessed by the elevated plus maze***

To examine the combined effects of BB_1 and BB_2 receptor antagonism, in a third experiment, rats were centrally injected with either vehicle (30% DMSO solution) or PD 176252 (0.621 nmoles) 15 min prior to testing in the EPM. Otherwise, the procedure was the same as the EPM portion of Experiment 1.

Histology

Following completion of the experimental procedures, rats received an overdose of pentobarbital and 25% India ink (1 μl) was delivered through the injection cannula. Animals were then sacrificed and their brains were removed and frozen. Location of the cannulae was verified histologically following thionin staining of the sections.

Statistics

Data for the open field test and EPM were analyzed using one-way analysis of variance (ANOVA) for each of the behavioral measures followed by Newman-Keuls multiple comparisons ($p<.05$). For fear potentiated startle, data were analyzed using a mixed measures ANOVA in which the drug condition was considered the between group variable, and trials (noise alone and tone + noise) as a within group variable. Newman-Keuls multiple comparisons ($p<.05$) of the simple effects were used to assess specific differences for significant interactions.

Results

Experiment 1: *Effects of central administration of BB_1 receptor agonist and antagonist on anxiety and/or fear responses.*

Analyses of behaviors in the open field test, revealed that the drug treatments influenced the number of entries as well as the time spent in the center of the arena ($F(2, 21) = 4.088$, $p < 0.03$ and $F(2,21) = 3.688$, $p < 0.02$, respectively) (see Table 1). The follow-up tests indicated that rats treated with the BB_1 receptor antagonist, BIM 27123, initiated a significantly greater number of entries and spent significantly more time in the center of the arena relative to vehicle treated rats. These effects were not likely due to increased locomotor activity, as BIM 23127 did not affect on the total number of squares crossed in the arena. In contrast to the effects of the antagonist, treatment with the agonist (NMB-30) did not affect open-field behaviors.

Table 1 Summary of the effects of BB_1 and BB_2 receptor agonists and antagonists on behavior as measured in the open field

Behavior	Vehicle		Agonist		Antagonist	
	GRP	NMB	GRP	NMB	GRP	NMB
Time Center (sec)	6.7 ± 2.7	4.8 ± 2.1	6.1 ± 1.4	4.3 ± 2.2	13.7 ± 4.2	13.4 ± 3.5*
No. Entries Center	2.3 ± 0.7	1.8 ± 0.5	2.2 ± 0.7	1.4 ± 0.6	4.3 ± 1.0	3.9 ± 0.7*
Squares Crossed Center	5.8 ± 2.3	41.0± 1.8	5.9 ± 2.2	3.7 ± 1.8	11.5 ± 3.0	9.4 ± 2.3
Time in Periphery (sec)	293.2 ± 2.7	295.2 ± 2.1	293.8 ± 1.4	295.7 ± 2.2	286. 1 ± 4.3	286.3 ± 3.7*
Squares Crossed Periphery.	122.2 ± 11.6	120.4 ± 12.9	103.6 ± 26.1	111.4 ± 10.6	143.3 ± 12.4	150.7 ± 11.4

Note. Data represent means ± SEM (s) for behavior as measured in the open field arena. Testing lasted for 300 s. Recorded behaviors included 1) time in the center of the arena, 2) number of entries into the center of the arena, 3) the number of squared crossed in the center, 4) the time spent in the periphery of the arena and 5) the number of squared crossed in the periphery.

* Significantly different from respective vehicle condition at $p < 0.05$

Analyses of the elevated plus maze behaviors indicated that the drug treatments affected the time spent on the open arms ($F(2, 20) = 3.34$; $p < 0.05$), time spent in the closed arms ($F(2, 20) = 3.96$; $p < 0.05$), the frequency of open arm entries ($F(2,20) = 5.456$; $p < 0.05$) and the number of unprotected head dips ($F(2,20) = 3.56$; $p < 0.05$) (see Figures 1a-d). The follow-up tests revealed that rats injected centrally with the BB_1 receptor antagonist BIM 23127, initiated a significantly greater number open arm entries, spent significantly more time on the open arms, and significantly less time in the closed arms relative to vehicle or NMB-30 treated rats. In addition, rats treated with either BIM 23127 or NMB-30 prior to testing showed significantly more unprotected head dips (risk assessment behavior) in comparison with vehicle treated controls.

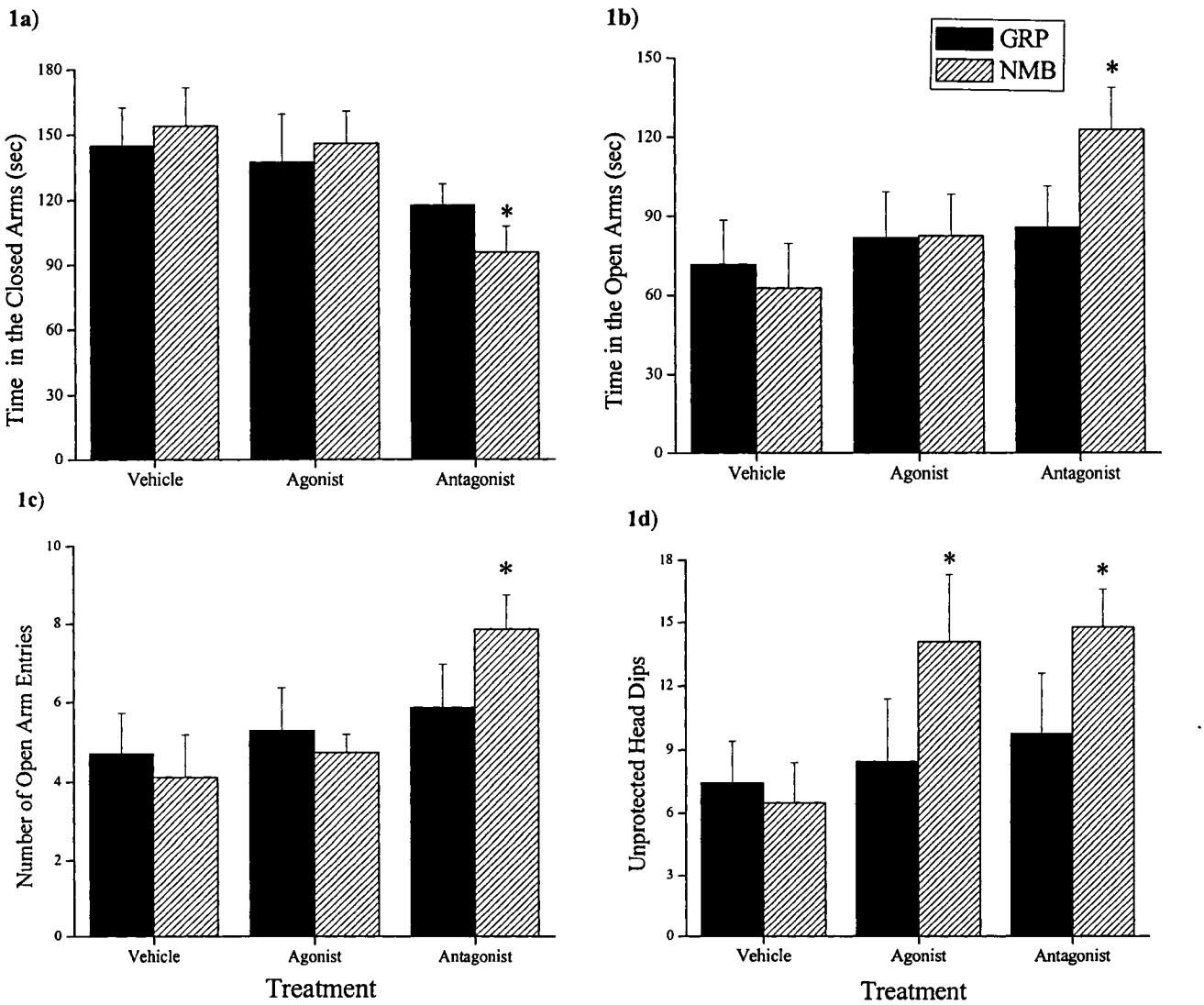


Figure 1 Each column represents the mean ± SEM for each behavior on the EPM for animals treated with either vehicle, BB_1 and BB_2 receptor agonists (NMB-30 or GRP), and BB_1 and BB_2 receptor antagonists (BIM 23127 or [Leu^{13}-(CH_2NH)Leu^{14}]-BN). Solid columns represent animals that participated in the GRP study and hatched columns represent animals that participated in the NMB study. a) the amount of time (in seconds ± SEM) animals spent on the closed arms of the EPM, b) the amount of time (in seconds ± SEM) animals spent on the open arms of the EPM c) the mean number of times (± SEM) animals entered onto the open arms of the EPM and d) the mean number of unprotected head dips (± SEM) while the animals were on the open arms.
* significantly different from respective vehicle condition at $p < 0.05$.

Analysis of the startle amplitude scores revealed a significant Drug Condition x Trial interaction, $F(2, 31) = 4.799$; $p < 0.01$ (see Figure 2a). The follow-up comparisons revealed that there were no differences in startle amplitude between drug conditions during the noise alone trials; however, in the presence of the cue (tone), the fear potentiated startle response was significantly attenuated in animals treated with BIM 23127 compared to vehicle animals, whereas NMB-30 treatment had no effect.

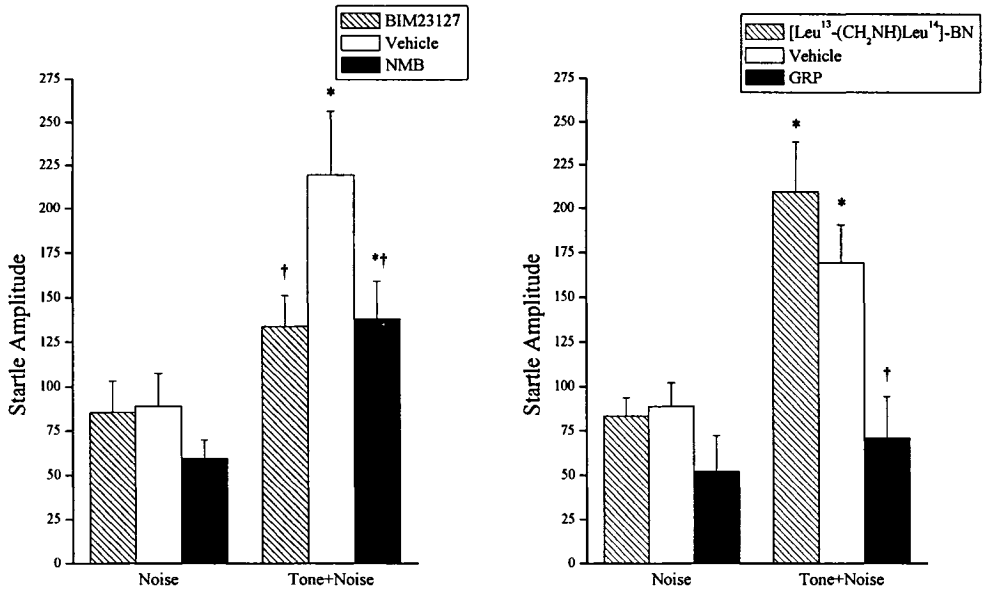


Figure 2 Columns represent mean startle amplitude (in arbitrary units) (± SEM) for noise alone and tone + noise trials in rats treated with either a) BIM 23127 (hatched columns), vehicle (open columns) or NMB-30 (solid columns) or b) [Leu^{13}-(CH_2NH)Leu^{14}]-BN (hatched columns), vehicle (open columns), or GRP (solid columns).
* significantly different from group-matched noise alone trails $p < 0.05$.
† significantly different from trial-matched vehicle condition at $p < 0.05$.

Experiment 2: ***Effects of central administration of BB_2 receptor agonist and antagonists on anxiety and/or fear responses.***

The BB_2 receptor manipulations were found not to significantly influence performance in the open-field and the elevated plus maze (see Table 1and Figures 1a-d). In the fear potentiated startle paradigm, startle amplitude varied as a function of the Drug Condition x Trial interaction, $F(2, 23) = 6.85$; $p < 0.004$ (see Figure 2b). The follow-up tests revealed that during the noise alone trial there were no differences in startle amplitude between groups. In contrast, during the cued (tone + noise) trials, animals treated with GRP showed significantly lower startle amplitudes compared to vehicle-treated rats, whereas the groups did not differ on the non-cued trials. The response to the tone + noise in the [Leu^{13}-(CH_2NH)Leu^{14}]-BN treated rats was comparable to that of vehicle treated animals.

Experiment 3: ***Effects of central administration of a mixed BB_1/BB_2 receptor antagonist on elevated plus maze performance***

As depicted in Table 2, time spent in the open arms was increased by treatment with the mixed BB_1/BB2 receptor antagonist PD 176252, ($F(1, 13) = 5.28$; $p < 0.03$). In addition, the frequency of open arm entries was increased somewhat in rats treated with PD 176252, although this affect was just shy of statistical significance ($p < .06$). None of the other EPM measures were affected by the treatment.

Table 2 Summary of the effects of the mixed BB_1/BB_2 receptor antagonist, PD 176252, on behavior as measured in the EPM

Behavior	Vehicle	PD 176252
Time spent in closed arms	174.8 ± 7.9	184.5 ± 19.6
Time spent in open arms	43.8 ± 7.25	71.6 ± 9.9*
Number of open arm entries	5.8 ± 0.9	8.5 ± 0.8
Number of unprotected head dips	12.3 ± 1.9	15.8 ± 1.8

Note. Each cell represents the mean ± S.E.M (s) for behavior as measured in the EPM. Testing lasted 300 s. Recorded behaviors included 1) time spent on the open arms, 2) time spent of the closed arms, 3) the number of open arm entries and 4) the number of unprotected head dips.

* Significantly different from vehicle condition at $p < 0.05$

Discussion

Despite the accumulating pharmacological and neurochemical evidence supporting involvement of BLPs in the mediation and/or modulation of the stress response, the potential involvement of this family of peptides in the regulation of fear- and/or anxiety-like behavioral responses is not well characterized. Thus the main objective of the present investigation was to assess the effects of BB_1 and BB_2 receptor agonists and antagonists in validated models of anxiety that involve unlearned (open field and elevated plus maze exploration) and learned (fear-potentiated startle) responses. Consistent with the involvement of BLPs in the stress response [297], both mammalian analogues of BB, NMB and GRP, influenced fear and/or anxiety related responses. However, whereas NMB appeared to play a role in both anxiety and conditioned fear responses, GRP seemed to be selectively involved in the conditioned fear response. These findings suggest that these two peptidergic systems subserve distinct roles in stress-related responses. Of course, these findings are mitigated by the scope of this study, including the limited number of doses and behavioral paradigms tested. The proposed differentiation will need to be verified across a wider array of behavioral paradigms and of doses.

In the open field test, rats treated with BIM 23127 (BB_1 receptor antagonist), entered the central portion of the arena more readily and spent more time in this area than did vehicle treated rats. As BIM 23127 did not increase exploration of the field (square crossings), the anxiolytic actions of the compound did not appear to be secondary to motoric effects. Similarly, in the EPM, BIM 23127 markedly reduced anxiogenic

behavior, reflected by an increase of open arm entries, time spent in the open arms and risk assessment behavior. Consistent with the anxiolytic action of BIM 27123 in the open field and elevated plus maze, this BB_1 receptor antagonist attenuated the fear potentiated startle response. In addition, central administration of PD 176252, which is primarily a non-peptide BB_1 receptor antagonist (with modest effects on BB_2 receptors), increased time spent on the open arms. Taken together, the present findings implicate BB_1 receptor-related processes in anxiety and fear.

With the exception of the present experiment, all other attempts to investigate the role of the BB_1 receptor in anxiety-related behavior have been based on mutant mice deficient of BB_1 receptors. However, some of these studies using genetically modified strains failed to detect differences between BB_1 receptor deficient mice and their wild type counterparts, on either the EPM or light dark box test.[517, 519, 522] In contrast, using the marble burying test, Yamada et al. [520] found that mice deficient of BB_1 receptors displayed decreased marble burying indicative of diminished anxiety. The data from studies using knockout mice are difficult to reconcile with those of the present study that involved pharmacological blockade of receptors. However, it ought to be considered that aside from the inconsistent results provided by studies using knockout mice, it is possible that life-long receptor deficiency in knockout models may instigate compensatory mechanisms distinct from those observed in pharmacological models of receptor blockade.[350]

Just as the BB_1 antagonist, BIM 23127, provoked an anxiolytic responses in the EPM, administration of the agonist, NMB-30, was unexpectedly found to increase unprotected head dips (risk assessment), a behavior commonly associated with a decreased level of anxiety. Furthermore, administration of NMB-30 reduced the fear potentiated startle response, a finding reminiscent of the anxiolytic action of the agonist in the EPM test. Thus, beyond effects on anxiety, the diminished fear potentiated startle implicates NMB involvement in fear-related responses. As indicated earlier, the dose response function pertaining to anxiety and fear responses is not known and hence firm conclusions regarding the effects of NMB agonists are not possible.

Although the present findings suggest that NMB may play a role in mediating both anxiety and fear responses, the mechanism(s) by which this peptidergic system promotes such actions is unknown. One possibility involves interactions with the serotinergic system(s) as NMB has a stimulatory effect on 5-HT neurons at the dorsal raphé nucleus.[374] Indeed, anxiety has been linked to the over activity of central 5-HT system(s) [75, 76], and increased release of 5-HT has been reported in association with anxiety and/or fear.[130, 495, 514, 526] In contrast, drugs with anxiolytic properties tend to reduce endogenous levels of 5-HT.[74, 396, 397] Like other anxiolytic compounds, it is possible that blockade of BB_1 receptors might reduce levels of 5-HT, resulting in decreased levels of anxiety and/or fear.

There is some evidence to suggest that in addition to its effects on BB_1 receptors, BIM 23127 may have antagonistic actions on urotensin receptors.[180] As i.c.v.

administration of urotensin promotes anxiogenic behavior in rats [285], it is difficult to ascertain whether the anxiolytic and fear-reducing effects observed in the present investigation with BIM 23127 are purely a BB_1 effect or may in part have been attributable to its action on urotensin receptors. Importantly, however, we observed similar results on the EPM with PD 176252, which is considered a mixed BB_1/BB_2 receptor antagonist (although its highest affinity is for BB_1 receptors), with no known affinity for urotensin receptors. Given that the selective BB_2 receptor antagonist was ineffective in the open field and EPM, it is likely that the anxiolytic effect observed with PD 176252 in the EPM was a result of is action on BB_1 receptors rather than urotensin receptors.

Although behavior in the open field and EPM was modified following the administration of BB_1 receptor agonists and antagonists, the same did not hold true for BB_2 receptor agonists and antagonists. Indeed, administration of either GRP or [Leu^{13}-(CH_2NH)Leu^{14}]-BN were ineffective in modifying behavior reflecting anxiety in these two paradigms. Consistent with these findings, studies using mutant mice have shown that a deficiency of BB_2 receptors was not associated with behavioral changes in either the light/dark box [517] or on the EPM.[445, 517] In contrast, in the fear potentiated startle paradigm, injection of GRP reduced the fear potentiated startle response, whereas administration of the BB_2 receptor antagonist, [Leu^{13}-(CH_2NH)Leu^{14}]-BN provoked a modest (nonsignificant) enhancement of this response. These findings are consistent with the finding of an enhanced conditioned emotional response among BB_2 receptor deficient mice [445] and together support the notion that the GRP peptidergic system may be

preferentially involved in fear responses and less so with respect to other types of anxiety responses.

The fear potentiated startle and the conditioned emotional response paradigms are generally believed to measure learned (classically conditioned) fear responses.[158, 415] In contrast, the open field and EPM measure behaviors reflecting unconditioned responses and thus innate anxiety.[94, 158, 415, 525] It seems probable that fear and anxiety-type responses are mediated through distinct neuronal systems.[93, 292, 415] While the neurocircuitry involved in anxiety responses observed during exposure to the open field and EPM has not been well delineated, there are data regarding neuronal correlates of conditioned fear responses. For instance, it was reported that lesions to the central nucleus of the amygdala (CeA) blocked the fear-potentiated startle response.[60, 183] This same pattern of results was observed in other models of conditioned fear, including the conditioned emotional response.[251, 415] In addition, the lateral amygdala, located upstream from the CeA, is thought to be critically involved in the expression of learned fear to auditory stimuli.[404, 445] Shumyatsky et al (2002) reported that the gene which encodes GRP is highly expressed in the lateral amygdala, and BB_2 receptors are located on GABA interneurons at this site. Upon stimulation with GRP, excitation of these interneurons increased inhibition of principal neurons. Indeed, mice deficient in BB_2 receptors showed decreased inhibition of principal neurons, increased long-term potentiation and enhanced long term memory of fear.[445] Although the present study only examined the effects of i.c.v. drug administration and therefore can not specify the site(s)

of drug action, the lateral amygdala is a likely candidate given its role in learned fear as well as the prevalence of BB_2 receptors at this site.

In summary, the results of the present investigation implicate mammalian BLPs, GRP and NMB, in anxiety and/or fear responses. However, whereas NMB appeared to play a generalized role in mediating both fear and anxiety responses, GRP seemed to be selectively involved in fear responses. It remains to be determined whether this profile is evident in other paradigms that assess anxiety versus fear. Such a distinction may have important implications for the development of novel therapeutic interventions for clinically significant anxiety or fear reactions.

Preface to Chapter IV

Since the behavioral studies tended to support the contended role for NMB in anxiety and fear-related responses, we next undertook studies aimed an elucidating potential involvement of serotonergic mechanism(s) underlying NMB's distinct responses. Specifically, using *in vivo* microdialysis approach we assessed whether microinfusion of NMB or GRP into the dorsal raphé nucleus (a primary projection site of 5-HT neurons) altered the release of 5-HT at the ventral hippocampus. In addition we examined if BB_1/BB_2 receptor blockade elicited the opposite effects to those of the peptide agonists.

Chapter IV

Bombesin receptors as novel anti-anxiety therapeutic target; non-peptide antagonist PD 176252 reduces anxiety and 5-HT release through BB_1 receptors

Abstract

The effects of PD 176252, a non-peptide bombesin BB_1/BB_2 receptor antagonist, on anxiety-related behaviors were assessed in several ethologically relevant tests in rats, including social interaction, approach to food in a novel environment, and potentiated startle tests of anxiety. As well, vocalization of guinea pig pups following separation from their mother was evaluated. Consistent with a role for the bombesin (BB) family of peptides in subserving anxiety-type behaviors, the antagonist increased social interaction (3.75 and 7.5 mg/kg; i.p.), dose-dependently reduced the number of vocalizations made by guinea pig pups separated from their mother for 5 min (1-30 mg/kg, i.p.), reduced response latencies to approach and eat a palatable snack in an unfamiliar environment, and reduced the fear-potentiated acoustic startle response (5 and 10 mg/kg., i.p. and 100-200 ng per rat i.c.v.). When administered directly to the dorsal raphé nucleus (DRN), PD 176252 (20-500 ng) increased social interaction under aversive conditions, as did the $5\text{-}HT_{1A}$ receptor agonist 8-OH DPAT (50 ng), implicating the DRN as a potential site of action of the BB_1/BB_2 antagonist. Furthermore, systemic and intra-DRN microinfusion of the peptide antagonist (PD 176252) suppressed, whereas its agonist (NMB-30) promoted, the *in vivo* release of 5-HT in the ventral hippocampus (a projection area of 5-HT neurons originating in the DRN). Taken together, these findings indicate that endogenous BB-like peptides at the DRN stimulate 5-HT release in limbic brain regions, and that BB receptor (BB_1 and/or BB_2) antagonists may represent a novel class of anxiolytic compounds potentially counteracting these effects.

Introduction

Although the benzodiazepines were a major advance over the barbiturates, in terms of their margin of safety and toxicity, they are fraught with side effects including motor effects and the tendency to induce tolerance and dependence. This has prompted the search for alternatives to benzodiazepine receptor agonists.[127, 129] One line of inquiry in the quest for alternative pharmacotherapy involves drugs affecting 5-hydroxytryptamine (5-HT) neurotransmission, prompted to a large extent by the clinical success of buspirone (5-HT_{1A} receptor agonist) that blunts the release of 5-HT from terminals of neurons originating in the dorsal raphé nucleus (DRN).[110] It is of interest to note that the 5-HT_{1A} receptors exist both pre- and postsynaptically and direct injection of 5-HT_{1A} agonists into areas containing presynaptic (DRN) and postsynaptic (hippocampus and amygdala) 5-HT_{1A} receptors can elicit opposing effects on 5-HT release and anxiety-like behaviors.[12, 181, 182] Such findings suggest that behavioral changes may reflect a balance between anxiogenic versus anxiolytic actions of 5-HT in these different regions. In addition, postsynaptic 5-HT likely contributes to the expression of anxiety-like behavior through other 5-HT receptor subtypes, as direct injection of the 5-HT_{2C} receptor agonist mCPP into the ventral hippocampus induces anxiogenic-like behavior.[507] Thus, development of pharmacological tools that selectively affect specific 5-HT projections may yield anxiolytic agents with a better therapeutic profile.

Bombesin (BB), an amphibian-derived peptide, evokes several effects in mammals through actions at a heterogeneous population of receptors.[24] Of BB's mammalian counterparts, neuromedin B (NMB) binds to the BB_1 receptor with higher affinity than does gastrin-releasing peptide ($GRP_{1\text{-}27}$) or neuromedin C (NMC ($GRP_{18\text{-}27}$), whereas GRP and

NMC have a greater affinity than NMB for the BB_2 receptor. In relation to cell function, Pinnock et al. [374] showed that BB and NMB increased the firing rate of 5-HT cells in the DRN. Since the reduction of 5-HT release has been linked to anxiolytic drug action, it might be expected that antagonism of the excitatory action of BLPs on 5-HT cells in the DRN would decrease anxiety levels.

In view of the potential role of NMB and GRP in mediating anxiety, the effects of BB agonists and antagonists were assessed on several behavioral paradigms thought to reflect anxiety. In particular, we demonstrate that systemic administration of PD 176252, which is primarily a non-peptide antagonist of the BB_1 receptor (with modest effects on BB_2 receptors) [16], influenced anxiety-like behaviors in ethologically relevant tests of anxiety. These included analysis of social interaction [128], approach to a familiar palatable snack in rats placed in a novel (anxiogenic) environment [298] and in the guinea pig pup vocalization test [321], all of which are sensitive to anxiolytic and/or antidepressant compounds [128, 298, 321] and are uncontaminated by the effects of painful stimuli. In addition, the effects of PD 176252 on fear-potentiated startle were assessed to determine the effects of the agent on learned fear responses [255]. Given the potential role of 5-HT in mediating anxiety [12, 181, 182], we examined the effects 5-HT lesions (using 5,7-DHT) on BB_1/BB_2 binding sites at the DRN. Finally, we examined the effects of PD 176252, administered systemically or locally at the DRN, on the *in vivo* release of 5-HT at the ventral hippocampus, a projection site of the 5-HT neurons emanating from the DRN.

Materials and Methods

Animals

All test subjects were group housed in a holding room with lights on from 07:00 - 19:00 h., for at least 2 weeks prior to testing. Rats for microdialysis experiments were housed in a holding room lit by a reversed light cycle (lights on from 19:00 - 07:00 h). Sprague-Dawley rats (300-375g; Charles River, St. Constant, Quebec) were used in the Canadian studies, whereas the Cambridge studies utilized male hooded Lister rats (300-375g; Harlan Olac, Bicester, UK). Female Dunkin Hartley guinea-pigs (Dave Hall, U.K.) with litters of 1 to 3 day old pups were used for the vocalization studies. They were housed in mother + litter groups under a 12 hour light/dark cycle (lights on at 7:00). All animals received food and water *ad libitum* and the temperature (23°C) and humidity (60%) were kept constant. All procedures were approved by the appropriate Local Ethics Committees and met the guidelines set out by the Canadian Council on Animal Care. All attempts were made to minimize the number of animals used considering the variability in responses associated with the treatments.

Apparatus

The social interaction arena was circular (diameter 70 cm) and made of white Perspex with walls 30 cm high. For all studies the arena was lit by a bright light source (350 lux) located directly above the arena. A camera, linked to a video recorder in an adjacent room was also located directly above the arena to allow the test sessions to be recorded for later analysis.

The test cage for the guinea pig vocalization testing consisted of a sound-attenuated box with a white interior and white illumination and vocalizations were recorded on DAT-tape by means of a microphone and a DAT recorder.

To assess the behavioral responses associated with the presentation of a palatable snack, animals were tested in either a familiar (home) or unfamiliar (novel) cage. The home cage consisted of a shoebox-style clear Plexiglas container (24 x 30 x 18 cm) with the bottom lined with bedding material (beta chips) to a depth of approximately 1 cm, and a removable, grilled top. The novel environment (test cage) was identical to the home cage, but was freshly cleaned, and was devoid of wood chip floor bedding.

For the fear potentiated startle experiments, all animals were trained and tested in acoustic startle response monitoring systems (MED Associates Inc, St. Albans, VA), which were located in individual, ventilated, sound attenuated chambers (30 x 55 x 50 cm). Each chamber was equipped with a wideband speaker (1 kHz - 16 kHz), which provided the acoustic startle stimuli, as well as background noise. During the training and testing sessions, the rats were placed in individual cages (19 x 9 x 8.5 cm) with conductive floors (made up of 6 stainless steel rods of 4.9 mm diameter) that were placed on top of the startle sensor platform. Each sensor platform (25 x 11.5 cm) was linked to a signal transducer and load cell amplifier. The downward pressure exerted by the animal's startle response was converted to an analogue signal that was amplified (by the load cell amplifier) and then digitized on a scale of 0-2047 arbitrary units. The presentation and ordering of all stimuli were controlled by startle reflex software (MED Associates, St. Albans, VA).

Surgery

Microinjection and Microdialysis Experiments

Rats under halothane (3% in oxygen) anaesthetized were positioned with leveled skull, in a stereotaxic frame (Kopf Instruments) for cannula implantation. For DRN placements (social interaction and microdialysis experiments), 11 mm long steel guide cannulae were positioned at 7.4 mm posterior to bregma, 2.1 mm lateral to the midline and 6.5 mm ventral from dura at an angle of 19°, thus sitting it 1 mm above the target area. For microdialysis at the ventral hippocampus, guide cannulae (BAS Inc., West Lafayette, IN, USA) containing removable obturators were positioned 5.3 mm posterior to bregma, 4.85 mm lateral to the midline and 3.0 mm ventral from the dura. For cannulation of the 3rd ventricle (fear potentiated startle experiments) rats were anaesthetized with pentobarbital (65 mg/kg; i.p.) and stainless steel guides were positioned 4.3 mm posterior to bregma, 0.0 mm lateral to the midline and 4.3 mm ventral to the skull surface. All cannulated rats were housed individually, and allowed 7 days recovery prior to the test day. The cannulae were kept patent using stainless steel dummy cannulae, which were manipulated daily by gently wrapping the rat in a cloth and rotating the dummy cannulae.

5,7 DHT Lesioning

Having been injected with desmethylimipramine (25 mg/kg; i.p.) 1 h previously, the rats were anaesthetized and positioned in the stereotaxic instrument as above. A hole was drilled 1 mm posterior of bregma and 1.4 mm to the right of the midline and a needle, connected to a glass syringe via polyethylene tubing, was lowered 3.5 mm below the dura, thus positioning the needle directly within the lateral ventricle. An injection of

either vehicle (5µl of 0.2% ascorbic acid in artificial cerebrospinal fluid; n=10) or 5,7 DHT (150 µg/5 µl vehicle; n=10) was made over a period of 30 s and the needle left in place a further 30 s to allow for any diffusion away from the tip. The wound was sealed with dental cement and the rat housed singly and allowed to recover for 13 days, during which time they were monitored for adverse reactions to the treatment (none were evident).

Behavioral testing

Social interaction

Rats were allocated to a partner on the basis of body weight, such that members of a pair did not differ by more than 10 g. In the first experiment, both rats of each pair were systemically (i.p.) injected with either vehicle (n = 10) or PD 176252 (3.75 and 7.5 mg/kg, n = 10/group) 60 min prior to being placed into the arena for a 5 min period. In the second experiment, which assessed the effects of microinfusion at the DRN, the rats were gently wrapped in a cloth and injected with either vehicle (50% cyclodextrin in aCSF, n = 12) or PD 176252 (100 ng/0.5µl, n = 14) using a needle that extended 1 mm below the tip of the indwelling cannula. Injections of 0.5 µl were made over a period of 30 s; the needle was left in position for a further 30 s to allow drug diffusion. Three minutes later the cannulated rat and an unfamiliar, non-cannulated partner were placed together in the arena and the behavior observed for 7 min. Time spent in active social investigation (specifically sniffing, following, and grooming the partner) was recorded by an observer blind to drug treatment. Testing was performed between 10:00 and 14:00 h, in an order randomized for drug treatment and the arena thoroughly cleaned between each trial.

Following DRN microinjection the rats were allowed a 7-day drug "washout" period. They were then randomly assigned to a re-test condition with a different partner from that experienced week 1 earlier. Rats received DRN microinfusion, as described earlier, of vehicle (aCSF, n = 10) or the 5-HT1$_A$ receptor agonist 8-OH DPAT (50 ng/0.5µl, n = 9). The social interaction test was the same as that conducted 1 week previously.

Guinea pig pup vocalization

Guinea pig pups were selected for the test groups using the criterion that they emit a minimum of 500 vocalizations upon separation from the dam in a 5 min test period on each of 3 consecutive days prior to the test day. On the test day, pups were submitted to a pre-test (baseline) that took place 30 min after a sham-injection. After this pre-test, the guinea pigs were injected with either vehicle or PD 176252 (1-30 mg/kg; i.p.) and tested again for 5 min, commencing 30 min after drug administration.

Approach to a palatable snack in a novel environment

Rats (n = 8/group) were habituated to a novel, highly palatable snack (Christie HoneyMaid Graham Crumbs) presented in their home cage (15 min snack access) for 8 consecutive days. The snack was presented in a 6 cm diameter ceramic dish placed in the center of the cage. During the last 3 (of the 8) days of this habituation period, when the approach and consumption parameters had stabilized (varying less than 20%) [298], baseline measures of the latency to initiate consumption as well as the amount consumed were recorded. On the test day, rats were administered either PD 176252 (10 mg/kg; i.p.) or 50% cyclodextrin vehicle (control condition) and returned to their home cage. Twenty min

following treatment, rats were transferred to the novel cage (freshly cleaned clear Plexiglas test cage without bedding) and presented with the now familiar palatable snack. The latency to initiate snack consumption and the amount consumed (over 15 min) were recorded.

Fear-potentiated startle

Training/conditioning

On each of 2 consecutive days, rats were first acclimatized to the darkened startle test chamber (with 50 dB ambient white noise), for 5 min. They were then presented with 20 light-shock pairings (per day) on each of 2 consecutive days. On each trial, a 0.65 mA shock was delivered through the grid floor (using a shock source and scrambler, ENV-410B and ENV-412S, respectively, MED Associates) during last 0.5 s of the 3.7 s CS (light); ITI = 60 s (range 45-75 s).

Pre-manipulation fear test

Rats were placed in the same startle cages (where they were trained), and 5 min later presented with 20 startle-eliciting auditory stimuli (110 dB; habituation session). Following this, 10 acoustic startle stimuli were presented, half of which were presented alone (unconditioned stimulus; noise alone trials) and the other half presented 3.2 s after the onset of the 3.7 s light (conditioned stimulus; CS-noise trials). Those rats that failed to show CS-elicited potentiation (i.e. less than 40 % increase in the CS-paired startle response as compared to matched non-CS associated responses) were omitted from subsequent studies (approximately 25%). The selected rats were then placed into groups based on similar fear levels based on difference scores [(CS + noise) – (noise-alone)]. The test procedure was

identical to that of the pre-manipulation fear test, except that the animals were first injected with the test drug or vehicle, and the appropriate vehicle (control) 20 min before the test. The effects of central (100 and 200 ng) and systemic (5 mg/kg and 10 mg/kg) drug administration of PD 176252 were tested using separate groups of animals (n = 9/group).

In-vivo microdialysis

An initial microdialysis experiment assessed 5-HT release following the systemic injection of PD 176252. Following implantation of the intracerebral guide cannula, a microdialysis probe (BAS Inc., West Lafayette, IN, USA) was lowered down the guide cannula and secured such that the active portion of the membrane (4 mm long) extended into the ventral hippocampus. While the animals (n = 6/group) recovered from surgery in the test chamber, the probe was connected via polyethylene tubing (protected by a metal tether connected to a swivel hooked onto a balance arm) to a precision pump (Harvard Apparatus, Endenbridge, UK) and perfused overnight at 1 µl/min with artificial cerebrospinal fluid (aCSF) consisting of 140 mM NaCl, 1.2 mM $CaCl_2$, 4mM KCl at pH = 7.0. Approximately 18 h post surgery, dialysate samples were collected every 20 min at a flow rate of 1.5 µl/min. After a 1 h stabilization period (previously shown to be a sufficient for stabilization [302]) and a stable baseline established, the rats were injected systemically (i.p.) with either vehicle (50% hydroxypropyl-beta-cyclodextrin in saline) or PD 176252 (10 mg/kg) and samples were collected for a further 3 hours. In a second microdialysis experiment, under halothane anaesthesia, rats (n = 8/group) were stereotaxically implanted with guide cannula aimed at the DRN. Once the cannula was set firmly in place, a 4 mm microdialysis probe was lowered in the ventral hippocampus

(AP: -5.3 mm; L: +4.85 mm; DV: -8.2 mm), and held in position using the second arm of the dual arm stereotaxic apparatus. An injector was lowered through the guide cannula such that it protruded 1 mm beyond the tip of the guide cannula, and into the DRN. The probes were perfused with aCSF at a flow rate of 1.5 µl/min, and sample collection initiated after a 1 h stabilization period. Samples were collected every 20 min for a maximum of 5 h. After collecting 5 baseline samples, animals were injected with vehicle (50% cyclodextrin in aCSF or aCSF; 0.5 µl) and 5 more samples were collected. Animals were then injected with NMB-30, GRP_{1-27} (Peninsula Laboratories Inc; 10 nmoles/0.5 µl) or PD 176252 (200 ng/0.5 µl) and 5 additional samples were collected.

Section and tissue preparations

Thirteen days after intracerebral injection of 5,7 DHT or vehicle, all rats were sacrificed and the brains immersed for 20 seconds in *n*-pentane, cooled to approximately -35°C. The brains were then wrapped in aluminum foil and stored at -70°C until required for sectioning or for High Performance Liquid Chromatography (HPLC) analysis. Brains for sectioning were mounted and sectioned (10 µm thickness) using a cryostat microtome. The sections were thaw-mounted onto gelatin coated glass microscope slides and stored at -70°C until required for autoradiography. Slides were prepared with sections for total binding and sections for non-specific binding being taken alternately as they were cut from the brain. Brains for HPLC analysis of neurotransmitter content were thawed and the dorsal and ventral hippocampus, striatum and frontal cortex dissected. Each separate brain area was placed in an Eppendorf tube with 500 µl of 0.1 M perchloric acid (containing 1 ng of an internal standard - dihydroxybenzylamine) homogenized

using ultrasound and centrifuged at 8000 g for 15 min. The supernatant was removed and stored at -70°C until required for HPLC analysis.

Sample analysis

Dialysate samples

Samples were immediately analyzed *ex-vivo* for their 5-HT content using HPLC (Agilent Technologies, Walbronn, Germany). A 50 µl volume of each dialysate was injected via an autoinjector (Agilent 1100 series Autosampler, Walbronn, Germany) into the HPLC system equipped with a single cell electrochemical detector (Antec Leyden Model Intro, Montreal, Que, Canada) with an applied potential of 0.650 nA, a filter of 1 s., and a range of 0.1 nA/V. Separation of these analytes was achieved by their passage through an ESA, 4.6 X 150 mm, 5 Micron analytical column (Zorbax Eclipse XDB-C18, Agilent Technologies, Walbronn, Germany). The column was equilibrated at a flow rate of 1 ml/min with mobile phase consisting of (in mM): 90 sodium dihydrogen phosphate (monobasic), 1.7 1-octane sulfonic acid (sodium salt), 50 citric acid (monohydrate), 5 KCl, 50 mM EDTA and 10% acetonitrile, final pH = 2.4. The quantification of the analytes was performed by comparing their area under the curve to those of known external standards (calibrated at 1.25 pg/50 µl, 5 pg/50 µl and 50 pg/50 µl) using computerized Agilent ChemStation chromatography data acquisition system (Agilent Technologies, Walbronn, Germany).

Tissue Samples

The effectiveness of the neurochemical lesions (for the autoradiography study) was confirmed by measuring the concentration of various catecholamines and indoleamines, at specific brain sites. On the day of analysis samples were filtered

through 0.2 μm syringe filters and 20 μl of the filtrate injected into the HPLC column, as described above.

Histology

Cannula placement verification

Following the experiments, animals were sacrificed and their brains were removed and frozen. Location of the 3rd ventricle cannulae (fear potentiated startle experiment), microdialysis probes and the microinjectors aimed at the DRN (microdialysis and social interaction experiment) were verified histologically upon thionin staining of the sections. For the microdialysis experiment, only animals with both the injector and the microdialysis probe correctly positioned were included in the experimental group.

Autoradiography Studies

Sections were slowly thawed and dried under a stream of cold air before being placed in TRIS.HCl buffer (pH=7.4 at room temp) for 20 min. They were then placed in either a "total binding" or "non-specific binding" incubation mixture for 90 min. The total binding incubation mixture comprised TRIS.HCl (pH=7.4 at room temp.) buffer containing bovine serum albumin (0.5%), bacitracin (40μg/ml), $MnCl_2$ (3mM), leupeptin (4 μg/ml), phosphoramidon (2μM), chymostatin (2μg/ml), D-Phe^6-ethylester (10μM) and ^{125}I-BB (0.1nM; specific activity 2000Ci/mmol). Non-specific binding incubation mixture was the same as for total binding except that BB (1 μM) was included. Following the incubation period, sections were washed 3 times in fresh, ice cold TRIS.HCl (pH=7.4 at 4°C) for 10 s and finally washed twice in ice cold, HPLC grade

water, and dried under a cold stream of air. Autoradiograms were generated by laying the labeled tissues against [^{3}H]Hyperfilms (Amersham, UK). Standards (radiolabeled polymer strips obtained from Amersham, UK) were also laid down at the same time (one per film) to enable quantification of the binding. The films were developed in Kodak D-19 solution after 3 days of exposure at room temperature.

Drugs

The non-peptide BB_1/BB_2 receptor antagonist, PD 176252 (3-(1H-indol-3-yl)-N-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(nitro-phenyl)ureido]propionamide) was dissolved in 50% hydroxypropyl-beta-cyclodextrine in saline using a heated sonicating water bath and administered i.p. at the appropriate doses in a 1 ml/kg volume. A 50% cyclodextrin injection was used as a control (vehicle) condition. For intracranial administration, PD 176252 was dissolved in 50% cyclodextrin and then diluted accordingly in aCSF. 8-OH DPAT (Sigma/Aldrich, Poole, UK) was dissolved in aCSF. Desmethylimipramine, 5,7-DHT and all standards for HPLC were obtained from Sigma (St. Louis, MO, USA), whereas SCH50911 was obtained from Tocris Ltd (Ellisville, MO, USA). For the microdialysis experiment, NMB-30 and GRP_{1-27} were obtained from Peninsula Laboratories Inc. (Belmont, CA, USA) and were dissolved in aCSF. Chemicals for the autoradiography and also for the mobile phase for the HPLC were obtained from Sigma (St. Louis, MO, USA) and were of HPLC grade. ^{125}I-BB (Specific Activity 2000 Ci/mmol) was obtained from NEN (Boston, MA, USA).

Data analysis

Data from the social interaction experiments were analyzed by unpaired t tests when only 2 groups were tested. When more than 2 groups were used, significance was determined through one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$). For the vocalization experiments the difference in the number of calls emitted before and after treatment was counted using Spike2 software. The reduction in the number of calls (as a percentage of baseline) was analyzed using a Kruskall-Wallis test followed by Mann-Whitney tests between vehicle and different doses. Data from the potentiated startle experiments and from the approach to a snack in the familiar and novel environments were analyzed by mixed measures analysis of variance (ANOVA) where dose served as the between-group factor, and stimulus condition was the within-group factor.

In the first microdialysis experiment, 5-HT content was analyzed as a percent change from baseline (mean of three samples) taken at 20-min intervals. Samples collected after treatments were compared to the mean basal value. For the second microdialysis experiment, 5-HT content was expressed as a percent change from baseline (mean of three sample) and analyzed using a mixed measures ANOVA with Treatment condition (Vehicle, PD 176252 or NMB-30) as the between-group factor and samples as the within-group factor. Follow-up comparisons were conducted using Newman-Keuls multiple comparisons ($\alpha < 0.05$). A similar analysis was conducted to measure 5-HT content following local infusion of GRP at the DRN. For the 5,7 DHT lesioning studies, each of the neurotransmitter peaks relayed from the HPLC, was converted by computer

into values representing ng/mg wet weight tissue based on the neurotransmitter standards of the day. A Student's t test was used to analyse between group differences.

For the autoradiography studies, a computerized image analysis system (MCID) was used to construct a standard curve of radioactivity from the standard polymer strip for each film. The optical density of selected brain regions was then measured and converted to nCi/mg protein using the standard curve. Three sections from each brain region were measured and the values averaged for each animal. The values for each region for each animal were then analysed by Student's t tests for differences between control and lesioned rats.

Results

Effects of systemically administered PD 176252on social interaction

Systemic injection of PD 176252 significantly influenced levels of social interaction of the rats ($F(2,27) = 7.20$, $p < 0.01$). The follow-up comparisons indicated that both the 3.75 and 7.5 mg/kg doses ($p < 0.01$ and $p < 0.05$ respectively) of the antagonist significantly increased social interaction (see Figure 1). In contrast, the drug treatment did not affect locomotor activity (data not shown).

Effect of Intra-DRN PD 176252 on social interaction

Direct injection of PD 176252 into the DRN, as shown in Table 1, significantly increased social interaction scores, $t(22) = 4.10$, $p < 0.05$. As the drug did not affect locomotor activity, the altered social interaction could not be attributed to motor effects. In

the subsequent study, using both a higher and a lower dose of PD 176252, it was found that 500 ng provoked an anxiolytic effect, whereas at 20 ng this was not the case. Finally, as shown in Table 1, administration of 8-OH DPAT (50 ng) significantly increased social interaction without affecting locomotor activity $t(25) = 27.77$, $p < 0.01$.

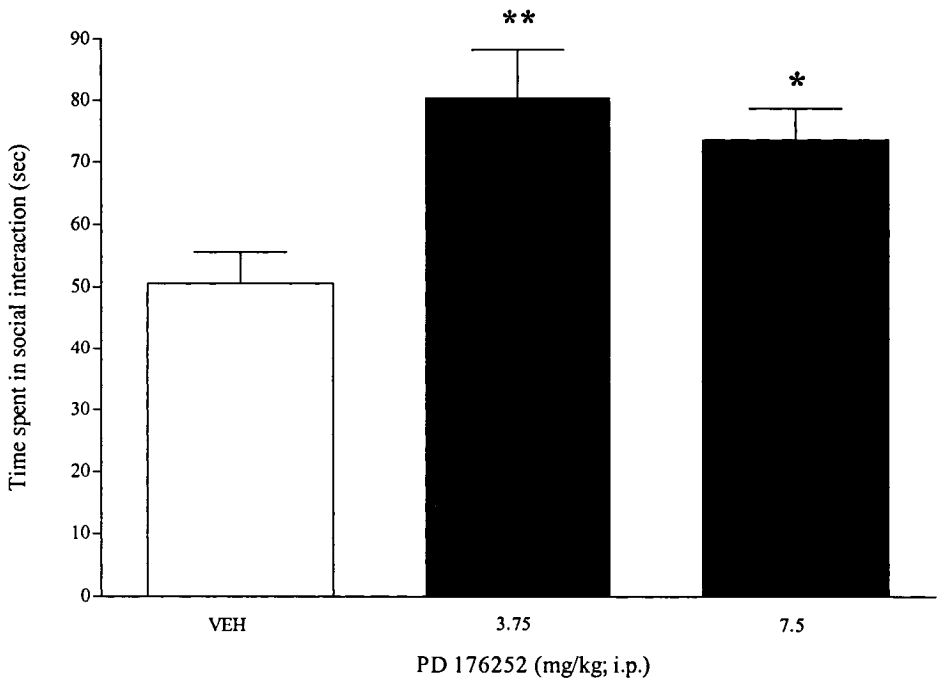


Figure 1 Effect of PD 176252 on rat social interaction. Each column represents time spent in social interaction during a 5 min test period (mean ± SEM) following injection of vehicle (open column), PD 176252 (3.5 mg/kg i.p.; hatched column) or PD 176252 (7.5 mg/kg i.p.; solid column).
* $p<0.05$ and ** $p<0.01$ from vehicle condition.

Table 1 Effect of intra-DRN administration of PD 176252 and 8-OH DPAT on rat social interaction (sec; mean ± SEM) and locomotor activity (beam breaks; mean ± SEM)

	Social Interaction Score	**Locomotor Activity**
Experiment 1		
Control (vehicle)	39.1 ± 4.6	144.3 ± 8.8
PD 176252 (100 ng)	50.1 ± 2.6*	152.5 ± 6.1
Control (vehicle)	47.5 ± 2.7	158.8 ± 9.9
8-OH DPAT (50 ng)	67.5 ± 3.4**	159.2 ± 7.8
Experiment 2		
Control (vehicle)	35.3 ± 3.3	172.5 ± 10.8
PD176252 (20 ng)	36.0 ± 4.4	169.8 ± 14.6
PD 176252 (500 ng)	47.4 ± 5.4*	153.2 ± 10.4

*$p<0.05$ and **$p<0.01$ respective vehicle controls (aCSF); post hoc Duncan's test after one way ANOVA.

Effects of systemically administered PD 176252 on guinea pig pup separation

A single administration of PD 176252, 30 min before the test, dose-dependently (1-30 mg/kg; i.p.) increased the percentage of reduction in the number of calls, with a median effective dose of 10 mg/kg (Figure 2). At the highest dose (30 mg/kg) a 4-fold change was observed relative to vehicle treated animals.

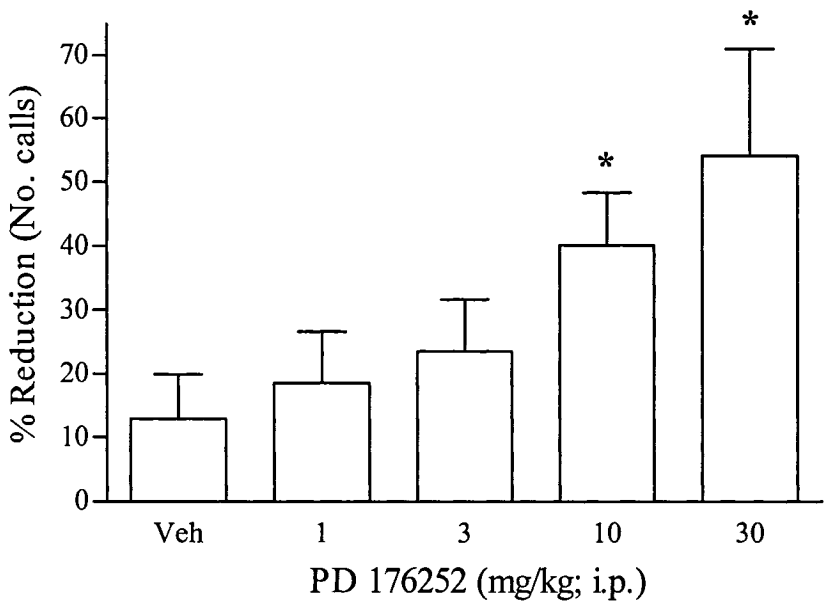


Figure 2. Effect of PD 176252 on guinea pig pup vocalizations following separation from the dam during a 5 min test period. Each column represents % reduction in the number of vocalizations made following PD 176252 (1-30 mg/kg; i.p.) or vehicle injection relative to the number of calls made following sham injection (mean ± SEM).
* $p<0.05$ from vehicle condition.

Effects of systemically administered PD 176252 on approach and consumption of a palatable snack in a novel environment

Repeated measures ANOVA of the amount of snack consumed revealed a significant Test condition (home vs. novel cage) x Drug treatment (PD 176252 vs. vehicle) interaction ($F(1, 14) = 4.82$, $p < 0.05$). Follow-up tests indicated that in the home cage there were no significant group differences in the amount of snack consumed. However, as depicted in Figure 3, a marked reduction in the amount of snack consumed was evident in the novel cage condition, and this effect was significantly attenuated by PD 176252. Similarly, analysis of the latency to consume snack food revealed a significant Test condition x Drug treatment interaction ($F(1, 14) = 29.38$, $p < 0.0001$), which was attributable to a markedly greater increase in novelty-induced latency in the controls, as compared to PD 176252-treated rats.

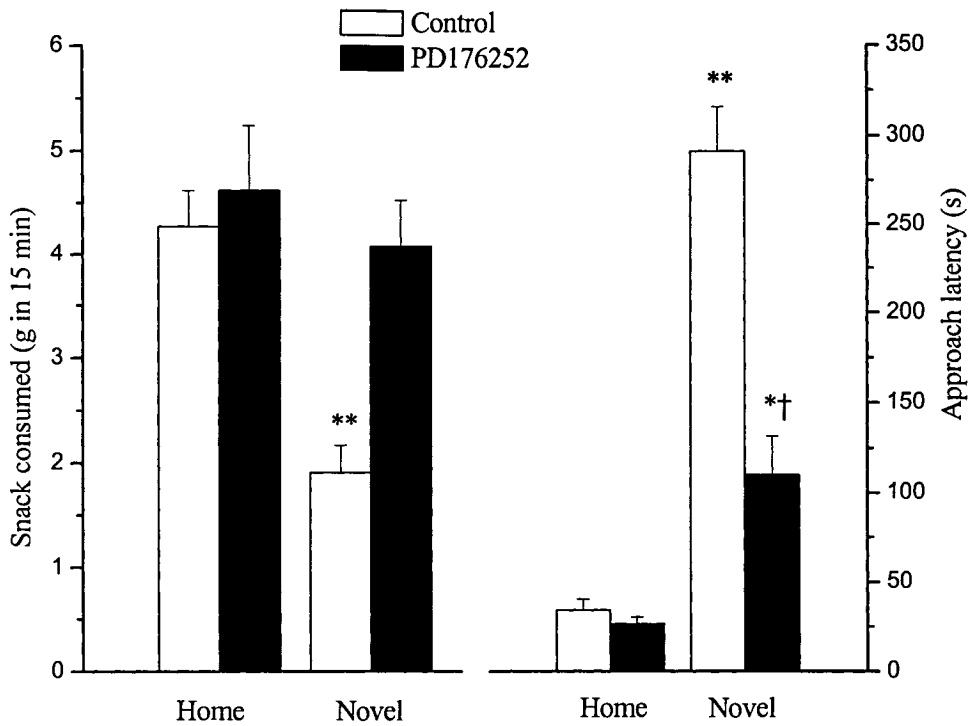


Figure 3 Effect of PD 176252 on snack consumption (g) and on the latency to approach the snack (sec) in the home cage and novel cage conditions. Each column depicts snack consumption (mean ± SEM over 15 min) or approach latency (mean ± SEM) under the home cage (open columns) or the novel cage (solid columns) conditions.
* $p<0.0.5$ and ** $p<0.01$ from respective home cage baselines
† $p<0.05$ from condition matched control

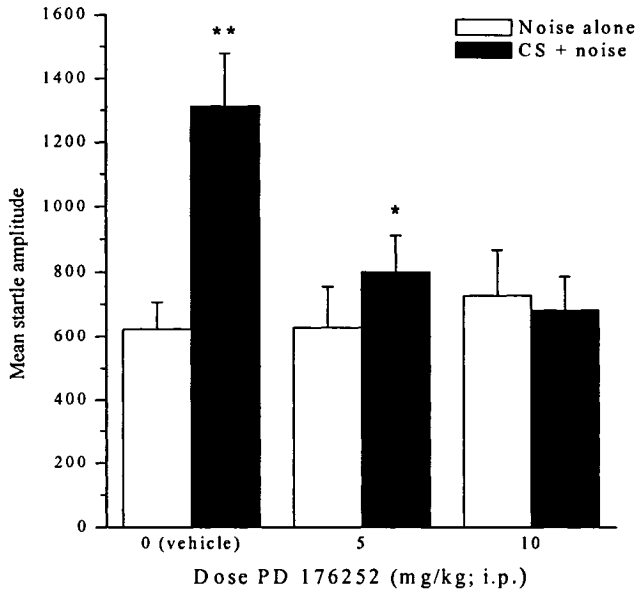
Effects of systemically administered PD 176252 on fear potentiated startle response

Analysis of the startle and fear potentiated startle responses revealed that responsivity varied as a function of the Treatment (doses) x Stimulus condition (CS+ or CS-) interaction ($F(2,24)=7.94$, $p < 0.005$). Comparisons of the means comprising the simple effects revealed in the control (vehicle treated) group, the startle amplitude in the presence of the CS (fear-related cue, solid columns) was markedly higher than that noted in the absence of CS (open columns) (see Figure 4A). Administration of PD 176252 did not alter the startle response in the noise alone condition (CS-); however, the lower dose of the drug attenuated the CS+ induced potentiation of the startle response, while the higher dose entirely eliminated the potentiation.

The effects of centrally administered PD 176252 on fear potentiated startle response

Analyses of the effects of centrally administered PD176252 revealed that responsivity varied significantly as a function of the interaction between the Stimulus condition (CS+ or CS-) and Treatment condition (Vehicle, PD 176252 (100 and 200 ng; i.c.v), ($F(2,24)= 4.533$, $p < 0.05$). As seen in Figure 4B, the startle amplitude of the control animals receiving vehicle (50% cyclodextrin; 3 µl i.c.v.) increased markedly in the presence of the fear cue (CS+). However, this fear-potentiated startle response was significantly attenuated in rats pre-treated with the lower dose of PD 176252 (100 ng; i.c.v.), and completely blocked in those receiving the higher dose (200 ng; i.c.v.) of the antagonist.

A



B

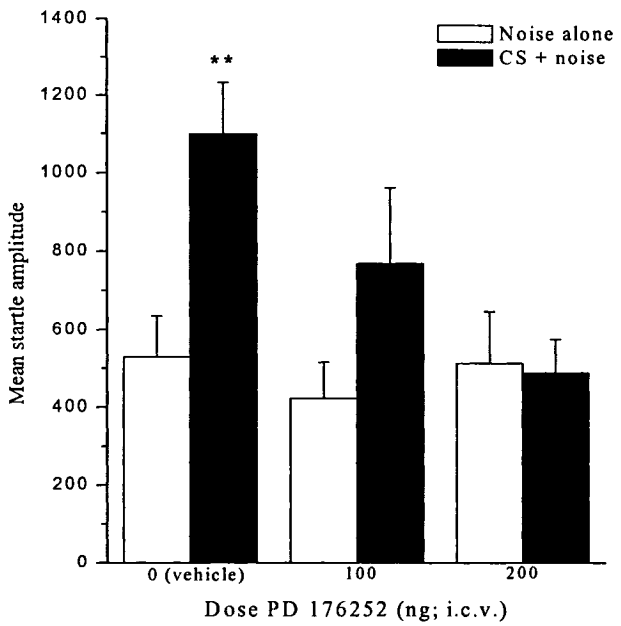


Figure 4 Effect of PD 176252 on fear-potentiated startle amplitude following systemic (i.p; A) and i.c.v. (B) administration. Each column depicts startle amplitude (mean ± SEM) under the noise alone (open columns) and conditioned stimulus + noise (CS + noise; solid columns) situations (see methods).
* $p < 0.05$ and ** $p < 0.01$ (CS + Noise vs Noise alone)

Autoradiography

Overall regional distribution of specific ^{125}I-BB binding to BB_1 binding sites

This was in general agreement with other authors [24, 325, 343] with the highest densities being found in olfactory and thalamic nuclei. Other regions displaying relatively high BB_1 binding included the ventromedial hypothalamus, striatum, septum, piriform cortex and amygdalo-hippocampal area. Binding in the hippocampus was mainly in the dentate gyrus with some diffuse binding present throughout. Binding was not strongly marked in CA1, CA2 or CA3 regions. BB_1 sites were also observed in the mid-brain raphé nuclei. In the DRN the binding sites were found to be located along the mid-axis running dorso-ventral i.e. low levels of binding being visible in the lateral "wings", whereas the binding in the median raphé nucleus (MRN) was distributed throughout the nucleus. Binding was found to be particularly strongly marked in the rhabdoid nuclei, which lie just dorsal of the MRN (see Figure 5).

Effects of 5,7 DHT lesioning on bindings of ^{125}I-BB to BB_1 binding sites

Binding of ^{125}I-BB to BB_1 receptors in the DRN (see Figure 5) was reduced by approximately 50% in the 5,7 DHT treated rats (veh = 14.9 ± 2.9 nCi/mg protein, lesion = 7.24 ± 0.49 nCi/mg protein; $p<0.01$). However none of the other regions analyzed (MRN; see Figure 5, frontal cortex and olfactory nuclei, dorsal or ventral hippocampus – not shown) were affected by the lesion.

Large reductions in 5-HT and 5-HIAA levels in the dissected regions of brains from 5,7 DHT-treated rats compared with those treated with vehicle, demonstrated that

the majority of the 5-HT system had been destroyed by the neurotoxin (Table 2). In addition, the fact that noradrenaline levels were not different between control and lesioned rats demonstrated the selectivity of the lesion for the 5-HT neurons.

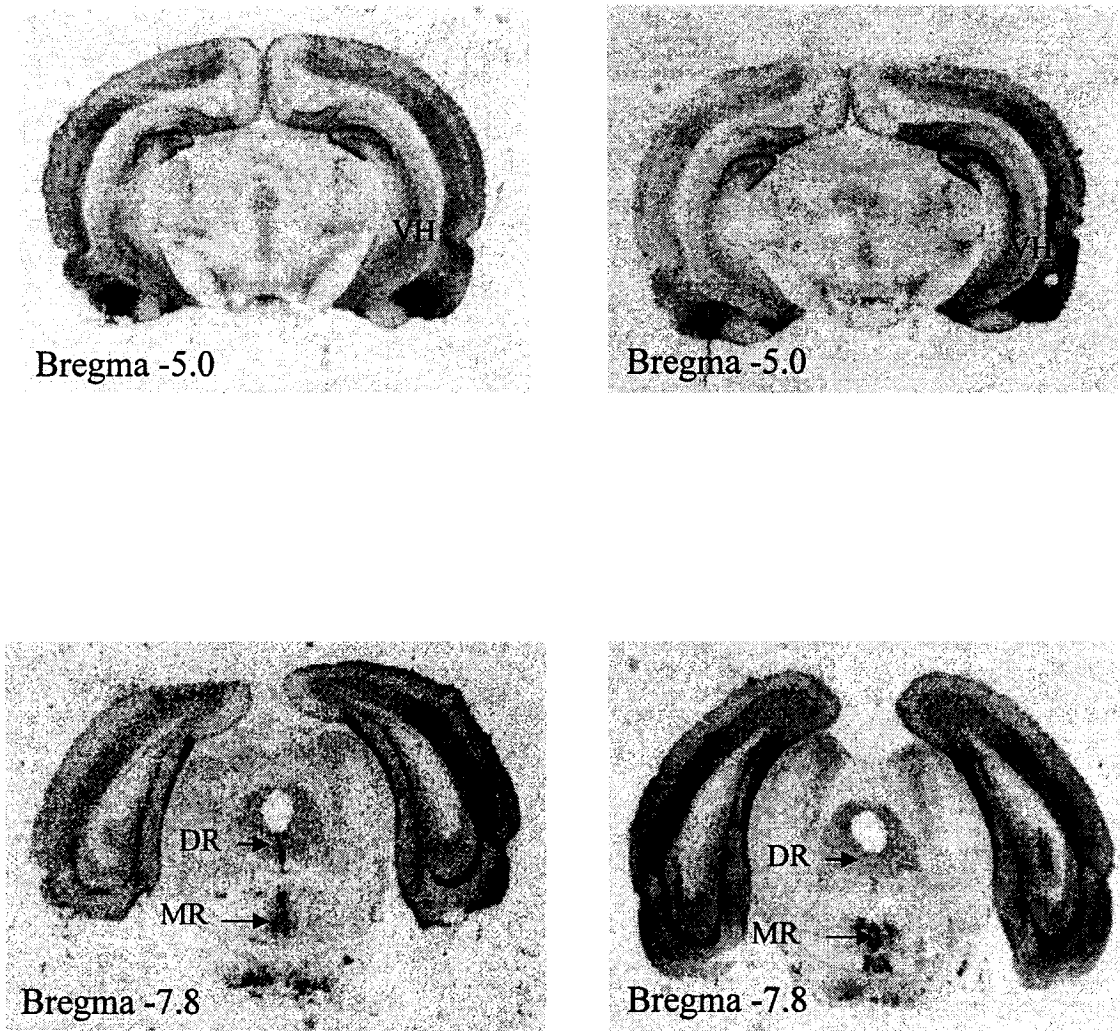


Figure 5 Representative autoradiographs depicting coronal sections of rat brain taken from rats injected i.c.v. either with 5,7 DHT or vehicle prior to sacrifice. For ease of comparison sections on the left are from vehicle treated rats and sections on the right are from rats treated with 5,7 DHT. Abbreviations: DR (dorsal raphé), MR (median raphé); VH (ventral hippocampal region)

Table 2 Effects of 5,7 DHT lesioning on bindings of ^{125}I-BB to BB_1 binding sites in the frontal cortex, striatum and dorsal and ventral hippocampus

	5-HT		5-HIAA		Norepinephrine	
	Vehicle	**5,7 DHT**	**Vehicle**	**5,7 DHT**	**Vehicle**	**5,7 DHT**
Frontal Cortex	0.94 ±0.02	0.10* ±0.03	0.28 ±0.01	0.04* ±0.01	0.39 ±0.07	0.29 ±0.04
Striatum	0.52 ±0.03	0.10* ±0.03	0.22 ±0.02	0.05* ±0.01	0.22 ±0.02	0.26 ±0.02
Dorsal Hipp.	0.40 ±0.04	0.04* ±0.009	0.26 ±0.02	0.02* ±0.009	0.24 ±0.02	0.20 ±0.02
Ventral Hipp.	0.61 ±0.09	0.03* ±0.008	0.31 ±0.009	0.02* ±0.009	0.50 ±0.10	0.43 ±0.04

Mean (±SEM) levels of 5-HT, 5-HIAA and NA (ng/mg wet weight tissue) in frontal cortex, striatum, dorsal and ventral hippocampus of rats, two weeks after i.c.v. administration (5 μl volume) of either vehicle (0.2% ascorbic acid; n=3) or 5,7-DHT (150 μg; n=3).
* $p < 0.01$ Students T-test

The effects of systemically administered PD 176252 on release of 5-HT at the ventral hippocampus

The average basal (pre-injection) level of 5-HT in dialysates recovered from the ventral hippocampus of all rats used was 14.2 ± 1.4 fmols/20 min (3 basal samples from each of 12 rats). Intraperitoneal injection of vehicle caused a transient increase in levels of 5-HT of around 10 - 15% of basal. However in rats injected with PD 176252 (10 mg/kg) levels of 5-HT were actually reduced such that 5-HT output was around 60% of basal levels (see Figure 6). The effect was maximal approximately 100 min post injection and remained significantly decreased for a further 80 min. The effect appeared not to be due to local inhibition of 5-HT in the hippocampus since PD 176252 (10 μM) did not alter 5-HT levels when perfused directly into the ventral hippocampus by reverse dialysis (≤ 10% reduction in basal levels over 60 min; n=2).

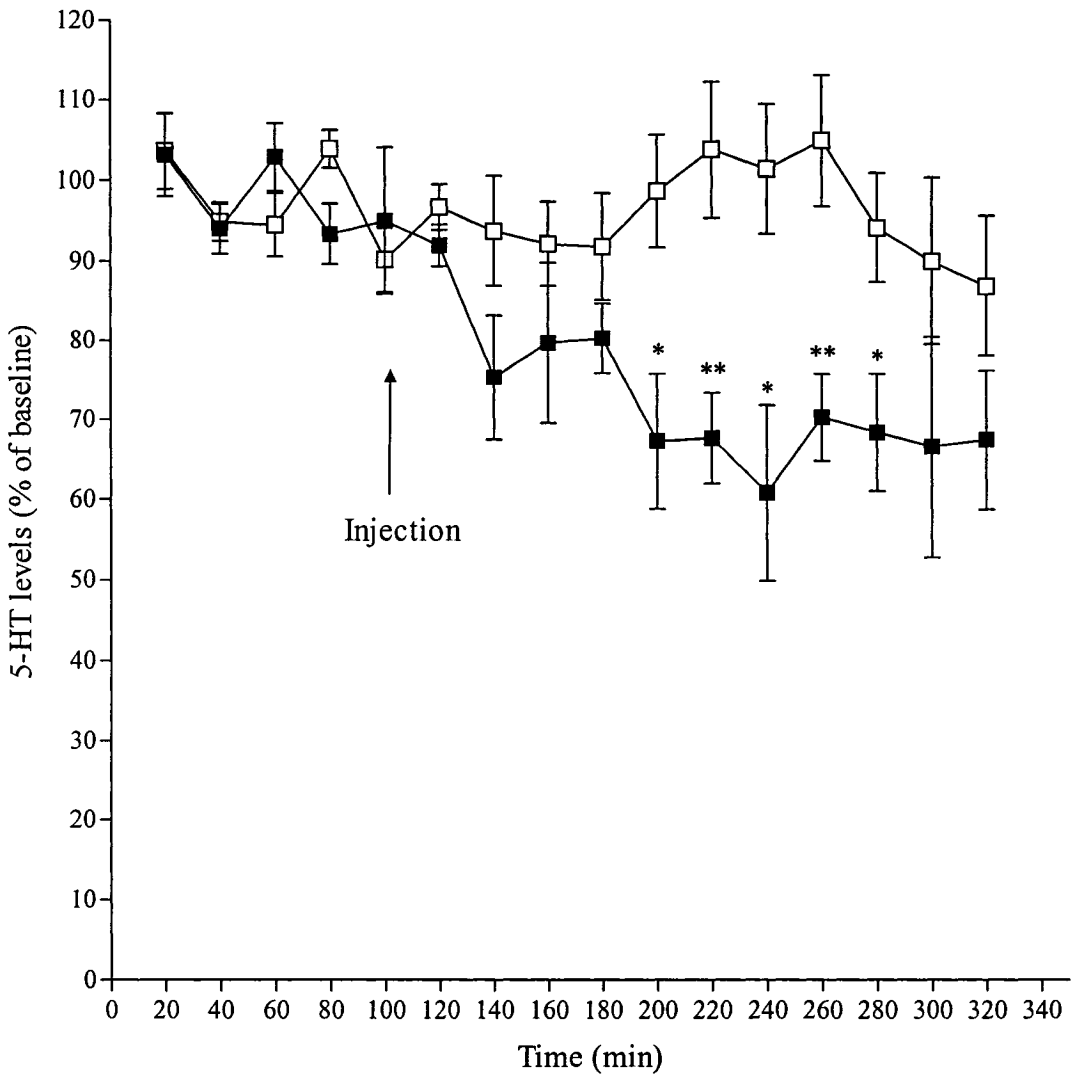


Figure 6 Effect of systemically administered PD 176252 (10 mg/kg) on extracellular levels of 5-HT as measured in the ventral hippocampus of the freely moving rat by in-vivo microdialysis. Open scquares represents saline injected animals; solid squares represent drug-treated animals (PD 176252)
* $p < 0.05$ and ** $p < 0.01$ Student's t-test vs data point t = 180 min
* $p < 0.05$ from time-matched sample post vehicle injection

Effects of intra-DRN PD 176252, NMB-30 and GRP_{1-27} on release of 5-HT at the ventral hippocampus

The average basal (pre-injection) level of 5-HT in dialysates recovered from the ventral hippocampus of all rats used was 9.1 fmols/20 min (3 basal samples from each of 24 rats). Levels of 5-HT post-vehicle, PD 176252 and NMB-30 injection are depicted in figure 7. For the effects of intra-DRN infusion of PD 176252, the ANOVA revealed a significant treatment effect on interstitial levels of 5-HT ($F(1,84) = 48.85$; $p < 0.0001$). Post hoc analyses revealed that interstitial levels of 5-HT following PD 176252 injection were significantly reduced as compared to 5-HT levels following infusion of vehicle ($p<0.05$). Similar to the findings observed with systemic injection of PD 176252, intra-DRN infusion of the antagonist resulted in a reduction in 5-HT output to around 60% of basal levels, an effect that was maximal 60 min post injection. For the effects of intra-DRN infusion of NMB-30, ANOVA revealed a significant Treatment x Time interaction ($F(1,84) = 2.58$; $p < 0.04$). Post hoc analyses revealed that the interaction was attributable to significantly higher levels of 5-HT following infusion of NMB as compared to the levels following vehicle injection ($p<0.05$). In contrast to the effects of PD 176252, intra-DRN infusion of NMB-30 resulted in a rapid and pronounced (around 200% of basal levels) increase in 5-HT output which peaked during the first 20 min post injection and remained elevated over the next 20 min. Intra-DRN infusion of GRP was without effect on interstitial levels of 5-HT at the ventral hippocampus (data not shown).

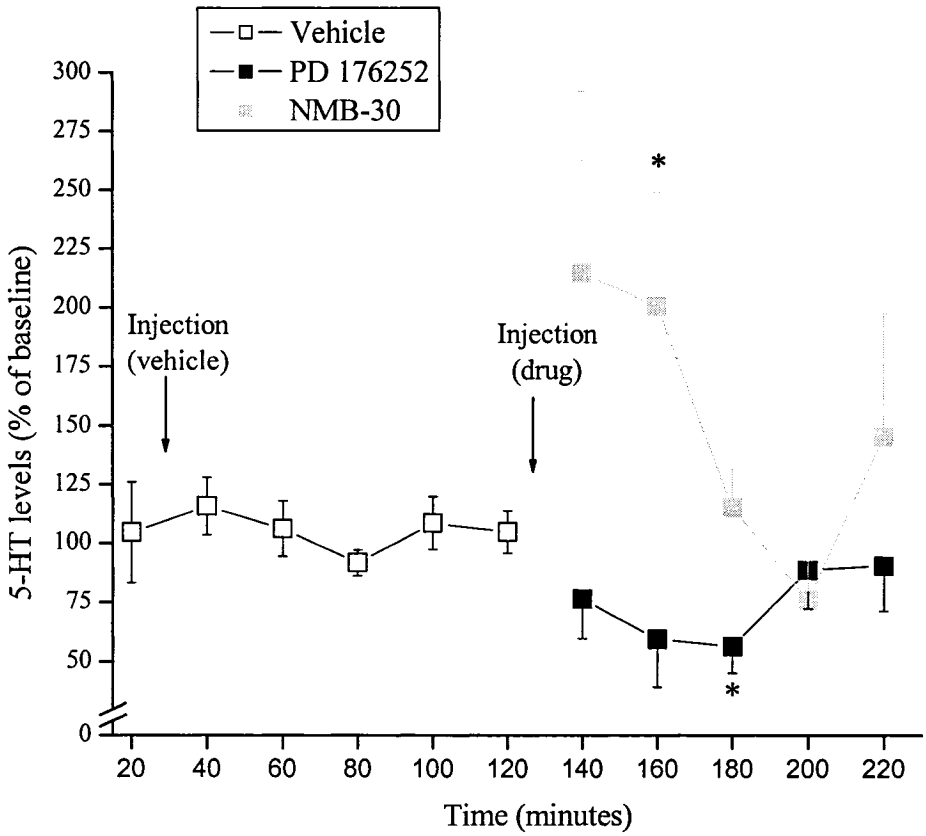


Figure 7 Effects of intra-DRN infusion of vehicle, PD 176252 (200 ng/0.5 µl) or NMB-30 (10 nmoles/0.5 µl) on interstitial levels of 5-HT as measured in the ventral hippocampus in anesthetized rats using in vivo microdialysis.
* significantly different from vehicle injection at $p < 0.05$

Discussion

Although BB was initially thought of as a satiety peptide [230, 300], it appears that like CRH, GABA/benzodiazepine, and CCK, BB-like peptides, and particularly BB's mammalian counterparts GRP and NMB, represent a class of neuromodulators that contribute to the integration and/or mediation of stress/anxiety responses.[297] In this regard, BB-related peptides and their receptors are highly concentrated in brain regions often associated with the stress response, including hypothalamic nuclei (e.g., parvocellular PVN, suprachiasmatic, supraoptic, preoptic and mammilary nuclei) [24, 497] as well as the nucleus of the solitary tract (NTS) and the parabrachial nucleus, the bed nucleus of the stria terminalis (BNST), amygdaloid nuclei, and the hippocampus.[72, 326, 356, 445, 509] Likewise, it has been reported that stressors were associated with alterations of endogenous levels of BLPs at the hypothalamus and medulla, as was the density of BB binding sites at the NTS, arcuate nucleus and PVN.[211] We previously observed that at limbic sites the changes of BLPs in relation to stressors were less notable than at hypothalamic structures [302], although *in vivo* analyses revealed that stressors provoked the release of BLPs from the central amygdala (CeA).[298, 302] As expected, like stressors, BB administration increased HPA functioning, eliciting release of median eminence CRH, pituitary ACTH and adrenal corticosterone, and further, the variations of ACTH elicited by BB could be attenuated by the CRH antagonist, α-helical CRH.[212, 213]

Despite the data indicating BB involvement in the central neurochemical stress response, limited data are available concerning the behavioral effects of BB manipulations. Distinct neuronal circuits may be activated by diverse stressors [298], and aspects of the stress

response (fear vs anxiety) may involve different brain structures [249, 255] and evoke distinct anxiety characteristics.[298] Thus, the present investigation of BB manipulations comprised utilization of several tests of anxiety involving ethologically relevant challenges, thereby avoiding confounding effects of painful stimuli.

As predicted, BB-like peptides influenced anxiety responses across the behavioural tests. In particular, central administration of BB dose-dependently enhanced locomotor activity in a familiar situation, but reduced exploration in a novel (presumably stressful) environment.[194, 296, 440] Furthermore, antagonism of BB_1 activity attenuated anxiety across a range of situations, including those that likely involved prewired circuitry (vocalization to removal of the dam, social interaction, approach to a snack in a novel environment), and those that involve conditioned fear (fear potentiated startle). In the latter instance, the increased reactivity was restricted to the fear potentiated response as startle intensity was unaffected in the absence of the fear cues. These findings clearly support a role for BB processes in anxiety, but this should not be misconstrued to suggest that BB-like peptides are necessarily involved in all anxiety-related behavioural outcomes. It remains to be established whether BB_1 manipulations affect anxiety in other common paradigms (i.e., elevated plus maze, open-field emergence, conflict situations) as well as whether BB differentially influences situation- or cue-specific anxiety versus generalized anxiety, or influences behaviors elicited by neurogenic stressors. However, it might be expected that BB administration elicits no effect on behaviour in the plus-maze since other anxiolytic compounds i.e. 5-HT_{1A} agonists acting via the DRN have been found to be inactive in such

anxiety paradigms.[182] Anxiety under these varied conditions may involve different processes, some of which may be unrelated to BB mechanisms.

It was shown that the BB_1 and BB_2 receptors are differentially distributed across brain regions.[24, 236] For instance, whereas the PVN, lateral amygdalar (LA), hippocampal and suprachiasmatic (SCN) nuclei primarily express the BB_2 receptor subtype, the DRN appears to have a preponderance of the BB_1 receptor subtype.[24, 236, 343, 373, 498] Indeed, intracellular recording studies revealed that the majority of DRN neurons were selectively sensitive to BB_1 (and not the BB_2) ligands.[373] It would appear from the results of the present investigation that these receptors are located on 5-HT neurons, as lesioning of the 5-HT system (using 5,7-DHT) markedly reduced BB_1 receptor binding in DRN (cell body/dendrite region). Furthermore, microinjection of BB_1 agonist (NMB) at the DRN, but not the BB_2 (GRP) agonist, provoked the release of 5-HT at the ventral hippocampus (terminal region). Conversely, microinfusion of the BB_1/BB_2 antagonist caused a diminution of extracellular 5-HT levels at the ventral hippocampus. Thus, it seems that at least a portion of the BB_1 receptors in the DRN are located on the 5-HT cell bodies of the neurons that project to the ventral hippocampus.

As already alluded to, anxiety has been associated with monoamine functioning, and particularly 5-HT and the $5\text{-}HT_{1A}$ receptors.[151, 260, 307] Consistent with this view, in the present study localized infusion of PD 176252 into the DRN which attenuated anxiety, provoked a marked reduction of 5-HT release (60% of baseline release) from the ventral hippocampus, and microinjection of the BB_1 agonist, NMB (but not $GRP_{1\text{-}27}$), stimulated the

release of 5-HT at this site. Importantly, the reduced 5-HT release was not engendered by the infusion of PD 176252 directly into the hippocampus, suggesting that the 5-HT variations were not related to the local hippocampal inhibition of 5-HT neurons. Interestingly, like our observations with NMB and its antagonist, it was reported that elevated 5-HT expression at the DRN was evident in mice with targeted disruption of the BB_1 receptor gene and the restraint-enhanced 5-HT expression noted in the wild-type mice was not evident in mice lacking the BB_1 receptor.[522] These data support the notion that these receptors are important in fine tuning subsets of 5-HT neurons. In this respect $5\text{-}HT_{1A}$-receptor gene expression appears to be downregulated in BB_1-deficient mice, although these analyses involved whole brain analyses.[520]

Unlike BB_1 receptors, it seems that in the lateral amygdala, BB_2 receptors are localized on GABAergic interneurons.[445] Moreover, manipulation of this peptidergic receptor subtype not only influenced long-term potentiation, but also affected the acquisition and performance of a conditioned fear response. Thus, while the present investigation did not support BB_2 involvement in anxiety processes, it is premature to dismiss a role for this receptor subtype within the lateral amygdala in affecting fear and/or anxiety.[445] Inasmuch as GABA subunits have been implicated in anxiety processes, and inter-relations exist between GABA and 5-HT functioning [446], the possibility exists that BB_2 manipulations indirectly influence 5-HT functioning and anxiety through actions on GABAergic processes. This contrasts with the effects of BB_1 receptors present on the DRN, as their manipulation appears to directly regulate ventral hippocampal 5-HT functioning and its effect on anxiety.

We have observed that several physiological or psychological stimuli that activate endogenous BB-peptidergic systems, also activate the CRH system(s) [298, 302], and that CRH antagonists can attenuate pharmacological effects of BB-like peptides.[213, 378] Inasmuch as CRH is thought to subserve fear and anxiety responses, it may well be that the BB-like peptides act through CRH to induce anxiety, and that CRH itself may affect anxiety through actions on 5-HT neuronal functioning. In this regard, it was reported that central CRH administration dose dependently increased extracellular 5-HT levels, whereas the CRH receptor antagonist (D-phe-CRH) reduced hippocampal 5-HT levels.[263] Also, enhanced hippocampal serotonergic neurotransmission was observed in CRH_1 receptor deficient mice.[366] Although such changes were not evident using a pharmacological approach comprising chronic administration of a CRH receptor antagonist.[350]

Taken together, it appears that the antagonism of NMB receptors by a compound that affects both the BB_1 and BB_2 receptor subtypes is effective as an anxiolytic in several different behavioral paradigms. Moreover, consistent with the view that 5-HT processes involving the DRN may contribute to anxiety [12, 181, 182], social interaction was increased by intra-DRN administration of the BB_1 antagonist and this effect was as pronounced as that induced by the 5-HT agonist 8-OH DPAT. It is posited that this effect of PD 176252 may occur via an action in the DRN to reduce 5-HT levels in areas such as the hippocampus, which are innervated by the serotonergic fibres originating in the DRN. Further studies may reveal whether the treatments of anxiety through compounds of this class will provide an alternative to the benzodiazepines. However, it may be important to employ agents that

differentially affect BB_1 and BB_2 receptors given that BB_2 knockout mice exhibited an exaggerated fear conditioned response.[445] In effect, it may be that the two receptor subtypes play different roles in mediating anxiety (or different types of anxiety), and the development of pharmacotherapeutic intervention strategies for various anxiety-related disorders ought to consider their relative contributions.

Preface to Chapter V

Although the use of relatively specific receptor antagonists in previous experiments have been helpful in investigating the intrinsic role of GRP and NMB in anxiety and fear, the field has been deficient in offering antagonists with high specificity and solubility. Another technique that circumvents such methodological predicaments, offering higher specificity to each of the different receptor subtypes, is the receptor knockdown strategy using antisense oligodeoxynucleotides. Therefore, Chapter V was an attempt to use this technique to further delineate the specific roles played by GRP and NMB in anxiety-related responses using different animal models.

Chapter V

Effect of bombesin-like peptide receptor knockdown using antisense oligonucleotides sequences directed against BB_1 or BB_2 receptors on anxiety-like behavior

Abstract

Bombesin-like peptides (BLPs) are thought to be involved in behavioral, endocrine and autonomic regulation of the stress response. Four BLP receptor subtypes have been characterized to date, and are identified as BB_1, BB_2, BB_3 and BB_4. Only two have been found to be expressed in the mammalian brain, namely BB_1 or NMB-preferring receptors and BB_2 or GRP-preferring receptors. The anatomical distribution of the BB_1 and BB_2 receptors is distinct throughout the brain and the gastrointestinal tract, suggesting that they might play a differential role in the mediation of the stress response. The specific functional role of BB_1 and BB_2 receptors is not well understood. It can be presumed that blockade of these receptor subtypes might shed some light on the intrinsic role(s) of these receptors. However, at the present time, highly selective and specific antagonists for the various receptor subtypes are not available. Therefore, an antisense oligodeoxynucleotide (ODN) approach was used to study the inherent role of BB_1 and BB_2 receptors in stress and anxiety. An ODN directed against the BB_1 and the BB_2 receptor mRNA was continuously infused over 7 days via a cannula aimed at the third ventricle. Behaviorally, animals administered with BB_2 ODN displayed higher lick frequencies in the punished conflict drinking test, increased frequencies in exploratory behavior in a novel environment as well as decreased grooming and scratching. Treatment with BB_2 ODN was also found to attenuate the bombesin (BB)-induced increases in blood glucose and corticosterone levels. Functional deletion of the BB_1 (NMB) receptors resulted in increased exploratory behavior in a novel environment. However, behavior of animals in the elevated-plus maze and locomotor activity in all treatment groups remained unaltered. Therefore, it appears that the BB_2 receptor subtype

might exert a more prominent and direct role in grooming, conflict drinking as well as glucose and corticosterone regulation than the BB_1 receptors. Taken together, these data support the contention that BB_1 and BB_2 receptors might be differentially involved in stress and anxiety-related behaviors.

Introduction

Two analogues belonging to the bombesin (BB) family of peptides have been identified in mammals to date, and have been labeled gastrin-releasing peptides (GRP), due to its effects on gastric tissues and the intestine [289] and neuromedin B (NMB).[312] These peptides and their receptors, GRP-preferring or BB_2 receptors and NMB-preferring or BB_1 receptors respectively, have distinct distribution patterns yet some co-localization at certain brain regions does exist.[24, 72, 313 325, 340, 343, 418, 497] Studies using immunohistochemical techniques have detected both peptides on neurons located in the dentate gyrus and the nucleus ambiguous, the nucleus of the solitary tract, the parabrachial nucleus, the bed nucleus of the stria terminalis as well as the preoptic and the supramammillary nuclei of the hypothalamus.[24, 72, 326, 356, 497] Radioimmunoassay and in situ hybridization studies have identified the presence of GRP in hippocampal areas (CA1, CA2, CA3, dentate gyrus), various nuclei of the amygdala (medial, lateral, basolateral, basomedial and cortical) as well as the brain stem.[356, 497] Conversely, immunostaining detected traces of NMB in olfactory regions, the central nucleus of the amygdala and a few thalamic nuclei, such as the lateral habenula and the reticular nucleus.[340, 343, 497] Similarly, BB_2 receptors are highly expressed in hypothalamic nuclei, including the paraventricular, anterior and arcuate nuclei and the median eminence [24, 236, 323, 325, 369]; whereas BB_1 receptors are found in greater quantity in the olfactory bulb, thalamic nuclei, dentate gyrus and dorsal raphé nucleus.[24, 343, 498] The disparity in their expression pattern suggests that these two peptidergic systems may play differential roles in the central nervous system.

One area of research which has gained increased attention in recent years is the role of bombesin-like peptides (BLPs) in stress-related responses.[297] While there is substantial evidence demonstrating that BB can affect the processes mediating and/or modulating the stress response [19, 211, 213, 274, 297], the specific roles of its mammalian counterparts, namely GRP and NMB, in stress-related responses are not well characterized. In this context, we have found that stressor exposure can provoke the release of both GRP and NMB at certain brain sites, including the anterior pituitary and central nucleus of the amygdala; supporting the notion that these peptides may play a physiological role in the modulation of the stress response.[302] There is some evidence that central GRP injection activates the HPA axis however, it is unknown as to whether central NMB infusion has the same effect.[19, 139, 140] The production of mutant mice lacking either BB_1 or BB_2 receptors has provided some insights into the roles of NMB and GRP in stress-related responses. For example, mice lacking BB_2 receptors showed no alterations in anxiety-type behavior as measured by the light dark box and elevated plus maze.[517] In contrast, Shumyatsky et al. [445] recently reported that BB_2 receptor deficient mice displayed a greater and more persistent long term memory of fear. Similarly, inconsistencies have also been reported in studies with BB_1 receptor deficient mice. Mice lacking BB_1 receptors show a dysregulated response to stress as indicated by enhanced plasma levels of corticosterone following restraint stress exposure.[518] In terms of anxiety-related behavior, mice lacking BB_1 receptors showed no differences as compared to wild type mice in the light dark box and elevated plus maze.[517, 522] In contrast, altered emotionality in another anxiety-related paradigm has been observed as the BB_1 knockout mice showed decreased marble burying behavior.[520] Results with

mutant mice should however be interpreted with caution as life long receptor deficiencies may be accompanied by compensatory mechanisms which may in fact mask the true function of these receptors.[350]

Aside from the use of genetic models to further differentiate the specific roles of these ligands and their receptors in the mediation of stress and/or anxiety type responses, another approach is the use of pharmacological manipulations using specific receptor antagonists to achieve a functional blockade of BB_1 or BB_2 receptors. However, this approach has been fraught with the lack of antagonists with high affinity, specificity and/or solubility. It is noteworthy that most studies exploring the role of BLPs in anxiety and stress have done so using currently available receptor antagonists. These antagonists have been shown to have a certain degree of cross-reactivity to the other BLP receptor subtypes as well as other family of peptides, such as urotensin II receptors [180], and are therefore not sufficiently specific. An adequate assessment of the contribution of each of the BLP receptor subtypes in anxiety and stress-related behaviors requires the use of antagonists that will specifically bind to one receptor subtype without affecting other types of receptors.

Another technique that offers a high degree of specificity to each of the different receptors is the receptor knockdown strategy.[6] This is done using antisense deoxyoligonucleotides which could be a very effective tool to further differentiate the specific role of GRP and NMB in stress and/or anxiety. To this end, we designed and synthesized various specific antisense oligodeoxynucleotide probes raised against the

BB_2 and BB_1 receptor mRNAs. Thus, in the present investigation we wanted to characterize the specific roles played by GRP and NMB (by altering the expression of their respective receptors) in the endocrine and behavioral responses to various stress/anxiety paradigms.

Material and methods:

Subject

Male Sprague-Dawley rats (weighing between 350 to 400 g) were individually housed in standard clear plastic cages placed in a temperature (23°C) and humidity (60%) controlled room. Animals were maintained on a 12 hr light/dark cycle (lights on at 07:00) and had *ad libitum* access to Purina rat chow and tap water. All experimental procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the research Ethics committee of the University of Ottawa. Rats were randomly assigned to a treatment group and the experimenter was blind.

Antisense oligodeoxynucleotides (ODN)

A 21-mer antisense ODNs was designed corresponding to the rat BB_1 receptor mRNA (5'- TGG GGG GCA TGG TGT CCT TTC- 3') together with a second antisense ODN corresponding to the BB_2 receptor mRNA (5'- ACA GGG GCG CAT GTA ACC AGC- 3'). A corresponding mismatch control sequence was also designed for each of the receptor antisense ODNs (NMB-R: 5'- TGG GCG GCT TCG TGA CCT TTC- 3'; GRP-R: ACC CGG CCG CAT GTT ACC AGC). Each sequence was checked for homology with all other known rat mRNA using Genbank sequence database. No homology was

found for any of the sequences used. The ODNs were custom synthesized and desalted (Sigma-Genosys, Houston, Texas, USA). All s-ODNs were comprised of a phosphodiester backbone in which the first and the last three bases were modified by inclusion of a phosphothioate to decrease degradation processes by endogenous nucleases, and to minimize potentially toxic effects. However, phosphothioate oligonucleotides have been reported to have some toxicity which can potentially confound the result.[367] Thus the sequences used in the current study were chemically modified to minimize such toxic effects, by adding the phosphothioate only on the first and last three nucleotides of the sequence.

Surgery

Rats were anesthetized using sodium pentobarbital, 60 mg/kg (i.p.) and stereotaxically implanted with a 5.5 mm 22-gauge connector/guide cannula (Plastics One, Roanoke, VA, USA) containing a removable obturator aimed at the third ventricle. The coordinates were A/P -4.4 mm; DV -4.4 mm; L/M 0 and were obtained from Paxinos and Watson.[361] The infusing cannula was fixed to the skull with four stainless steel screws and dental cement (Patterson Dental, Quebec, Canada). The cannula was equipped with a connector shaft allowing the use of an osmotic minipump while at the same time permitting acute injection. Two days following cannula implantation, rats were again anesthetized using halothane and an osmotic minipump was subcutaneously positioned between the shoulder blades of the animal. The pump was connected to the connector shaft of the cannula via a 35-cm piece of vinyl tubing that was coiled and placed in the

cavity. The wound was closed with sutures, and the rats allowed 2 recovery days before testing was initiated.

ODN dilution and infusion

On the day before implantation, the osmotic minipumps (Alzet model 2002; Durect Corporation, CA, USA) were filled with saline (0.9%) and primed overnight in a 37°C incubating chamber. On the day of surgery, the vinyl tubing was first loaded with 12 μl of saline followed by 95 μl of ODN solution, and the remainder of the tubing was filled with saline. A small air bubble separated each solution. The ODNs were dissolved in vehicle solution (0.9% saline) to a final concentration of 10 μg/μl and were infused at a rate of 0.5 μl/h. Thus, animals received a one-day infusion of saline, followed by 7 days of ODNs infusion. Behavioral testing started on day 2 of ODNs infusion. The groups were as follow: 1) BB_1 ODN group, 2) BB_2 ODN group, 3) BB_1 and BB_2 mismatched ODN group and 4) vehicle (0.9% saline solution) infusion group.

Behavioral testing:

Open Field

On Day 2 of ODN infusion, rats were tested in the open field prior to the elevated plus-maze test. The open field testing arena consisted of a light gray plastic box (57 x 57 cm; 30 cm high walls) equally divided into 36 squares. Each rat was placed in the center of the arena and its behavior was monitored for 5 min via a video camera mounted above the field. The total distance (number of squares crossed by the animals) traversed during the 5 min period was recorded. Before introducing the next animal, the arena was

thoroughly cleaned using a 70% ethanol solution. For acclimatization, animals were transferred to the testing room 1 hr prior to testing.

Elevated-plus maze (EPM)

Immediately following open field test, each rat was tested on the EPM, a test validated for the assessment of the responses to anxiogenic and/or anxiolytic compounds.[364] The apparatus consists of four arms positioned in the shape of a plus (+), elevated 50 cm from the floor. Two of the opposing arms were surrounded by walls (40 cm high), and the two other opposing arms were open planks (a relatively anxiogenic portion of the maze). All four arms connect in the middle by a 10 x 10 cm square platform. This test is based on the conflicting instinct of the animal to explore a new environment and its inherent fear of open "vulnerable" spaces.[364] To initiate the test, rats were placed onto the open central area facing one of the closed arms. The behavior was recorded for 5 min via a video camera system mounted above the maze and scored by a blind experimenter. The following behaviors were scored: 1) occurrence of entries in open arms (entry was counted only when all four paws were on an open arm), 2) time spent on the open arms, 3) occurrence of entries in the closed arms, 4) time spent in closed arms, 5) number of unprotected head dips (protruding of the head over the edge of an open arm), as well as 6) number of protected head dips (protruding of the head over the edge of the maze while the body was within the closed arm).

Vogel Test

Rats were tested in the Vogel Test on Days 3, 4 and 5 of ODN infusion. The testing chamber (Coulbourn Instruments) consisted of a 31 x 25 x 30 cm rectangular box with front and back walls made of Plexiglas and two side walls made of removable metal plates. The grid floor of the chamber was comprised of 16 steel rods (2 mm diameter) spaced 3 cm apart. The spouts of the drinking bottles protruded within the cage and were wired to a Coulbourn shocker, capable of delivering a mild shock every 5^{th} lick. When a subject licks the spout the tongue breaks a beam allowing the computer to quantify the number of licks emitted by the test subject.

Training: On the first day of Vogel test, rats were placed in the test cages and allowed to drink freely from the spout for 10 min, to familiarize them to the cage and water delivery system. Rats were then water deprived for 24 hrs, placed again in the testing cages for a second session, and given free access to water. The purpose of these 10-min no shock sessions were to allow animals to acclimate to the new environment and schedule of restricted access to water. Rats were again deprived of water for an additional 24 hrs. Testing: On the last day of Vogel test, subjects were reintroduced to the testing cages, but this time, licking of the drinking spout was simultaneously rewarded by water (every lick) and punished by an electric shock (0.5 mA; given every 5^{th} lick) delivered through the spout. The number of licks was recorded for each animal.

Behavioral recording

On day 7 of ODN treatment, animals were injected with BB (0.5 μg/3 μl/60 s) and the frequency of each of the following behaviors was recorded over a 30-min test period: grooming, scratching, digging, exploring, sleeping, sitting, eating, and drinking. The occurrence of each behavior during each 5 s time period was recorded, for the full 30-min period, using a computerized behavioral data-logging program.

Glucose and corticosterone

The blood samples for glucose and corticosterone measurement were collected at 0, 15, 30 and 60 min following BB infusion (0.5 μg/3 μl/60 s) by lancing the rat's tail close to the tip with a sterile surgical scalpel blade (size 10) and then wiping the first droplet of blood away. Subsequent blood droplets were collected (2 for each time point). One droplet was collected with a glucose strip and immediately analyzed for glucose content using a portable glucose meter Elite™ (Ames, Miles, Canada). A second droplet was blotted onto preprinted circles on S&S filter paper (Schleicher & Schuell, Mandel Scientific), allowed to dry at room temperature and were stored at –20°C until analysis for corticosterone levels. Blood was eluted from the filter paper by placing one 2.5 mm punch of filter paper in a 12 x 75 culture tube containing 100 μl of Dulbeco's phosphate buffered saline (Sigma, USA) and then shaking the tubes on an automatic shaker (50 rpm) for 1 hr at room temperature. Tubes are stored overnight at 4°C. On the following day, tubes are again agitated on the automatic shaker for 1 hr at room temperature and corticosterone levels in the eluted samples were determined using a commercial RIA kit (ICN Pharmaceuticals, CA).

Histology

At the end of the experiment, animals were anesthetized with halothane, decapitated and the brains were carefully removed and stored at -80°C. Subsequently, brains were sectioned to verify for placements. Only animals with correct placements were included in the analyses.

Statistical analysis

Data for the open field, EPM, Vogel test and behavioral occurrences were analyzed using one-way analysis of variance (ANOVA) with ODN treatment as a between subjects factor. Changes in glucose and corticosterone levels were analyzed using a mixed measures ANOVA in which the ODN treatment was considered the bewteen subjects factor and time as the within subjects factor. Post hoc comparisons were conducted using Newman-Keuls multiple comparison tests. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of BB_1, BB_2 or MM ODN treatment on behavior in the open field

No significant differences were found in the open field paradigm (figure 1). None of the ODN sequences used affected the locomotor activity of the animals in the open field.

Effects of BB_1, BB_2 or MM ODN treatment on behavior in the elevated-plus maze

The ANOVAs revealed no significant differences in the behaviors observed (time spent in the open and closed arms as well as occurrence of open and closed arm entries) in the EPM. There was a trend towards an increased time spent in the open arms with infusion of BB_2 ODN, although this difference did not reach statistical significance.

Effects of BB_1, BB_2 or MM ODN treatment on behavior in the Vogel test

Figure 2 shows the number of intermittently punished licks emitted by the animals. The ANOVAs revealed a significant effect of ODN treatment on the number of punished licks accepted by the rat ($F(3, 30) = 3.999$; $p < 0.05$). Post-hoc analyses revealed a significant increase in the total number of licks in the paradigm in animals treated with BB_2 ODN antisense, but not in those treated with BB_1 ODN antisense.

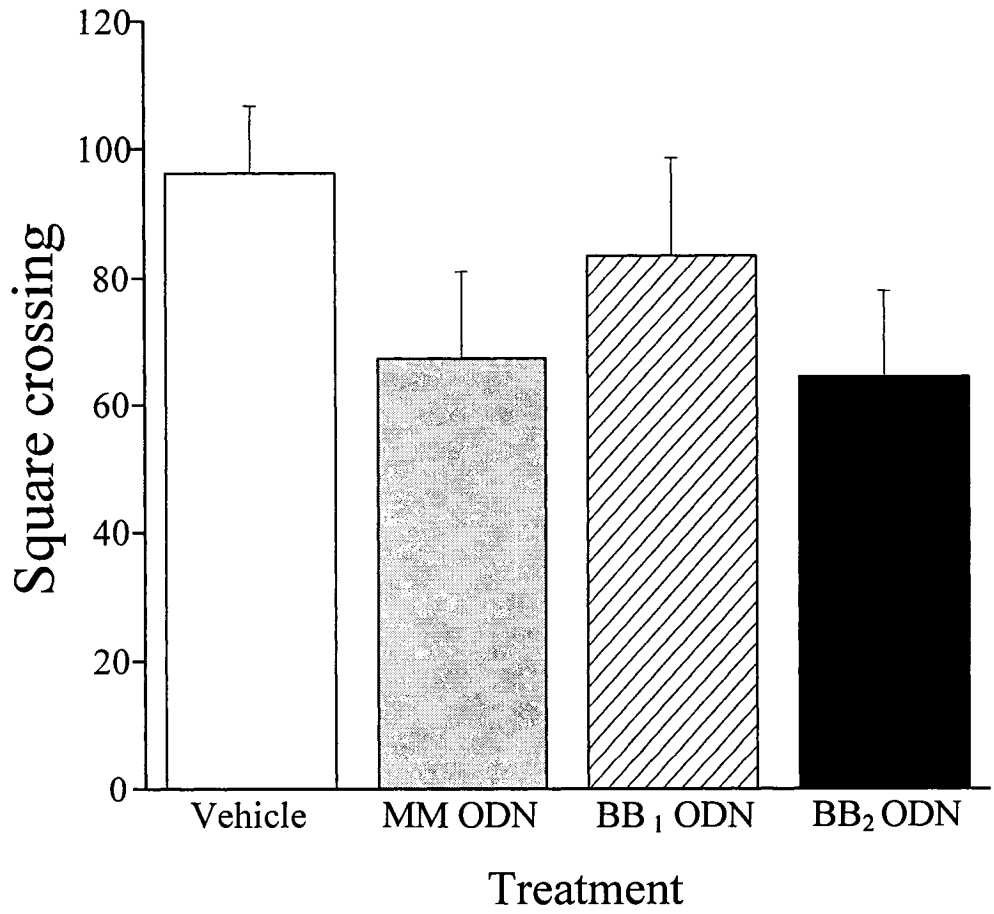


Figure 1 Locomotor activity (mean number of squares crossed in 300 sec ± S.E.M) in an open field arena of animals following central infusion of either vehicle (saline) solution (open columns), mismatch (MM) ODN (gray column), BB_1 ODN (hatched column) or BB_2 ODN (solid column).

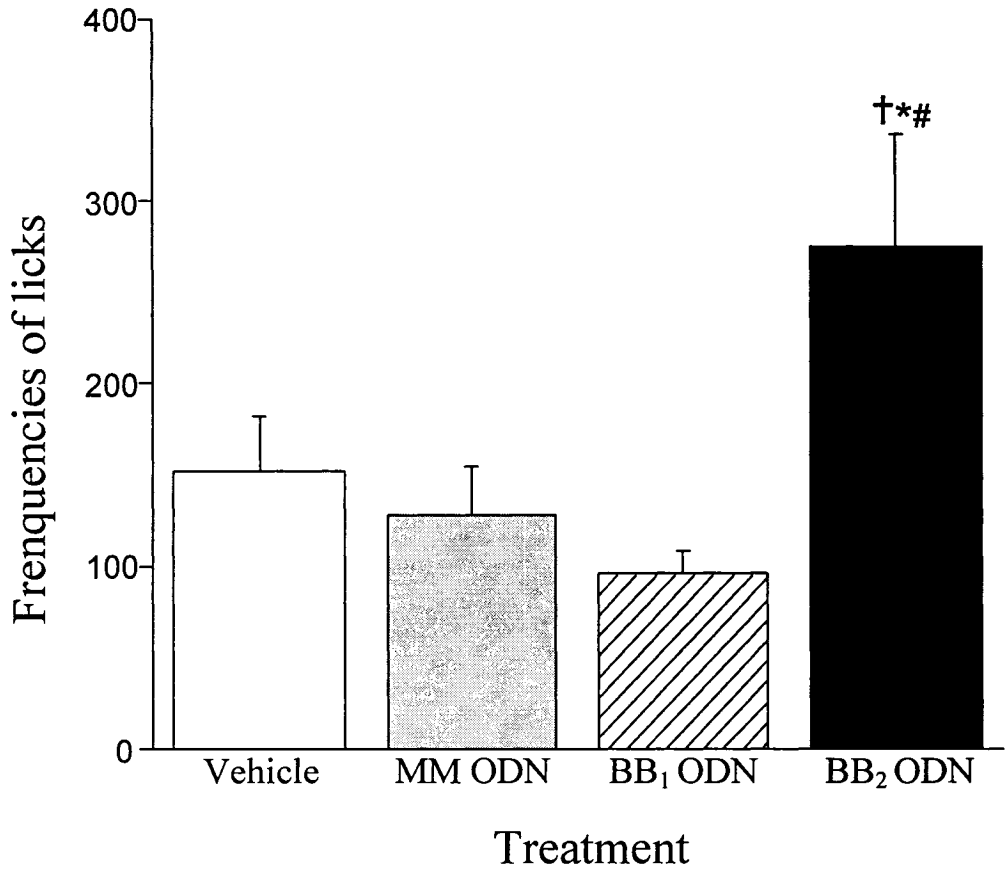


Figure 2 Number of licks (mean ± S.E.M.) in the punished drinking conflict test (Vogel) emitted by animals following central infusion of either vehicle (saline) solution (open column), mismatch (MM) ODN (gray column), BB_1 ODN (hatched column) or BB_2 ODN (solid column).

* Significantly different from vehicle control treatment group at $p < 0.05$

† Significantly different from MM control treatment group at $p < 0.05$

Significantly different from BB_1 ODN treatment group at $p < 0.05$

Effects of BB_1, BB_2 or MM ODN treatment on behavior

As expected, oligonucleotide treatment elicited changes in the behavior of the animals following central BB injection. ANOVA revealed a significant treatment effect for grooming and scratching occurrences ($F(3, 31) = 3.998$; $p < 0.05$)(see Figure 3a). Newmann-Keuls multiple comparisons further revealed that in animals treated with BB_2 ODN, the frequency of grooming and scratching behaviors was significantly decreased as compared to both the BB_1 ODN group and the vehicle group. Additionally, ANOVA showed a treatment effect on exploratory behavior ($F(3, 26) = 15.495$; $p < 0.05$)(see Figure 3b). Student-Newman-Keuls multiple comparisons revealed that ODN treated animals (MM, BB_1 and BB_2 ODN) exhibited more exploration as compared to the vehicle treated group. There were no significant differences in all other behaviors assessed.

Effects of BB_1, BB_2 or MM ODN treatment on plasma levels of glucose and corticosterone

A mixed measures ANOVA revealed that blood glucose levels varied as a function of the ODN treatment condition x Time interaction ($F(3, 62) = 3.256$; $p < 0.01$). There was a significant and sustained increase in glucose concentrations over time in all treatment groups. Student Newman-Keuls post-hoc comparisons of the mean of the main effects constituting this interaction revealed that amongst the BB_2 ODN animals, there was a significant blunting of BB-elicited rise in blood glucose levels at 15 min following BB administration (Figure 4). However, at 30 min post-injection, there was a significant blunting of the glucose response in both the BB_2 and MM ODN groups, relative to vehicle controls.

Figure 5 shows the time course of corticosterone levels following a central injection of BB. As anticipated, corticosterone levels varied as a function of Treatment condition x Time interaction ($F(3, 62) = 3.553$; $p < 0.01$). The multiple comparisons indicated that relative to basal corticosterone concentrations, elevations of this hormone were detected immediately following BB injection (15 min time point) and were sustained until the end of the experiment for each treatment group. However, only the BB_2 ODN treatment attenuated the BB-induced increases in corticosterone level (at the 15 min time point); whereas both BB_2 ODN and MM ODN animals showed attenuated BB-induced increases in corticosterone levels by 30 post-injection as compared to the BB_1 ODN and vehicle treated animals (Figure 5).

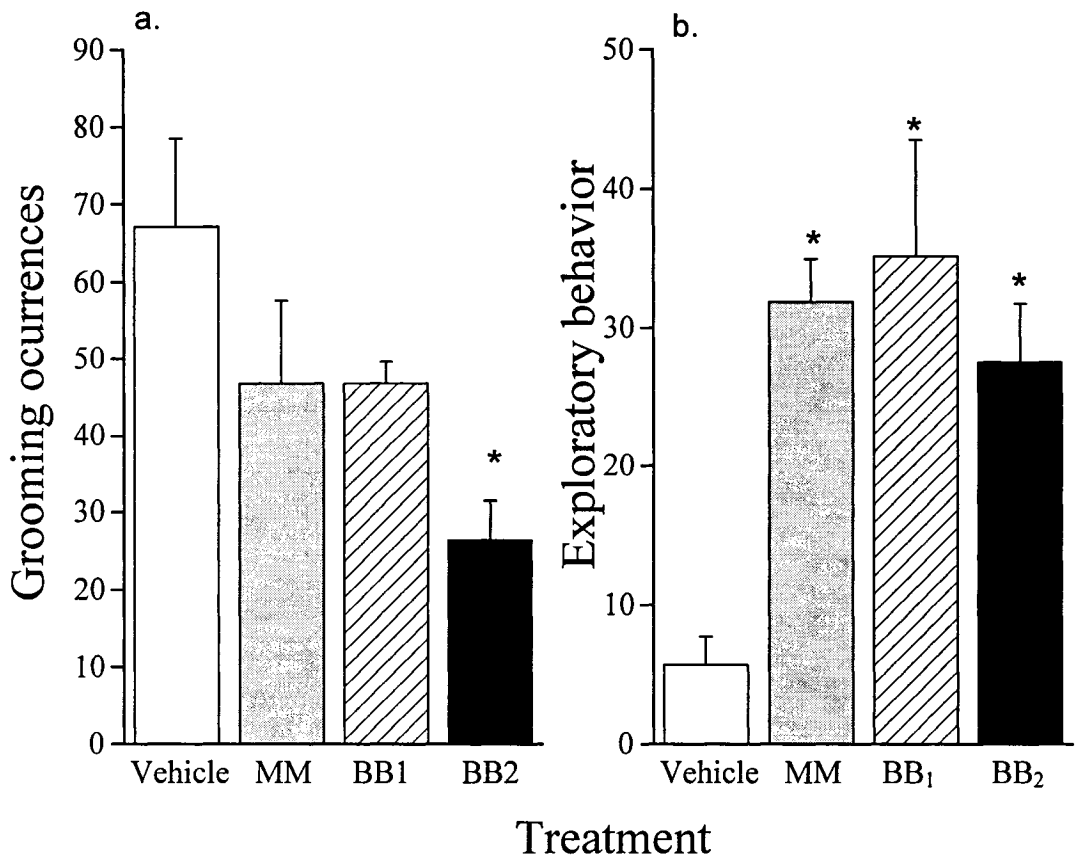


Figure 3 Grooming and exploratory behavior occurrences of animals treated with either vehicle (saline) (open columns), mismatch (MM) ODN (gray columns), BB_1 ODN (hatched columns) or BB_2 ODN (solid column) following central administration of BB (0.5 μg/3 μl/60 sec).

* Significantly different from vehicle treatment group at $p < 0.05$

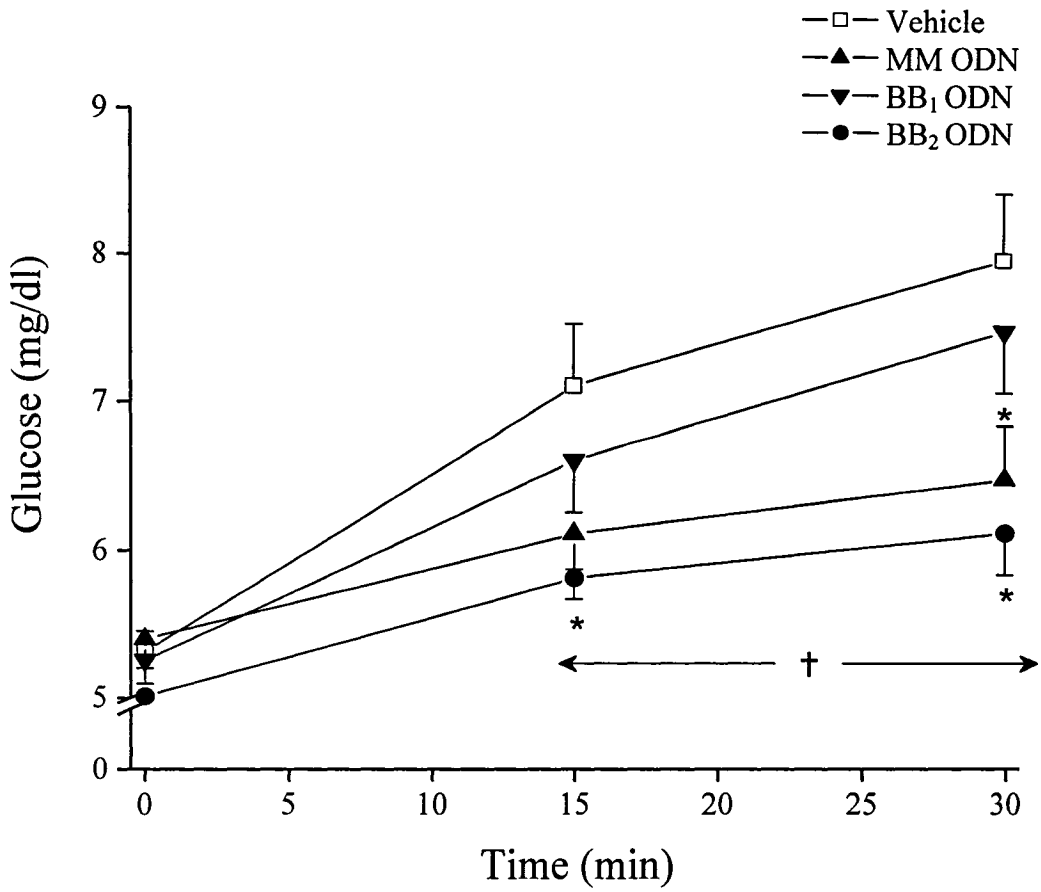


Figure 4 Blood concentrations of glucose (mean ± S.E.M.) at 0, 15 and 30 min time intervals in rats that were treated with either vehicle (saline, mismatch MM) ODN,BB_1 ODN or BB_2 ODN 30 min following central administration of BB (0.5 μg/3 μl/60 sec).
* Significantly different from (between-treatment condition) time-point matched vehicle values at $p < 0.05$
† Significantly different from (within-treatment condition) baseline values at $p < 0.05$

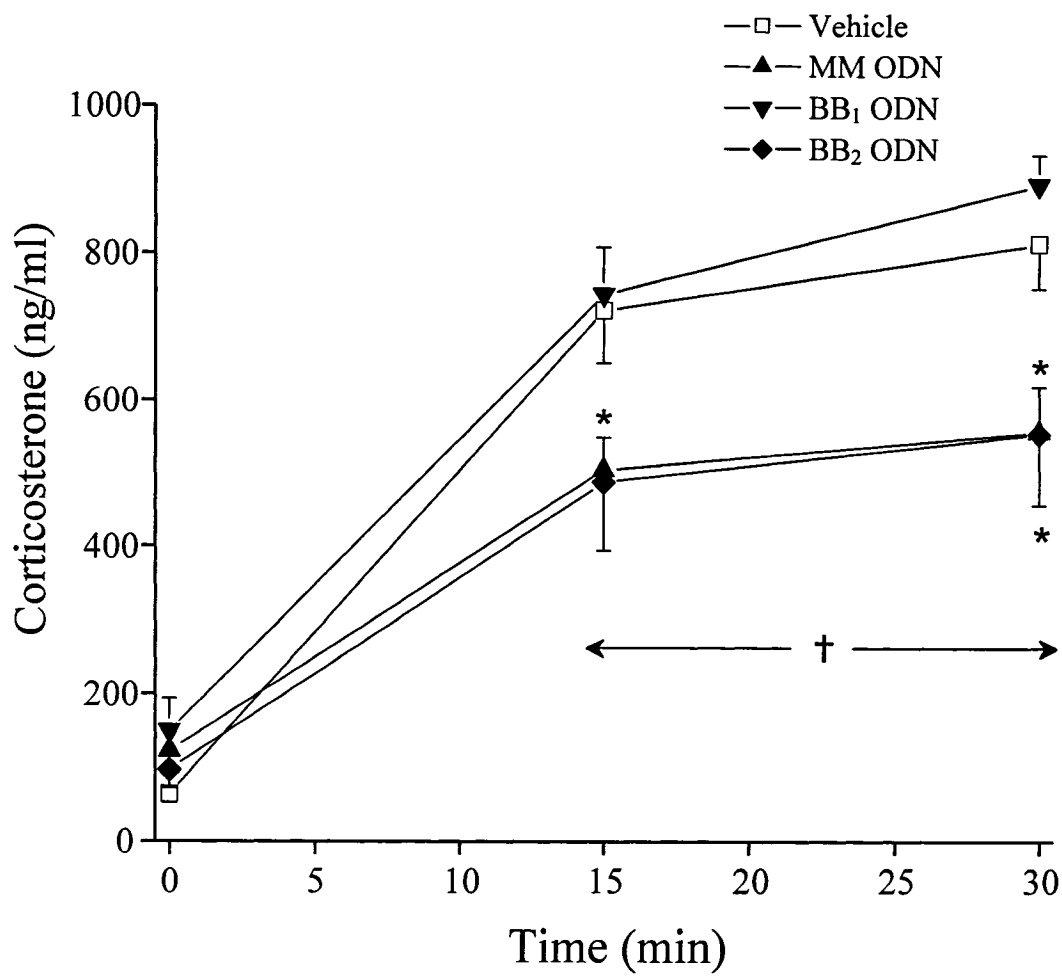


Figure 5 Blood corticosterone concentrations (mean ± S.E.M.) at 0, 15 and 30 min time intervals in rats that were treated with either vehicle (saline, mismatch (MM) ODN, BB_1 ODN or BB_2 ODN 30 minutes following central administration of BB (0.5 μg/3 μl/60 sec).

* Significantly different from (between-treatment condition) time-point matched vehicle values at $p < 0.05$

† Significantly different from (within-treatment condition) baseline values at $p < 0.05$

Discussion

In recent years, there has been mounting evidence suggesting the involvement of BLPs in anxiety and stress-related behaviors. For instance, BLPs and their receptors are present in various stress-relevant brain sites, including the hypothalamus, pituitary gland, and the amygdala.[189, 323, 497, 510] Furthermore, stressor exposure is not only associated with a rise in BLP immunoreactivity at specific brain regions, but also with enhanced release of BLPs at various stress-responsive brain sites [211, 298, 302]; these effects parallel those of CRH seen during stressor exposure. And last but not the least, central administration of these peptides alter behaviors [148, 194, 231, 300] and endocrine responses [140, 163, 213, 274, 277, 348] that are usually associated with a state of anxiety and/or stress.

The objective of the present study was to further test the hypothesis that BB_1 and BB_2 receptors might differentially be involved in the regulation of anxiety and stress-related responses. In order to attain this objective, we utilized the receptor knock-down strategy. We deployed two ODN sequences specifically targeting either the BB_1 or the BB_2 receptors. Both sequences included the start codon of each of the BB_1 or BB_2 receptor mRNAs where the first and last three nucleotides were modified to include a phosphothioate group to increase their stability, decrease the toxicity and protect the ODN from quick enzyme degradation.[6, 367]

We found that administration of various ODN sequences did not affect the locomotor activity, suggesting that they did not induce sickness behavior or toxicity in these animals. The lack of effect of the treatment on locomotor activity in all groups also

eliminates potential confounding effects on tests of anxiety where locomotion is involved.

Grooming and scratching behaviors, also known as displacement behaviors, are often associated with a state of stress in rodents [1,459], and thought to represent a coping strategy. Several studies have shown that central administration of BLPs markedly increases grooming and scratching behaviors of rodents.[82, 148, 198, 230, 303] Our data revealed that pretreatment with BB_2 ODN attenuated the BB-induced grooming and scratching behavior, whereas BB_1 ODN was without effect, suggesting that NMB is not involved in the mediation of this effect. Indeed, whereas central administration of GRP/NMC potently elicits grooming, NMB is relatively weak in that context [491] supporting the notion of a greater involvement of the BB_2 receptor subtypes in this behavior.

Another behavior typically affected by the state of stress is the reduction in exploratory activity in a novel (presumably stressful) environment. Centrally administered GRP and NMB have been reported to decrease locomotor activity in a novel environment.[194] In concordance with this, BB_2 ODN treatment was associated with an increase in exploratory behavior in a novel environment. In addition, we found that animals treated with the BB_1 antisense ODN also explored significantly more in a novel environment. However, since MM antisense ODN had the same effect as both BB_2 and BB_1 ODN sequences on exploration in a novel environment, it is possible that this effect

is attributable to the intrinsic properties of an antisense sequence rather than the functional knockdown of these specific receptors.

Furthermore, our result revealed that BB_2 ODN treatment significantly increased the number of punished licks accepted by the rats (in the Vogel test) as compared to the control, mismatch and BB_1 ODN groups, an indication of a decreased state of anxiety. These results are in keeping with previous studies investigating the effect of GRP agonist and antagonist administrations on animal behavior, and suggest a possible behavioral effect of this peptide and its receptors in anxiety and stress-related responses.[194, 297, 410] BB_1 ODN infusion failed to affect punished drinking behavior, relative to control treatments. Additionally, our results indicated that BB_1 or BB_2 ODNs did not significantly affect the behavior of the animals on the elevated plus maze. This is in keeping with earlier studies conducted with mutant mice deficient in either BB_1 or BB_2 receptors. For instance, two separate experiments failed to detect significant differences between BB_1 receptor deficient mice and the wild-type control, on the EPM (i.e. time spent in open arms, number of entries into open arms, time spent in closed arms, etc.).[517, 522] Similarly, Shumyatsky et al. also found that anxiety behavior in the elevated plus maze and light dark box was not affected in BB_2 receptor deficient mice.[445]

In addition to their behavioral effects, BLPs also exert physiological effects including activation of the hypothalamic-pituitary-adrenal axis. Several investigators were able to demonstrate a rise in ACTH and corticosterone levels following central infusion of GRP.[140, 348] In contrast, administration of a specific BB_2 receptor antagonist

was capable of blocking the GRP-induced increase in ACTH and corticosterone.[140] Similarly, NMB has also been shown to induce the release of ACTH from the anterior pituitary and corticosterone from the adrenal cortex [274, 277]; however this effect had never been demonstrated with central infusion of NMB.

Sympathetic activation, and consequently circulating glucose level, has also been reported to be affected by NMB and GRP.[45, 46, 213, 376] Of the two BLPs, effects of GRP on blood glucose levels appeared more potent (relative to those of NMB).[376] Consistent with this observation, down-regulation of the BB_2 receptor (with infusion of the BB_2 ODN), blocked the BB-induced rise in blood glucoses and corticosterone levels at 15 and 30 min post-injection. However the specificity of this effect was questionable since MM ODNs also attenuated the elevation in blood glucose and corticosterone levels (at 30 min post injection) normally seen following central BB administration. It is noteworthy, however, that the BB_1 ODN did not block the stress-induced increase in glucose or corticosterone. This finding seems inconsistent with data from BB_1 receptor deficient mice, which displayed elevated stress-induced corticosterone levels compared to the wild-type group, but had similar basal corticosterone levels.[522] However, in models deploying life-long knockout strategies, it is not unusual for compensatory neuropeptide/neurotransmitter mechanisms to come into play, and mask the specific peptide (or receptor)-related changes. However, on the whole, the possibility remains that BB_2 receptors are more directly involved (relative to BB_1 receptors) in endocrine, corticosterone and glucoregulatory processes.[376]

The relative lack of effect of the BB_1 ODN treatment in the different endocrine and behavioral paradigms was unexpected. It is possible that the ODN sequence selected, may not have been ideal, in that the targeted mRNA binding site may have been inaccessible to the ODN sequence used.[37, 38] Furthermore, ODN have also been known to have so called 'non-antisense effects', including stimulation of the immune system, toxicity and binding to non-targeted genes or mRNAs. Thus, one explanation for the absence of effect may be attributed to the choice of the sequence of the ODN used in our study. Furthermore, in the absence of actual quantification of the extent of the receptor expression, it remains possible that there may not have been adequate receptor down-regulation, to cause total functional shutdown of the receptor system.

Another limitation of this study was that the selected control condition (mismatched ODN sequences) was not devoid of action; it significantly affected the behavior, endocrine and glucose profile of animals treated. Our MM ODN sequence seemed to mimic some of the effects of the BB_2 ODN. It is, therefore, possible that not enough mismatched bases were changed in the original GRP sequence to construct the MM sequence, making our control sequence sufficiently similar to the BB_2 ODN to bind to the BB_2 receptor mRNA. However, this similarity does not seem to extend to the behavioral measures used in the experiment.

Despite some of the limitations, these results do lend further support for our hypothesis that BLPs are involved in the mediation of stress-related responses and that NMB and GRP may have distinct roles in this context. The present study indicated that

the down-regulation of BB_2 receptors had both behavioral and physiological effects. Knockdown of the BB_2 receptor decreased BB-induced grooming and exploration in a novel environment and increased the number of punished licks accepted by the rats in the vogel test. It also blocked the stressor-induced increase in blood glucose and corticosterone levels. On the other hand, the knockdown of BB_1 was virtually without effect. Although these observations could be interpreted as supporting distinct roles of these receptor subtypes in the mediation and/or expression of stressor-elicited effects, it remains possible that this may be related to the specific ODN sequence utilized. Indeed, if the sequence had mRNA binding, it could have resulted in an unsuccessful functional down-regulation of the BB_1 receptor subtype.

Preface to Chapter VI

Results from chapters III, IV and V provide evidence that NMB and GRP influence the expression of anxiety-related behaviors. Another response similar to stress and anxiety is the fear response. To our knowledge, this thesis project is the first to explore the involvement of BLPs in purely fear-related responses and to attempt to determine the comparative role of these peptides in fear. Thus we set out to determine if GRP and NMB are involved in the mediation of fear-related responses. We investigated if infusion of GRP, NMB and their respective receptor antagonists would alter the acquisition and expression of conditioned fear using the conditioned emotional response paradigm.

Chapter VI

The Roles of Gastrin-Releasing Peptide and Neuromedin B and their receptors in acquisition and expression of conditioned fear

Abstract

Classical fear conditioning or conditioned emotional response (CER) has been proposed and used as an animal model to study the underlying mechanisms involved in learned fear. Bombesin (BB) a tetradecapeptide of amphibian origin, has been shown to alter the stress response, suggesting that this peptide may be affecting some endogenous peptidergic systems. Indeed, recent studies have revealed that the receptors affected by amphibian BB may have endogenous ligands, including neuromedin B (NMB) and gastrin releasing peptide (GRP), which may be involved in mediation/modulation of anxiety and fear-type responses. The objective of the current study was to characterize the role(s) of GRP and NMB in fear learning and fear-related responses using the CER paradigm. The effects of central (i.c.v.) administration of GRP, GRP (BB_2) receptor antagonist, [Leu^{13}-(CH_2NH)Leu^{14}]-BN, NMB-30 and BB_1 receptor antagonist (BIM 23127) were assessed on the acquisition (drugs administered before training), expression (drugs administered before testing), and reconsolidation (recall test 24 hr after drug administration) of learned fear. Central administration of GRP (0.30 n moles) significantly attenuated the expression of both contextual and cued fear as reflected by a reduction in freezing behavior to both the context (cage were shock was received) and cue (tone paired with shock). Similarly, both NMB (0.30 nmoles) and BIM 23127 (1.70 nmoles) reduced fear expression in the contextual portion of the test. None of the compounds tested had effects on the acquisition or the recall of conditioned fear. These findings suggest that both GRP and NMB play some role in the expression of learned fear.

Introduction

Exposure of an organism to an adverse or fearful event results in the rapid acquisition and consolidation of this experience into long term memory.[436] This process pertaining to danger in the environment is vital for the survival of the organism. Classical fear conditioning or conditioned emotional response (CER) has been proposed and used as an animal model to study fear learning processes.[8, 215, 432] The CER paradigm consists of exposing an animal to neutral sensory stimuli such a tone (cue) or an environment (context) that have previously been associated with an noxious stimulus (footshock). When a rodent is reintroduced to the context or the cue (previously paired with the noxious event) in the absence of the conditioning stimulus (e.g. footshock), it consistently exhibits freezing behavior (defined as the complete absence of movement, except those associated with breathing).

The CER paradigm has gained considerable popularity in recent years to investigate the neurochemical and neuroanatomical underpinnings of learned fear. There are several reasons for the surge of interest in this technique. First, conditioned fear is a highly conserved form of behavior exhibited in both normal as well as laboratory environments and in all species ranging from rodents to humans.[248, 249] Indeed, neuroanatomical findings from animal studies using conditioned fear have shown parallel changes in humans, as revealed through functional magnetic resonance imaging techniques.[54, 233] Secondly, the CER paradigm has important clinical implications as it has been proposed as a model to study underlying mechanisms involved in many anxiety disorders including post-traumatic stress disorder.[247] Lastly, the relative simplicity of this

technique and ease at which it is performed makes it an ideal model to study the underlying mechanisms involved in learned fear. Although it has long been recognized that the amygdala is critically involved in fear learning [28, 95, 122, 249, 279, 357, 371, 463], the progress in our understanding of the neurochemical underpinnings of acquisition, expression and/or extinction of conditioned fear have in large part, been attributable to the CER paradigm. Indeed, a better understanding of the underlying mechanisms involved in learned fear might be expected to lead to the development novel therapeutic interventions for certain anxiety disorders.

The CER paradigm is not only amenable to the assessment of the fear response [118], but also of fear memory consolidation and reconsolidation [334] processes. It has been postulated that consolidated fear memories can be reactivated during retrieval, thus returning the memory to a flexible/adaptable state during which time it can be altered before being stored as a new memory (reconsolidation).[334] This consolidation process requires the production of new proteins.[91, 202, 335] In this context, bilateral administration of anisomycin, a protein synthesis inhibitor, into the lateral and basal nuclei of the amygdala caused a reduction in fear expression in response to a cue (a tone) as compared to vehicle treatment.[335]

Increasing evidence suggests the involvement of the bombesin (BB) family of peptides in the mediation of anxiety and fear-type responses.[277, 297, 348, 410] In support of this contention, a number of studies measuring bombesin-like peptide (BLP) immunoreactivity have detected high concentrations of these peptides in stress-sensitive

brain sites, including the hypothalamus, the amygdala and the pituitary gland.[189, 323, 325, 326, 356, 445] Although, BB is not a peptide present in mammals, two endogenous mammalian counterparts for BB have been identified namely, gastrin-releasing peptide (GRP) and neuromedin B (NMB). The high affinity GRP preferring receptors are designated as subtype 2 (BB_2), whereas those preferring NMB are classified as receptor subtype 1 (BB_1). These receptor subtypes as well as their respective ligands are located thoughtout the central nervous system including in limbic structures such as the bed nucleus of the stria terminalis, various amydgaloid nuclei and the hippocampus.[24, 325, 445, 497] Limbic structures such as the amygdala have consistently been shown to be involved in auditory fear conditioning. Therefore, the high levels of GRP expression in these structures might suggest a potential role for this peptide in auditory fear learning.[445] However the specific roles of the two peptides in fear responses remain unclear. The effects of NMB have been examined primarily in anxiety-related paradigms; however, studies have produced conflicting results.[517, 520, 522] Using the marble burying behavior as an indicator of anxiety levels, it was reported that mice lacking the BB_1 receptor exhibited decreased marble burying (anxiolytic state) relative to the wild-type control mice. These results suggest that BB_1 receptor-deletion rendered mice *less* 'anxious' than the non-mutant control group.[520] However, these mice exhibited less risk assessment behavior in the light-dark box and the elevated-plus maze, suggesting that mice lacking the BB_1 receptor are *more* anxiety prone.[517] Such apparent inconsistencies may suggest that peptides affect specific forms of anxiety and/or fear, and underline the need to further assess the involvement of NMB in anxiety and fear-related responses.

It is of interest to note that GRP gene and BB_2 receptors have been found to be highly expressed in the lateral nucleus of the amygdala, a brain region involved in auditory fear conditioning.[445] Furthermore, BB_2 receptor-deficient mice exhibited increased fear expression in both the cued and contextual fear tests.[445] While the BB_2 receptor-deficient mice did not show altered anxiety responses in the elevated-plus maze test, these animals did display a greater and more persistent long term memory of fear, suggesting that GRP plays a role in the regulation of amygdala-dependent fear-related learning and less so in anxiety related behaviors.[445] In contrast to these findings, Roesler and his colleagues [408] showed that microinjection of a selective BB_2 receptor antagonist directly into the basolateral amygdala, impaired memory retention on an inhibitory avoidance task indicating that blockade of BB_2 receptors impairs aversive memory.

Thus, while evidence from the above-mentioned studies suggests a role for both NMB and GRP in anxiety and/or fear-type responses, current data on this topic is not only scarce, but also fraught with inconsistencies. In the current study, our objective was to further characterize the role of GRP and NMB in fear learning and fear-related responses. These experiments examined the effects of central administration of BB_1 and BB_2 receptor agonists and antagonists on the acquisition of learned fear and expression of fear conditioning using the CER paradigm.

Materials and Methods

Subjects

Male Sprague Dawley rats obtained from Charles River Laboratories (St-Constant, Quebec) were used in all experiments and weighed approximately 225 g at the time of their arrival. Animals were permitted a 5-day acclimatization period during which time they were housed in pairs; they were subsequently housed individually in clear plastic cages (45 x 25 x 20 cm) in a temperature (23°C) and humidity-controlled (60 %) room, and were maintained on a 12-hour light/dark cycle (lights on at 07:00). Animals had *ad libitum* access to food and tap water. All experiments took place during the light phase of the light-dark cycle and were conducted in accordance with the University of Ottawa Animal Care guidelines.

Surgery

Subjects were anaesthetized using halothane (2.5%) and surgically implanted with a 22-gauge guide cannula (5.5 mm; Plastics One) aimed at the 3rd ventricle (AP: -4.3mm; DV: -4.3mm; L: 0; obtained from Paxinos & Watson.[361] A removable obturator was inserted to keep the cannula patent. The guide cannula was anchored to the skull using 4 stainless steel screws and dental cement. Rats were allowed a 5 day recovery-period during which animals were habituated to the injection procedure before testing began.

Drugs and Injection

Subjects were randomly assigned to one of five treatment groups as follows: gastrin-releasing peptide antagonist (GRPa) (n= 8), neuromedin B antagonist (NMBa)

(n= 7-9), gastrin-releasing peptide agonist (GRP) (n= 7-8), neuromedin B agonist (NMB) (n=7-8), or control (vehicle) (n=8-10). The GRPa, [Leu^{13}-(φ(CH_2NH) Leu^{14}]-BN, (Bachem; 1.26 n moles), the NMBa, BIM 23127 (Bachem; 1.70 n moles), GRP (Phoenix; 0.30 n moles) and NMB-30 (Phoenix; 0.30 n moles) were dissolved in Krebs Ringer Buffered Saline Solution (KRB) 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.[319] To facilitate central injections, rats were placed in a smaller cage (28 x 18 x12 cm), and the obturator replaced with a 28-gauge injector (Plastics One, VA, USA) that protruded 0.5 mm beyond the tip of the guide cannula.

Microinjections were delivered via the injection cannula using a 1.0 ml Hamilton glass syringe connected to an infusion pump (Harvard Apparatus, MA) via polyethylene tubing. All drugs were infused into the 3rd ventricle over a period of 1 min, at a flow rate of 3.0 µl/min. Following infusion, the injector was left in place for 30 s to ensure diffusion of the drug away from the cannula tip. Once the injection was complete, the obturator was replaced and rats were returned to their home cages until testing began.

Experimental protocol

Experiment 1: Effects of central administration of NMB, GRP, NMBa and GRPa on acquisition of fear memory

We used the Conditioned Emotional Response (CER) paradigm to assess the effects of drug treatment on acquisition and expression of fear memory in animals. Day 1 consists of a training day (conditioning), followed by 2 days of testing. The conditioning chamber (Coulbourn Instruments) was a 31 x 25 x 30 cm rectangular box with front and

back walls made of Plexiglas and two side walls made of removable metal plates. The grid floor of the chamber was comprised of 16 steel rods (2 mm diameter) spaced 3 cm apart and wired to a Coulbourn shocker.

On the training day, rats were infused with one of the five drugs (3 μl/min). Fifteen minutes after drug administration, rats were placed in the shock chambers and given a 1 min habituation period. Subsequently, animals were presented with the conditioned stimulus (CS) (a 20 s, 85dB tone), paired with the unconditioned stimulus (US), (1.0 mA continuous foot shock of 1.0 s duration) delivered during the last second of the tone. The US was paired with the CS 6 times, using a randomized intertrial interval (ITI) over a period of 10 min.

On Test Day 1 (24 h post training), freezing behavior was assessed both in the presence of the context (conditioning chamber) and the specific cue (CS, the tone), individually. Initially, contextual fear was evaluated by placing the animals in the conditioning chamber in which they had previously received the foot shock (no shocks were administered during this test). Freezing behavior, defined as the complete absence of movement except that associated with respiration, was recorded by trained experimenter(s), blind to the treatment conditions. The behavior was scored every 5 s for a total of 4 min. Immediately following contextual fear testing, rats were transferred to a novel environment (a clean cage identical to their home cage) for the cued fear testing. After a 1 min habituation in the novel cage, rats were presented with 20 tones with a 20-s

duration and a 1 min ITI. Again, the occurrence of freezing behavior was assessed every 5 s for a total duration of 20 min.

On Test Day 2 (48 h post training), animals were tested as on Test Day 1 in the absence of drugs treatment. This procedure was carried out in order to assess whether the peptide agonists or antagonists had any effect on memory reconsolidation or extinction processes.

Experiment 2: Effects of central administration of NMB, GRP, NMBa and GRPa on expression of fear memory

The design of Experiment 2 was almost identical to that of Experiment 1, with the exception of the drug administration regiment. Animals were administered one of the 5 drugs 15 min prior to contextual and cued fear expression on Testing Day 1 only. No drugs were administered before training, or contextual or cued fear testing on Day 2.

Histology

Following completion of the experimental testing, rats received an overdose of pentobarbital and 25% India ink (1 μl) was delivered through the injection cannula. Animals were then sacrificed and their brains removed, immediately frozen and kept at -80°C. Location of the cannulae was verified histologically upon thionin staining of the sections. Data from animals with incorrect cannula placement were excluded from the study.

Statistical Analysis

To test the effects of the various treatments on contextual and cued fear, the raw freezing scores were transformed to a percentage of freezing occurrences. The baseline values were averaged and defined as 100%. All subsequent values were then expressed as a percentage of those average baseline values. Repeated measure ANOVA with time as a within measure and treatment as a between factor were performed independently for contextual and cued freezing for both the acquisition and expression of learned fear. Post hoc comparisons were conducted using Newman-Keuls test ($p < 0.05$). For cued fear, the percentage of freezing during 2 consecutive tones including the minute following the tones (the intertrial intervals) was averaged and considered as one of 10 time bins for cued freezing.

Results

Experiment 1: Effects of central administration of NMB, GRP, NMBa and GRPa on acquisition of fear memory

Results from the ANOVAs revealed no significant effect of Treatment (with NMB, NMBa, GRP or GRPa) on the acquisition of contextual or cued fear learning, on day 1 following training.

Experiment 2: Effects of central administration of NMB, GRP, NMBa and GRPa on the expression of fear memory

The repeated measure ANOVA did not reveal any Drug x Time interaction. However, there was a significant effect of drug Treatment on freezing behavior ($F(2, 9) =$

3.619; $p < 0.04$). Follow up comparisons revealed that pretreatment with GRP significantly decreased freezing behavior expressed in response to the contextual cues ($p < 0.05$) (Figure 1). Indeed, the percentage of freezing during contextual fear testing following GRP treatment remained at basal levels for the full 4 min of testing. In contrast, treatment with the GRPa failed to affect expression of this behavior, as compared to vehicle treated controls.

Figure 2 shows the contextual freezing occurrences on day 1 (injection day) in rats centrally injected with NMB, NMBa or vehicle solution. The results from the repeated measure ANOVA revealed a significant Treatment effect on freezing behavior ($F(2, 69) = 5.067$; $p < 0.002$). Consequently, pretreatment with either NMB agonist or antagonist significantly decreased freezing from the first minute, however the effect seemed to strengthen over the course of the test, such that by the 3rd min, NMB-induced freezing was back to basal levels. Freezing behavior was reduced to 20% at the 3rd min, and 10% by the end of the 4th min (end of the session). Although the decrease in freezing behavior of animals treated with NMBa was not as robust as seen with NMB, there was a significant overall reduction in freezing behavior occurrences as compared to vehicle treated animals. The freezing behavior in the NMBa and the vehicle treated animals tended to increase over time.

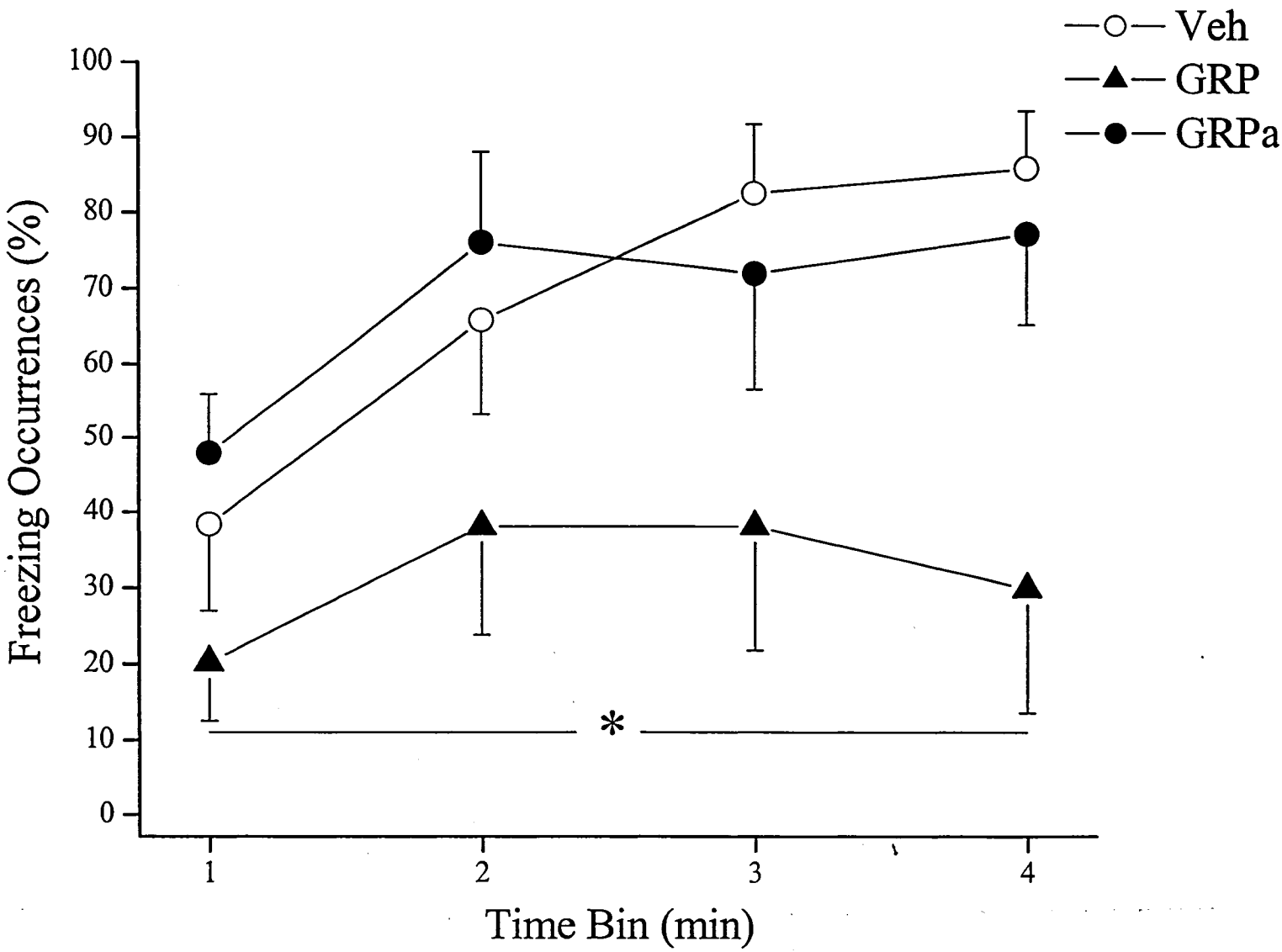


Figure 1. Percentage of freezing occurrences (± S.E.M) on day 1 during the 4 min contextual fear expression test following central administration of vehicle (3 µl; i.c.v.), GRP agonist (0.30 n mol; i.c.v.), or GRP antagonist (GRPa; 1.26 nmol; i.c.v.). The open circles represent the vehicle group, the filled triangles represent the GRP agonist group, and the filled circles represent the GRPa group.
*Significantly different from control (vehicle) group, $p < 0.05$

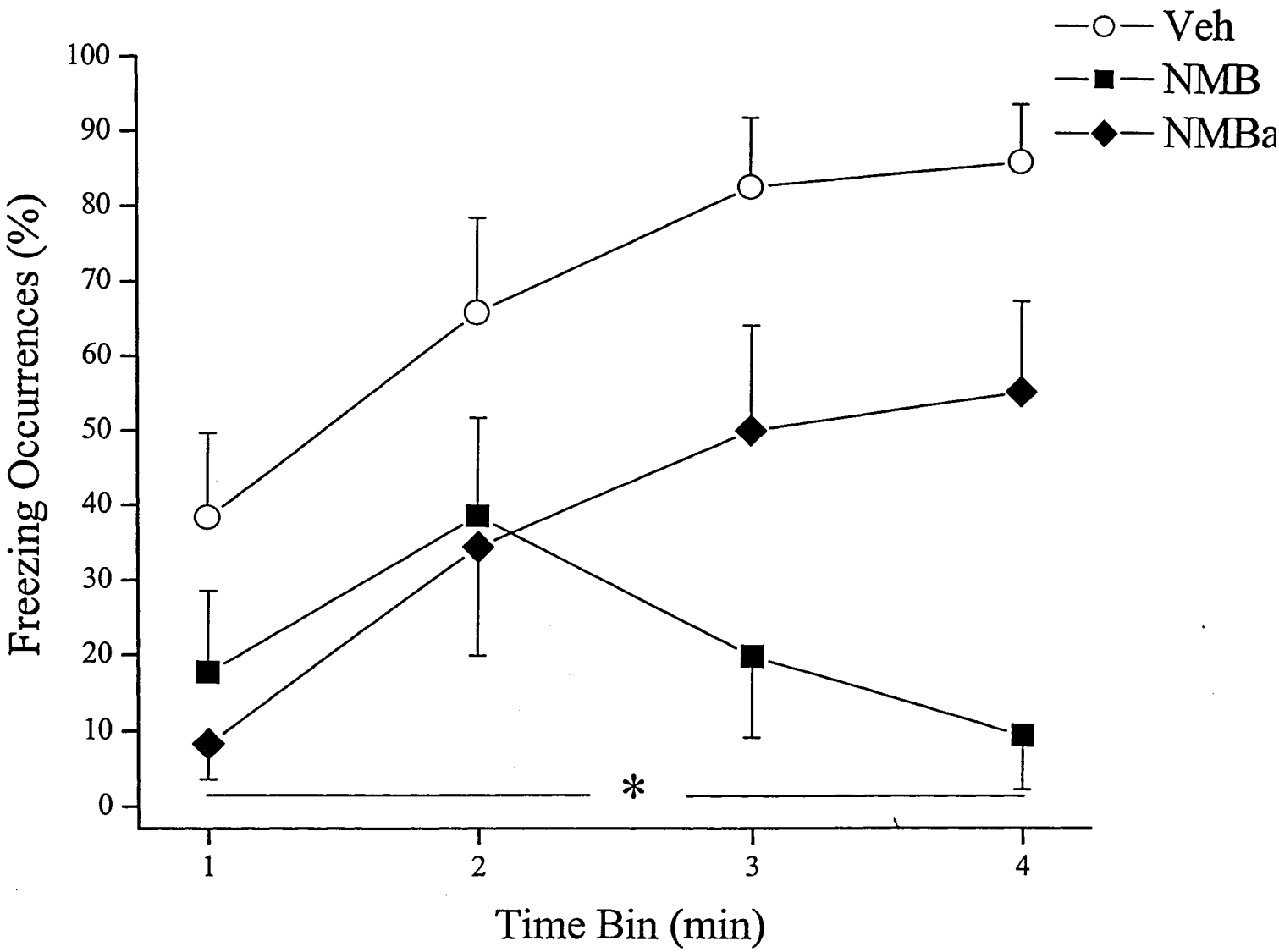


Figure 2 Percentage of freezing occurrences (± S.E.M) on day 1 during the 4 min contextual fear expression test, following central administration of vehicle (3μl; i.c.v.), NMB agonist (0.29 n mol; i.c.v.), or NMB antagonist (NMBa; 1.70 n mol; i.c.v.). The open circles represent the vehicle group, the filled squares represent the NMB agonist group, and the filled diamonds represent the NMBa group.
*Significantly different from control (vehicle) group, $p < 0.05$

Freezing behavior of animals exposed to the cue (tone) on day 1, 15 min following administration of GRP, GRPa or vehicle is depicted in figure 3. The overall repeated measure ANOVA revealed that freezing behavior varied as a function of Treatment x Time interaction ($F(2, 20) = 1.762$; $p < 0.026$). Newman-Keuls multiple comparisons of the simple effects for this interaction indicated that freezing behavior of animals treated with GRP significantly decreased with presentation of the first tone. The general trend towards a reduction in the occurrence of freezing behavior in GRP treated animals remained apparent for the first 8 min of tone presentation, although it did not reach statistical significance. No effects were found with GRPa or vehicle injections, although freezing behavior did decrease in these two groups over time.

Similarly, freezing behavior of animals exposed to the tone on day 1, 15 min following NMB or NMBa administration is depicted in figure 4. The overall repeated measure ANOVA revealed that freezing behavior varied as a function of Treatment x Time interaction ($F(2, 20) = 2.465$; $p < 0.0008$). Newman-Keuls multiple comparisons of the simple effects for this interaction revealed that freezing behavior of animals treated with NMBa was substantially lower than that of vehicle-treated animals, however, the reduction reached statistical significance only at the 3rd and 4th tone presentations. Freezing behavior following treatment with NMB appeared to follow the same pattern as NMBa, an overall reduction in freezing, although it did not reach statistical significance.

Results of the ANOVAs revealed no significant effect of NMB, NMBa, GRP or GRPa on freezing behavior of animals on day 2 of contextual or the cued fear testing,

whether drugs were administered before training (acquisition) or immediately preceding day 1 of testing (expression).

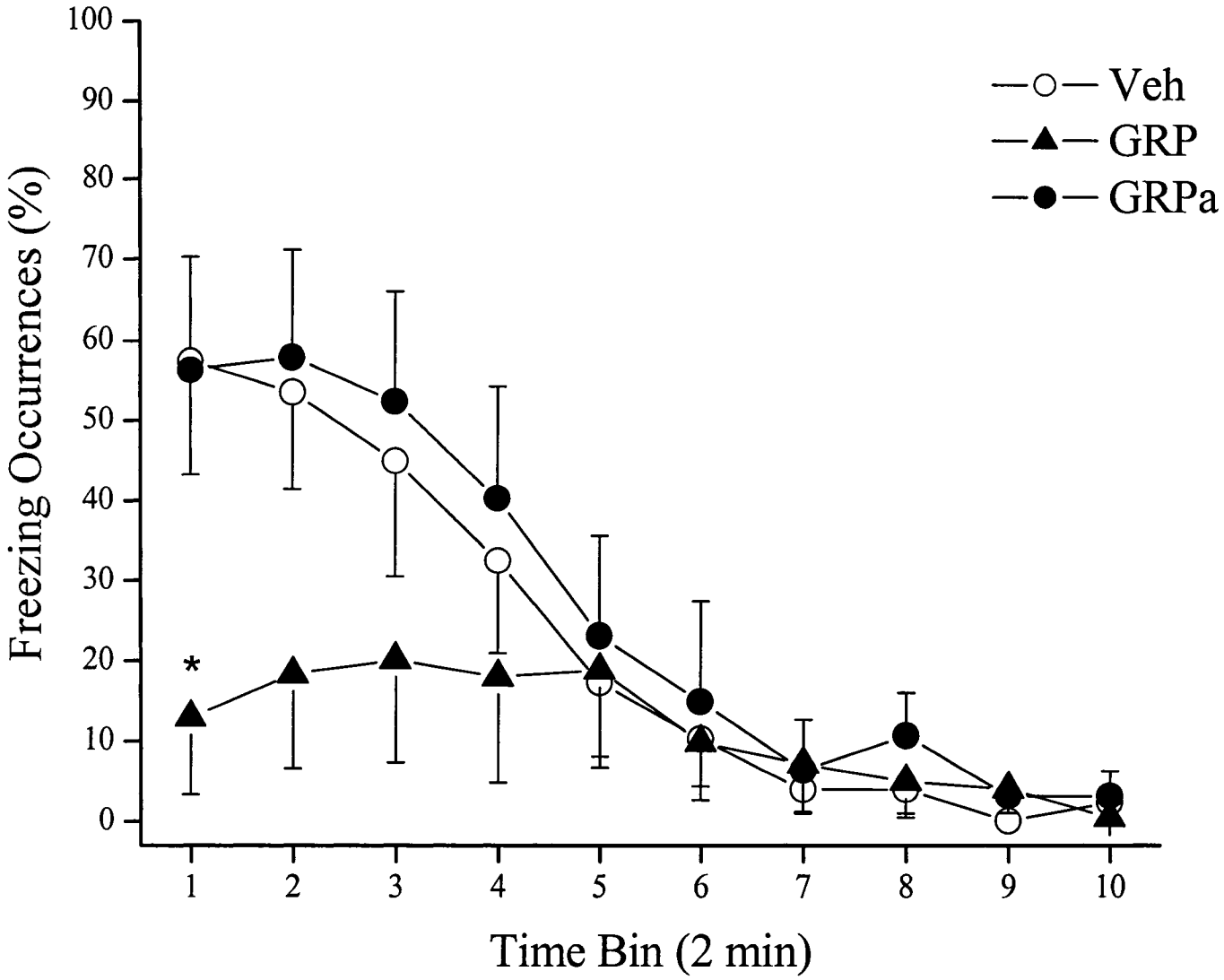


Figure 3 Percentage of freezing occurrences (± S.E.M) on day 1 during the cued fear expression test (presentation of tones) following central administration of vehicle (3 µl; i.c.v.), GRP agonist (0.30 n mol; i.c.v.), or GRP antagonist (GRPa; 1.26 n mol; i.c.v.). The open circles represent the vehicle group, the filled triangles represent the GRP agonist group, and the filled circles represent the GRPa group.
*Significantly different from control (vehicle) group, $p < 0.05$

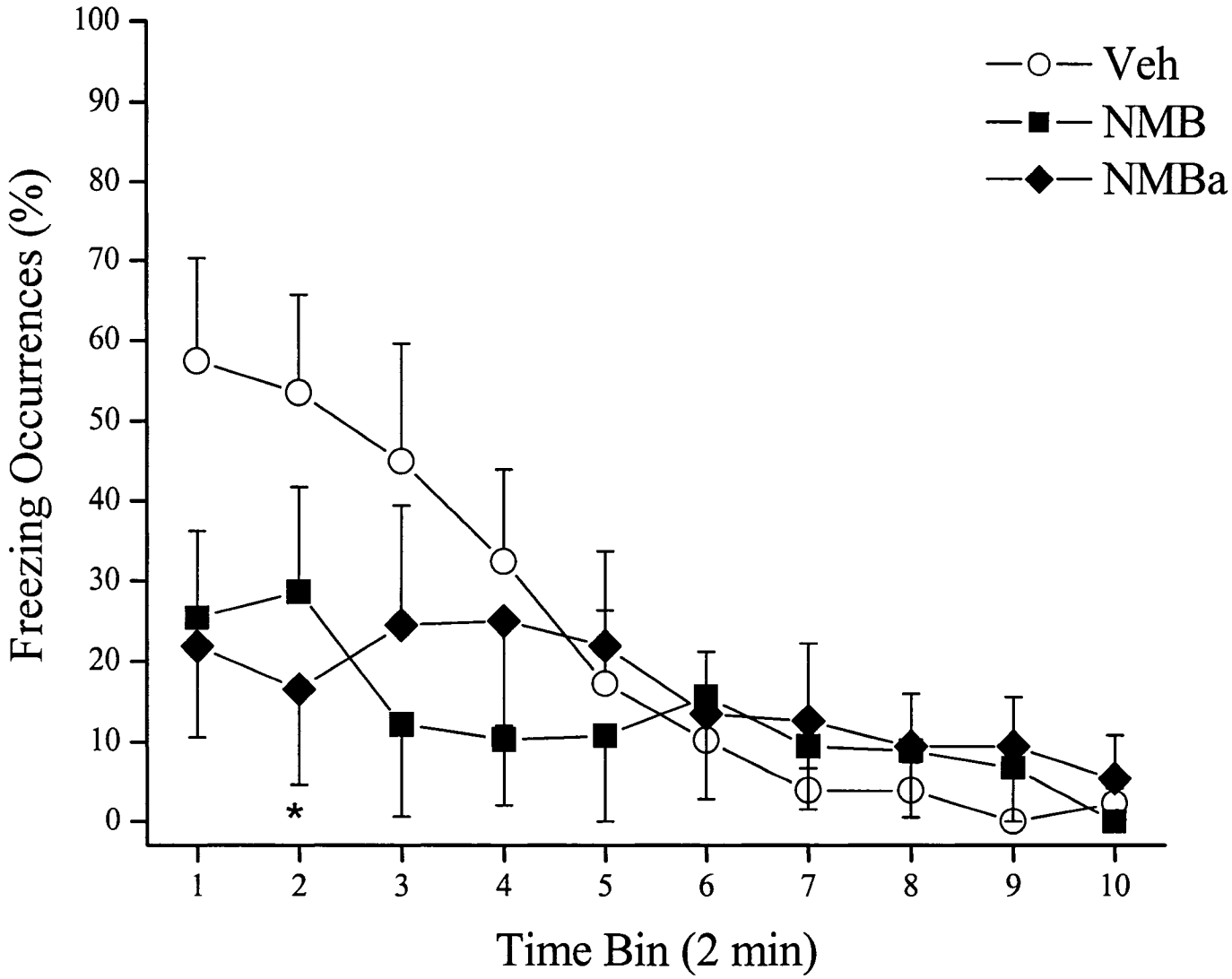


Figure 4 Percentage of freezing occurrences (± S.E.M) on day 1 during the cued fear expression test (presentation of tones) following central administration of vehicle (3 μl; i.c.v.), NMB agonist (0.29 n mol; i.c.v.), or NMB antagonist (NMBa; 1.70 n mol; i.c.v.). The open circles represent the vehicle group, the filled squares represent the NMB agonist group, and the filled diamonds represent the NMBa group.
*Significantly different from control (vehicle) group, $p < 0.05$

Discussion

Accumulating evidence tends to support the notion that BLPs, namely NMB and GRP, may represent important mediators or modulators of the stress response.[297] However, the specific role of these two peptides in fear-related responses has not been well characterized. In the current study we investigated the acute effects of BLP agonist and antagonist administration on both acquisition and expression of conditioned freezing behavior in rats. Results revealed that central administration of GRP (0.30 n moles), significantly attenuated the *expression* but not the *acquisition* of contextual and cued conditioned freezing. Similarly, both NMB (0.30 n moles) and its antagonist (NMBa; 1.07 n moles) reduced the expression of contextual freezing, but failed to affect the acquisition of fear memory. In terms of expression of fear in response to a specific cue previously paired with a noxious event (footshock), GRP and NMBa administration decreased freezing response to the tone. All treatments failed to significantly affect freezing behavior provoked by the context or the cue on the second day of testing.

Administration of the BB_2 agonist (GRP) markedly attenuated fear expression elicited by contextual as well as well as specific cues, on the first test day. Although this is the first experiment to directly assess the effects of GRP on fear conditioning, the findings are concordant with those of others using alternate approaches. For instance, using GRP receptor-knockout mice, Shumyatsky et al. (2002), found that mice lacking BB_2 receptors showed increased contextual and cued fear expression. GRP deficient mice displayed no alterations in their short-term memory or the hippocampal-based memory functioning, although they showed impaired amygdala-dependent long-term fear

memory.[445] These results suggest the involvement of GRP or BB_2 receptors distinctively in long-term memory processes.

The involvement of BLPs in both non-emotional and emotional memory has previously been demonstrated. For instance, systemic administration of GRP following scopolamine- or hypoxia-induced amnesia improved subsequent memory performance as compared to control animals.[433] In studies with BLPs, it has been found that the memory-enhancing effects of systemic BB administration can be attenuated by unilateral inactivation of the amygdala.[395] Results from the present experiment also suggest a potential role for GRP in memory processes. However, previous findings investigating the role of BB_2 receptors on fear memory have yielded conflicting results. Indeed, while Shumyatsky and his colleagues showed *enhanced* fear memory following BB_2 receptor knock out, Santo-Yamada and colleagues [433] showed that peripheral injection of the BB_2 receptor antagonist in mice *impaired* inhibitory avoidance learning in a light-dark box. Similarly, Roesler et al. (2003) found that bilateral infusion of the GRP antagonist directly into the hippocampus significantly *impaired* both long- and short-term inhibitory avoidance learning in rats.[409] Similarly, when administered systemically, the BB_2 receptor antagonist impaired memory for aversive stimuli (such as a footshock), but did not impair neutral recognition memory.[407] Finally, one study also reported impairment of conditioned fear acquisition following BB_2 receptor antagonist administration, whether the drug was injected systemically or centrally into the basolateral amygdala.[408] Despite these conflicting results, they do provide evidence supporting the involvement of GRP in aversive memory formation and its potential involvement in emotionally-based learning.

Several differences exist between our study and these former experiments that may explain the discrepancies. For instance, the drug doses used in the Roesler et al [408] experiment are considerably higher. As indicated earlier, various compounds often display an inverted-U dose-response curve.[115, 290, 354, 408] It is possible that the dose of GRP antagonist used in our experiment was relatively low and therefore failed to affect conditioned fear acquisition and/or expression. Secondly, it is possible that the drug treatment with the GRP antagonist did not reveal any effects due to the simultaneous activation of excitatory and inhibitory brain sites involved in the same response. Very specific microinjections of the drug (GRP agonist and antagonist) into targeted brain regions known to be involved in the expression of fear and/or anxiety, for example the amygdala, might overcome the shortcoming of intracerebroventricular injections, which permit activation of diverse brain sited due to volume distribution. In this context, the GRP system may be a suitable candidate since GRP has been located in various amygdalar nuclei and have been shown to exert an effect in fear conditioning involving specific amygdaloid nuclei.[408] Finally, the inhibitory avoidance paradigm used in the aforementioned studies is markedly different from the CER paradigm. The inhibitory avoidance paradigm may have diminished severity because animals have a degree of self-control over the delivery of the shock, whereas in the CER paradigm, the shock will be delivered irrespective of their behaviors. Thus, we could expect a greater degree of fear in the CER paradigm, compared to the inhibitory avoidance paradigm. Thus, it is likely that the two tasks rely on different cortical systems, which could explain why different effects were observed.

Other potential explanations for the lack of effect of the GRP antagonist may lie in the level of freezing displayed by animals in the control condition. The control condition typically serves as a baseline representation of behavioral responses against which every group is compared, and thus the ability to detect the effects of the treatments administered may be affected by the behavioral responses emitted by the control group. In this experiment, the mean freezing for the control animals was approximately 70%, a very high basal level making the detection of any further increase in freezing difficult to achieve (ceiling effect). In previous studies showing an increase in the percentage of freezing, using GRP knockout mice, the level of freezing of the control group was about 55%, rendering the detection of increases possible.[445] In future experiments, it might be preferable to use less severe training (for instance by reducing it to a single trial of 2 s 0.70mA foot shock [445]), to achieve moderate levels of freezing.

Paralleling the GRP effects on fear conditioning, both NMB agonist and antagonist markedly attenuated fear expression on day 1 of the contextual fear test. Many studies have shown the involvement of the NMB peptidergic system in stress and anxiety-related responses [276, 517, 520], although these results are unique given that NMB has never been investigated in fear-related paradigms. BB_1 receptor-deficient mice exhibit less marble-burying behavior which suggests that BB_1 receptor-deficient mice are less anxious.[520] The fact that both the NMB agonist and antagonist produced effects in the same direction was unanticipated. This effect might be attributable to the drug dose used. As mentioned previously, studies have demonstrated that some drugs display an inverted-U dose-response curve where specific intracerebroventricular (i.c.v.) drug doses are

optimal while low and high-end doses are ineffective or counter effective.[115, 290, 354, 408] In the same vein, Price and his colleagues (1998) showed that an intracerebroventricular administration of low doses of CRH (0.1 and 0.3 μg) decreased the release of 5-HT in the dorsal raphé nucleus, whereas a higher dose (3.0 μg) increased 5-HT release.[388] The use of a dose-response curve for the peptide agonists and antagonists in future research might give insight on the effect of these compounds on fear conditioning. Nonetheless, these data suggest that the NMB system is not only involved in the expression of anxiety-type responses, but may also play a role in fear-related responses.

In the contextual and cued fear tests, fear expression on day 2 reflected the extinction process. Indeed, on day 1, rats were exposed to the shock-cues, but did not receive additional shocks. Thus repeated exposure to the non-consequential cues on day 1 and day 2, provided an opportunity to un-learn or extinguish the previous pairing. Freezing levels on day 2 were generally quite low, suggesting no long-term effect of the treatments on expression of conditioned freezing or fear memory. It has been proposed that consolidated fear memories can be reactivated during retrieval, thus returning the memory to a flexible/adaptable state during which time it can be altered before being stored as a new memory (reconsolidation).[334] Further research is necessary to determine the effect of BLPs on memory reconsolidation, given that no treatment was given prior to testing on day 2. We hypothesize that GRP and NMB systems would increase memory reconsolidation, as seen with expression of conditioned freezing on day 1 since Shumyatsky et al [445] have shown that BB_2 receptor deficient mice exhibit fear memory up to 15 weeks following training.

The mechanism(s) through which GRP and NMB exert their effects on expression of conditioned fear is/are poorly understood. Previous studies, however, provide evidence suggesting that GRP might work through the GABAergic system.[11, 445] Indeed, Andrews and colleagues (2000) have demonstrated that hippocampal administration of GRP produced a 40% increase in extracellular levels of GABA as measured in dialysates.[11] Furthermore, the presence of BB_2 receptors has been identified on GABAergic interneurons.[445] These receptors are thought to indirectly regulate the activation/inhibition of amygdalar principal neurons through the control of GABA release from the interneurons. Correspondingly, in BB_2 receptor deficient mice there is a reduction in neuronal inhibition coupled with increased long-term potentiation, which ultimately translates into enhanced fear memory.[445] This contention is further supported by the evidence of the involvement of both GABA and GRP in anxiety-related responses and disorders.[297, 302, 445] In contrast, NMB appears to operate through the serotonergic molecular network. Previous researchers have consistently shown the anxiolytic effect of serotonergic neurotransmission facilitation [55, 168, 193, 502] and the involvement of BLPs in anxiety-related responses.[297] Expression of BB_1 receptors have been established in the dorsal raphé nucleus, the main source of 5-HT in the brain.[498] Two studies conducted using NMB deficient mice have reported a disruption in the serotonergic functioning in these animals. For instance, the NMB knock-out mice displayed increased tissue 5-HT levels in the dorsal raphé nucleus in conjunction with a reduction in the marble burying behavior occurrences as compared to their wild-type counterparts.[520, 522] Furthermore, under stressful conditions BB_1 receptor mutant mice did not show enhanced 5-HT levels following restraint stress exposure, suggesting an important role for NMB in modulating

the serotonergic system in an attempt to adaptively respond to homeostatic threats.[522] Thus, taken together these physiological and behavioral results provide evidence supporting a possible interdependence between the NMB and the serotonergic systems.

In conclusion, the results of the present study propose that NMB not only has a role in the mediation of anxiety-type responses, but also affects fear-related responses, suggesting that this peptide is involved in the undifferentiated stress response system, including fear and anxiety. In contrast, GRP appears to be selectively involved in fear and may participate in the supplementary fear-related processes of fear memory. Interestingly, BLPs appear to be selectively implicated in expression of conditioned fear, and less so in the actual formation or consolidation of fear memories as suggested by the lack of effect of this family of peptides on acquisition of conditioned fear. Future research is needed in order to assess if, in addition to the role played in expression of fear memories, these peptides also modulate or regulate reconsolidation processes of new memory formation. Studies should also be designed to further clarify the biological functions of these two BLPs with specific attention towards novel pharmacological interventions in the attempt to prevent fear-related disorders, such as post-traumatic stress disorder.

General Discussion

The general stress response is a well orchestrated cascade of events that prepares the body to attend and react to a threat (real or anticipated). The "stress response" can be defined into several "sub-components" including neurochemical, endocrine, emotional and behavioral responses. Within the context of the behavioral manifestation of the stress response, further subdivisions are recognized including those involving both anxiety and fear-type responses. Anxiety is thought to represent a long-lasting response that is associated with enhanced state of vigilance which is not necessarily tied to specific cue(s) and does not involve conditioning. Anxiety responses thus do not cease when a specific cue disappears. In contrast, fear responses often involving conditioning are usually associated with explicit cues or stimuli and are short lived.

It is known that exposure to a stimulus deemed to pose a threat (perceived or real) to homeostasis, activates the general stress response [180], however the temporal sequencing for when differentiation into fear- or anxiety type-responses occurs, remains unknown. There is evidence to support the view that anxiety- and fear-type responses activate different neural circuits and/or transmitter systems which eventually converge to activate the HPA axis and the autonomic nervous system, to evoke the prototypical stress response. Conversely, it has been suggested that exposure to a potentially threatening stimulus might first activate the stress response (to prepare the individual to react to a precarious situation), which would then be differentiated into specific anxiety- or fear-type responses, whichever is most appropriate or adaptive to the threat posed. These

contentions might also serve to explain the apparent overlap in brain sites and neurotransmitter systems that are activated by anxiety- or fear-eliciting stimuli.

The underlying mechanisms modulating (or differentiating) these stress-related responses have yet to be fully elucidated. In recent years, research has provided evidence drawing attention to BLPs as representing one of many classes of regulators or modulators of stress-related responses. In support of this contention, many stress-relevant brain sites express GRP and/or NMB and their receptors.[211, 297] Furthermore, administration (systemic or central) of mammalian BLPs activate the HPA axis, as reflected by elevated plasma ACTH and corticosterone levels.[19, 140, 213, 277]

The purpose of the first set of experiments was to explore the role of the two endogenous mammalian BLPs (GRP and NMB) in the mediation and/or expression of the generalized stress response. Initially, pharmacological techniques were used to establish if GRP and NMB activate the HPA axis through central mechanisms. Subsequently, physiological parameters were monitored, to determine if stressor exposure elicited changes in the endogenous systems using BLPs. Finally, we investigated the potential impact of prior stress history on the release and utilization pattern of BLPs. Using this multidisciplinary approach, we demonstrated that BLPs are not only capable of pharmacologically modulating some aspects of the stress response(s), but that NMB and GRP appear to serve differential roles (Chapter I). Subsequently, we demonstrated that the endogenous GRP and NMB peptidergic systems were altered when animals were exposed to stressors. Thus, stressor exposure (either appetive (snack consumption) or

aversive (airpuff exposure)) provoked the release of CRH and AVP (primary activators of the HPA axis) and was accompanied by the release of GRP and NMB at the anterior pituitary (Chapter II). In addition, our studies revealed that, like in the case of CRH and AVP, the animal's previous stressor history significantly altered the release pattern of GRP and NMB (Chapter II). Since these peptides appear to modulate the HPA axis, and consequently the stress response, we then explored in the second part of this thesis, the behavioral involvement of GRP and NMB in specific stress-related responses, namely anxiety- and fear-type behaviors. This was accomplished using a variety of animal models/paradigms of anxiety and fear-related behaviors. In the subsequent studies, we investigated the effect of both systemic and central administration of BLPs and their receptor antagonists, on behavior in different models of anxiety and fear, including open field, elevated-plus maze, Vogel, novel cage exposure, maternal separation, fear-potentiated startle, and conditioned emotional response. Results from these experiments showed that administration (either systemic or central) of the BB_1 receptor antagonist consistently produced both anxiolytic and/or fear-reducing effects in all of the animal models tested (Chapters III and IV). In contrast, GRP effectively attenuated fear-related responses but not the anxiety-type responses. Due to the lack of availability of highly specific antagonists, we attempted to address this issue further by exploiting the ODN receptor knockdown strategy. Although this approach of suppressing the BB_1 and BB_2 receptor expression using specific antisense oligonucleotides yielded some interesting and confirmatory results, it was confounded by methodological problems (Chapter V). In a final set of experiments, we explored the effects of manipulation of the BLP system(s) on specific fear-related responses using CER paradigm (an animal model of conditioned

fear) (Chapter VI). BLPs were found to affect the expression, but not necessarily the formation, of conditioned fear memories. Results revealed that acute administration of GRP significantly attenuated the expression of contextual and cued conditioned freezing. Similarly, acute injection of both NMB and BB_1 receptor antagonist reduced fear expression in contextual freezing testing. In addition, both GRP and BB_1 receptor antagonist elicited alterations in fear expression in the cued fear test, decreasing the occurrence of freezing behavior to the tone. Since results from the behavioral studies suggested a role for BLPs in anxiety and fear-related responses, we then attempted to assess the underlying mechanism through which these peptidergic systems mediated the noted effects. Thus, in chapter IV we monitored the effects of NMB, GRP and a mixed BB_1/BB_2 receptor antagonist on the *in vivo* release of 5-HT, using microdialysis technique. Our results revealed that systemic and intra-DRN microinfusion of BB_1 receptor antagonist (PD 176252) reduced the release of 5-HT at the ventral hippocampus; while administration of NMB (but not GRP), increased the release of 5-HT at the same site. This experiment provided evidence supporting the notion that whereas GRP may mediate its effects by affecting CRH and GABAergic systems, NMB may impart its effects by affecting serotinergic neurotransmission. Taken together, these data provide evidence for the involvement of BLPs in the mediation of stress-related responses via distinct actions of NMB and GRP in anxiety versus fear. NMB appears to be involved in the expression of both anxiety- and fear-type responses, suggesting that this peptide plays a role in the undifferentiated stress response, including fear and anxiety. In contrast, although GRP appears to be selectively involved in the mediation of fear-related

memories and responses, it may also participate in the supplementary mechanisms regulating the HPA axis.

Role of BLPs in the general stress response

Previous research exploring the potential role of BLPs in stress-related responses primarily utilized the amphibian peptide BB. Relatively few studies have assessed the differential involvement of the mammalian analogs of BB, NMB and GRP, in the general stress response. Garrido et al. [139] reported that GRP (at moderate doses) stimulated the release of ACTH and corticosterone, suggesting activation of the HPA axis. Additionally, administration of BB or GRP elevated plasma ACTH and corticosterone levels *in vitro* and *in vivo*.[19, 138, 140, 348] Another study demonstrated that systemically administered NMB elevated circulating levels of ACTH and corticosterone.[276] Prior to our experiment, however, neither the endocrine effects of *centrally* administered NMB nor the comparative effects of GRP and NMB on HPA axis had been carried out. Thus, in Chapter I we addressed several relevant questions: 1) do similarities exist between administration of BLPs and stressor exposure? 2) Does the blockade of BLP receptors elicit changes in the levels of stress-related hormones (corticosterone and glucose)? Results from the first experiment revealed that GRP administration attenuated the stressor-elicited rise in blood glucocorticoid levels, whereas injection of the GRP antagonist exacerbated this response. It would appear that these results are inconsistent with previous findings reported by other laboratories.[138, 139, 140, 348] However, this may be related to different doses deployed across various studies; the GRP doses used by other laboratories [138, 140] were typically higher than the ones used in our study. In Chapter V,

the effect of blockade of BB_2 receptor synthesis was also explored using a different method of antagonism. Functional deletion of BB_2 receptors in animals by knockdown strategy attenuated the BB-induced increase in blood glucose and corticosterone levels. The results obtained in these two set of experiments (Chapter I and V) appear to be divergent; however, the noted discrepancies might be related to the technique utilized in assessing the effect of BLPs. For instance, it is possible that the BB_2 receptor knockdown strategy used in our experiments (Chapter V) has an effect similar to that of administration of high doses of the BB_2 receptor antagonist used by others[138, 140], leading to similar results. The latter techniques might elicit pharmacological, rather than physiological or endogenous effects. In contrast, the doses applied in the first set of experiments (Chapter I) are much lower and might depict a better picture of the endogenous effect of the BLPs. Therefore, the differences in techniques might account for the disparity in the results obtained in these experiments (Chapters I and V). Furthermore, results obtained through genetic manipulation and pharmacological techniques have been shown to be quite different. Indeed, it has been well documented that genetic models induce compensatory mechanisms that are not observed in pharmacological manipulations.[350] More studies looking at the effects of different doses (dose-response curves) of GRP, NMB and their respective receptor antagonists are needed to determine the specific endocrine effects of these peptides on HPA activation.

Unlike GRP, NMB failed to alter HPA activity in both sets of experiments (Chapter I and V). While only one dose of NMB was examined, it is our contention that although NMB does play a role in the stress response, it might not do so directly through

affecting the central components of the HPA axis. High levels of NMB mRNA are found in the anterior pituitary [189, 313, 325], the source of ACTH. It is possible that NMB influences anterior pituitary functioning through autocrine or paracrine action, rather than through central mechanisms.[189, 363] In support of this contention, Pazos-Moura et al (2003) demonstrated that NMB exerts a local effect on thyrotropin-releasing hormone at the level of the pituitary.[363] Indeed, as mentioned earlier, previous research has demonstrated that NMB exerts its effect at the level of the pituitary gland in animals as well as in humans providing evidence for an paracrine function.[172, 189, 199, 276, 363] These findings support the contention that this peptide may be released from the pituitary gland to modulate pituitary function at a proximal site to where it is released. Alternatively, NMB may reach the pituitary through the systemic circulation.

If a peptide is physiologically relevant in mediating the stress response, then one might expect to see endogenous changes in the release of this peptide upon stressor challenge. Thus, in Chapter II we investigated whether 1) exposure to an acute stressor altered the availability of GRP and NMB at a selected stress-relevant brain site, and 2) if this response to stressor exposure was influenced by the animal's prior stress history? We, therefore, investigated the effects of stressor exposure (aversive or appetitive) on the *in vivo* release of BLPs (GRP and NMB) at the anterior pituitary using push-pull perfusion. This was a unique approach as the bulk of the existing evidence had measured only GRP fluctuations and utilized only static techniques. The anterior pituitary was selected as a target site for this set of experiments, as it is a central component of the HPA axis and has been shown to be rich in the expression of BLPs and their receptors.

[189, 313, 325] While stressor exposure is generally viewed as a negative experience, if defined in a broader context as a shift from homeostasis, stressors can also include appetitive events. Exposure to air puff stress was used as an aversive stressor whereas ingestion of a palatable snack was considered as an appetitive 'stressor'. Our results demonstrated that acute exposure to air puffs as well as the ingestion of a palatable snack was associated with a marked increase in the release of BLPs. In parallel, we also noted enhanced release of CRH and AVP, at the anterior pituitary. Since response to stressors is believed to be influenced by prior stress history [477], another objective of chapter II was to assess the long-term impact of acute or chronic stressor exposure on subsequent stressor-elicited changes in the interstitial levels of BLPs. Therefore, the *in vivo* release of GRP and NMB was assessed via push-pull perfusion in animals exposed to 14 daily sessions of restraint stress or 1 (20 min) session of restraint followed by the passage of time (14 days). Results showed that basal levels of interstitial GRP and NMB were increased in animals previously exposed to chronic or acute restraint (followed by the passage of time) as compared to control animals. In response to a novel stressor exposure (air puff stress or palatable snack ingestion), control (naïve) animals displayed a rapid and pronounced increase in the availability of BLPs. Although rats with previous stress experience also showed enhanced interstitial levels of GRP and NMB following air puff exposure or snack ingestion, the rise was more delayed and less pronounced as compared to the rise observed in the naïve control rats. These findings have several potential implications. First, the observation that the basal levels of BLPs are elevated following previous stressor exposure suggests that these peptidergic systems may become up-regulated (or sensitized) following conditions of prior stress experience. Thus, it is

possible that GRP and/or NMB 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. Secondly, the BLP peptidergic systems were also influenced by exposure to a positive or appetitive stressor (consumption of a palatable snack). These results indicate that BLPs are not only involved in the modulation of a response to an aversive stimulus, but are also implicated in modulation of the response to appetitive stimuli. In fact, many peptides thought to be involved in feeding and satiety have also been found to have a direct or indirect influence on the stress response; the most important of which are CRH, CCK, neuropeptide Y, as well as certain classical neurotransmitter systems, including NE, 5-HT and DA.[302] Taking an evolutionary standpoint, the concomitant involvement of peptides/neurotransmitters in regulation of both the stress response and food intake might be seen as having an adaptive value. Indeed, when an animal is exposed to a threatening stimulus (real or anticipated), cessation of other acutely non-essential activities, such as feeding, is advantageous. Furthermore, as suggested previously, consumption of a palatable snack might be viewed as a metabolic stressor. Indeed, our systems must be prepared to process and digest the food and nutrients that are ingested, and therefore this represents a threat to homeostasis, as does the potential influx of toxins. Furthermore, hunting and ingestion of food leaves the animal vulnerable to predator attack, therefore the animal is forced to keep a constant and sustained state of arousal and vigilance. In brief, these systems may serve as physiological "get ready" signals for biologically relevant situations, including feeding and responding to a more eminent stressor.

It was hoped that by exploring the effects of each of the BLPs on HPA activity we might get some indication of the individual or differential roles played by GRP and NMB. Certainly, the differential pattern of distribution of GRP and NMB suggests that these peptides might have a distinct role in stress-related and other responses.[24, 236, 326] We demonstrated that central GRP administration attenuated the stress-induced increase in blood corticosterone. On the other hand, central administration of BB_2 receptor antagonist exacerbated this stress-induced elevation of corticosterone. In contrast, NMB failed to exert a central effect on HPA axis hormone levels. It is also notable that in Chapter II the stressor-induced rise in NMB was more rapid as compared to GRP. Thus, it appears that GRP and NMB might contribute in a different way in the regulation the HPA axis. While GRP seems to mediate its effects on the stress response through central mechanisms, NMB appears to exert its effect on HPA activity peripherally, perhaps at the level of the anterior pituitary.

Role of BLPs in Anxiety and Fear

In the following four chapters, a different perspective was taken. In Chapters I and II we demonstrated that BLPs may elicit effects similar to those caused by stressor exposure (Chapter I) and that stressor exposure is accompanied by endogenously released BLPs (Chapter II). Next, we investigated whether BLPs are involved in mediating specific stress-related behaviors, namely anxiety and fear-type responses. There are several animal models that can be used to study anxiety-type behaviors including the open field exploration, elevated-plus maze, punished drinking test (Vogel), maternal separation elicited vocalization, and novel cage exposure.[52, 94, 158, 364, 365, 485, 524] These

paradigms have been shown to be sensitive to drugs with anxiolytic as well as anxiogenic effects [52, 364, 365, 485] and usually relate to a general distress state induced by unspecified danger. The FPS and CER paradigms have been validated as animal models of learned fear.[158, 415] These paradigms generate a response that is elicited by an identifiable stimulus or situation representing an eminent danger (i.e. footshocks). The fear-response elicited by the specific cue dissipates when the provoking cue is no longer present. The investigation of potential similarities or differences in the role of GRP and NMB in fear and anxiety type responses was investigated for the first time with the use of these different behavioral paradigms listed above.

Role of BLPs in Anxiety

As neurochemical and/or pharmacological evidence points to involvement of GRP and NMB in the general stress response, the question arises as to whether these peptides also affect stressor-related behaviors. More specifically, we assessed whether centrally administered GRP or NMB would alter anxiety-like behaviors in different animal models. Secondly, we hypothesized that if these endogenous peptides were important in the mediation or expression of stressor-elicited behavioral responses, then the blockade of their receptors (BB_1 and BB_2) would have the opposite effect. In the subsequent experiments we assessed whether activation (agonistic action) or the blockade (antagonists) of BB_1 and BB_2 receptors altered the behavior of animals using diverse anxiety paradigms, namely the open field and the EPM (Chapter III). Results revealed that animals treated with a BB_1 receptor antagonist spent more time in the central arena of the open field and in the open arms of the EPM, reflecting reduced levels of anxiety.

Moreover, NMB-30 administration (BB_1 receptor agonist) also increased the number of unprotected head dips (risk assessment behavior) in the EPM. These observations suggest that the BB_1 receptor subtype plays a role in anxiety-related behaviors. Furthermore, consistent with the contention that NMB plays a role in anxiety-type behaviors, we were able to demonstrate a dose-dependant increase in social interaction, reduction in the ultrasonic vocalization (emitted by pups removed from the dam) and in latencies to approach and consume a palatable snack following administration of a BB_1 receptor antagonist (Chapter IV). Other studies have found similar results using NMB receptor knockout mice.[520] NMB receptor deficient female mice engaged less in marble burying behavior indicating lower levels of anxiety compared to the wild-type mice.[520] Moreover, NMB receptor knockout males exhibited altered behavior in the EPM, supporting an anxiety-related role for the NMB/NMB receptor system.[517] In contrast, central activation or blockade of BB_2 receptors failed to alter the behavior of animals in the open field or the EPM. Consistent with these findings, studies using mutant mice have shown that deficiency of BB_2 receptors did not affect behaviors in either the light/dark box [445] or the EPM.[445, 517]

Taken together these results suggest that these two peptidergic systems may exert their effects on behavior in a parallel, yet distinct manner. Although the use of relatively specific receptor antagonists has been helpful in investigating the intrinsic role of GRP in anxiety and fear, the field has been lacking NMB antagonists with high affinity, specificity and/or solubility. Another technique that offers a better specificity to each receptor subtype is the receptor knockdown strategy using antisense

oligodeoxynucleotides. Down regulation of BLP receptor expression by means of oligodeoxynucleotide sequences (Chapter V) provided further insight into the roles of the BB_1 and BB_2 receptor subtypes in anxiety-related behaviors. Results from this set of experiments revealed that animals administered BB_2 receptor ODN displayed lower levels of anxiety as reflected by acceptance of a greater number of punished licks in the Vogel test, increased frequencies of exploratory behavior in a novel environment, as well as decreased grooming and scratching occurrences. Similarly, increased exploratory behavior was observed in BB_1 ODN treated animals. Although the results obtained with BB_1 ODN treatment are consistent with our previous findings, the anxiolytic response observed following functional suppression of BB_2 receptors in the Vogel test at first glance, appears contradictory. However, this might be attributable to several factors that make the Vogel paradigm perceptually different from other conventional anxiety paradigms (open field, EPM, social interaction and maternal separation-induced ultra-sonic vocalization). First, the Vogel test incorporates an active shock component at the time of testing, which is not used in other anxiety paradigms. Second, animals in the Vogel test have a certain degree of control over the situation as they are presented with a choice; drink and receive a shock or don't drink and avoid the punishment. Finally, this model might also encorporate a tolerance of pain component and not just anxiety- or fear-type behaviors. Interestingly, Roesler and colleagues [407, 408, 409] also observed decreased levels of anxiety following BB_2 receptor blockade using an anxiety model that involve similar factors as those involved in the Vogel test, the inhibitory avoidance task. Indeed, systemic administration of a BB_2 receptor antagonist impaired aversive memory possibly indicating a reduction in the anxiety level. It should also be noted, as previously

mentioned, that genetic deletion of receptors might not necessarily parallel or mimic effects of pharmacological manipulation or natural functioning of the system altered, due to the compensatory (and at times masking) changes.[350] In these experiments we did not find any differences between groups following BB_1 ODN treatment using several anxiety and fear paradigms. The lack of effect of this treatment might be explained by the ODN sequence used to knock down the BB_1 receptors. As mentioned before, the design of an appropriate ODN sequence that will bind exclusively to the mRNA targeted is very difficult. The binding site targeted on the specific mRNA can be inaccessible to the ODN sequence infused in order to knockdown the receptor.[38] Furthermore, they can produce non-antisense effects, including stimulation of the immune system due to its toxicity and binding to non-targeted genes or mRNA.[38]

Collectively, our findings from the behavioral approach (Chapters III, IV and V) provided support for the contention that the two mammalian BLPs, GRP and NMB, may have different endogenous roles in mediating/modulating anxiety-related behaviors. NMB appeared to have a more prominent role in anxiety behaviors, as compared to GRP. We consistently showed a reduction in anxiety in animals treated with either NMB or the BB_1 receptor antagonist. While both activation and blockade of BB_1 receptors showed the same effect behaviorally, they did not do so physiologically. In contrast, in the behavioral models utilized in these experiments (open field and EPM), while GRP and its receptor antagonist failed to affect anxiety-type behaviors (behavioral effect), these treatments did have a physiological effect and the effects (agonist versus antagonist) seem to be opposite.

Role of BLPs in fear

The objective of the next set of experiments was to investigate the role(s) of GRP and NMB in fear-type responses, using two validated animal models of conditioned fear, namely FPS and CER. Specifically, we wanted to examine whether BLPs differentially influence anxiety- versus fear-type responses. To achieve this objective, we assessed the effects of GRP, NMB or their respective receptor antagonists on the FPS response (Chapter III) as well as the acquisition and expression of conditioned fear in the CER paradigm (Chapter VI). Our results showed that BB_2 agonist (GRP) attenuated the FPS response. Similarly, FPS was also attenuated by BB_1 receptor blockade (Chapter III), suggesting a role for both NMB and GRP in conditioned fear. In keeping with this contention, in Chapter VI we further explored the involvement of BB_1 and BB_2 receptor subtypes in fear-related responding, using a different behavioral paradigm: CER. Animals treated with either BB_2 agonist (GRP) or BB_1 (NMB) receptor antagonist exhibited significantly attenuated freezing behavior. This decrease in fear-related behaviors was apparent in both contextual and cued fear tests. Our results are in keeping with those of other investigators suggesting a role for GRP in conditioned fear. Shumyatsky et al. [445] demonstrated that BB_2 receptor deficient mice displayed enhanced freezing behavior in contextual and cued fear testing; suggesting that BB_2 receptor activation should have an opposite effect. Interestingly in this same set of experiments, Shumyatsky and colleagues further demonstrated that behavior was not altered in the EPM and light dark box in mice devoid of BB_2 receptors. These findings, in combination with ours, lend support to the notion that the GRP peptidergic system may be selectively involved in fear type responses. In contrast, the NMB system appears to play an

undifferentiated role in both anxiety and fear type responses. Thus, these experiments have served to demonstrate an anxiolytic effect of GRP administration as seen by attenuation of the FPS response and a reduction in freezing behaviors in two animal fear paradigms. We also showed that NMB is involved in fear-related behavior as blockade of the BB_1 receptors decreased FPS and freezing behaviors. While BB_2 receptor antagonist tended to enhance freezing, NMB infusion showed a trend in the same direction as its antagonist, eliciting a decrease in freezing behavior occurrences. The fact that the NMB agonist and antagonist both produced effects in the same direction might be attributable to the doses used. As stated previously, studies have demonstrated that some drugs display an inverted-U dose-response curve where drug doses above or below and optimal dose window are ineffective or counter effective.[290, 354, 408] For instance, a similar bimodal effect was observed following either i.c.v or intraraphé CRH administration.[388, 390] Low doses of CRH reduced whereas high doses enhanced extracellular levels of 5-HT (5-HT) at forebrain regions. These bimodal effects of CRH on 5-HT release were attributed to the heterogeneous interaction of CRH with neuronal processes in the dorsal raphé nucleus (a major projection site for 5-HT neurons).[388, 390] Interestingly, Pinnock et al (1991) showed that NMB increased the firing rate of 5-HT cells in the dorsal raphé nucleus.[374] As in the case of CRH, the effect of NMB on 5-HT release might be bimodal, depending on the dosage employed. Therefore, in future research the use of different doses of the peptide agonists and antagonists might give better insight on the effect of these compounds on anxiety and fear. This holds true for GRP agonist and antagonist as well. While our results showed no effect of GRP or its antagonist on anxiety-related behavior, only one dose was assessed in our experiments. Furthermore,

intracerebroventricular administration of drugs results in volume diffusion of these compounds throughout the brain, hence potentially activating both excitatory and inhibitory brain sites involved in the response being investigated. Thus, it would also be beneficial to explore the effect of localized microinjections of these compounds into specific brain regions of interest.

Overall, our experiments investigating the role of the GRP and NMB systems in anxiety and fear do suggest a differential role of these peptides in these responses. These results propose a role for NMB in anxiety as well as fear, suggesting that this peptide is involved in the undifferentiated stress response system. In contrast, GRP appears to be selectively involved in fear-responses and memories.

Potential Mechanisms of Action

While the above-mentioned physiological and behavioral findings suggest a related, yet differentiated, role for the two BLPs in the regulation of stress-related responses, including anxiety and fear, they provide little insight into the neural mechanisms through which this family of peptides mediates their effects. Thus, the main objective of the experiments in chapter IV was to investigate potential interactions between BLPs and other stress relevant neurochemicals.

Serotonergic (5-HT) neurons projecting from the dorsal (DRN) and median raphé nuclei to limbic structures such as the hypothalamus, amygdala and hippocampus are thought to play a role in the mediation of the stress response.[218] Interestingly, NMB and

related BB_1 receptors have been identified in the DRN.[24, 497] Moreover, Pinnock et al. [374] showed that NMB increased the firing rate of 5-HT cells in the DRN. Recently, Yamada and colleagues (2002) provided further evidence supporting a possible link between the serotonergic system and NMB. Their results show that BB_1 receptor deficient mice display: 1) increased 5-HT expression at the dorsal raphé nucleus, 2) downregulated 5-HT_{1A} receptors, and 3) an attenuated stressor-induced elevation in 5-HT levels.[520, 522] Thus, in Chapter IV we wanted to further investigate potential interactions between NMB and the serotonergic system(s). Our data provide novel evidence supporting the contention that NMB might mediate its effects through modulation of the serotonergic system, in the regulation of the stress responses. Indeed, using microdialysis, we were able to show increased release of 5-HT at the ventral hippocampus following NMB administration in the DRN. We also found that administration of a non-peptide BB_1 receptor antagonist decreased the release of 5-HT at the same site. Many studies have demonstrated the importance of the serotonergic system in the modulation of both fear and anxiety [70, 151, 160, 260, 265, 329], and indeed a reduction in 5-HT release has been linked to anxiolytic action. It is therefore possible that the anxiolytic effects observed following administration of the BB_1 receptor antagonist may have resulted from decreased 5-HT release.

Collectively, the findings from Chapter IV provide support for the contention that NMB mediate its effects, at least in part via the serotonergic system. Many questions, however, remain unanswered particularly with respect to the exact nature of these indolamine-peptidergic interactions. In fact, several possible models could provide a

framework to explain our pharmacological findings. For instance, since BB_1 receptors are located on serotonergic cell bodies and/or dendrites it is possible that following stressor exposure (or exogenous BB or related peptide administration), BLP would bind to these specific receptors to provoke the release of 5-HT at the DRN. It can also be argued that NMB and 5-HT function in a cooperative and interdependent parallel systems. This model may be similar to one proposed by Cooper and Dourish (1990) to explain pharmacological interactions between CCK and 5-HT in the regulation of satiety. In this model, the relationship between CCK and 5-HT is collaborative in that the release of one would enhance the release of the other and vice-versa.[110] Moreover, it was also suggested 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, activation or blockade of one system will affect the ability of the other to induce satiety. We have shown in Chapter IV that manipulation of the NMB system elicits parallel changes in the serotonergic system. Finally, it is possible that NMB is involved in the fine tuning of the serotonergic system.[154, 265, 520, 522] Graeff and colleagues [154] have provided evidence supporting the "fine-tuning" role of 5-HT in defence and anxiety-type behaviors. They demonstrated that increases in 5-HT levels at the DRN caused enhanced fear learning whilst decreasing the innate fear in animals using the elevated T-maze. In this context, it is possible that as a neuromodulator, NMB facilitates the 5-HT response, whether this response is to enhance learned fear or to cause a reduction in behavior related to innate fear.

Unlike NMB, no interaction was found between GRP and 5-HT (Chapter IV) in our experiments. Results showed that activation of the GRP system at the DRN did not elicit changes in the release of 5-HT at the ventral hippocampus. There is limited evidence in the literature supporting a relationship between GRP and the serotonergic system. However, one study revealed a possible interaction between these two compounds.[138] Central administration of GRP evoked the release of 5-HT at the PVN in a dose dependent matter, as shown by increases in 5-HIAA levels, a 5-HT metabolite.[138] While these findings do support a possible relationship between GRP and 5-HT is should be noted that these effects were only observed only at the PVN. In addition, levels of 5-HT were extrapolated from measures of 5-HIAA concentrations, a 5-HT metabolite, which may not necessarily reflect enhanced transmitter release.[83, 200] Thus, while interactions between GRP and serotonergic systems can not be ruled out, more research is needed for a definitive answer.

There is sufficient research suggesting a potential interaction between GRP and GABA in mediating stress responses.[11, 445] Indeed, Andrews and colleagues (2000) have demonstrated that hippocampal administration of GRP produced a 40% increase in extracellular levels of GABA as measured in dialysates. Furthermore, the presence of BB_2 receptors has been identified on GABAergic interneurons.[445] These receptors are thought to indirectly regulate the activation/inhibition of amygdalar principal neurons through the control of GABA release from the interneurons. Correspondingly, BB_2 receptor deficient mice display a reduction in neuronal inhibition coupled with increased long-term potentiation which ultimately translates into enhanced fear memory.[445] This

contention is further supported by the evidence of the involvement of both GABA and GRP in anxiety-related responses and disorders.[14, 297, 298, 445] This interaction between GRP and GABA is also manifest in feeding related responses in the GI tract,[359] in seizure prone animals [11] and in the control of circadian rhythm.[317] Although these findings do support a possible interaction between the GRP and the GABA systems, more research is necessary in order to get clear insight in this cooperative relationship.

In summary, it would appear that NMB and GRP do play differential roles in the regulation or expression of anxiety- and fear-type responses. While NMB seems to affect both anxiety and fear possibly through its interaction with the serotonergic system; GRP appears to act on GABA to exert its effect more specifically on fear.

Implications

The findings presented in this thesis support the contention that 1) NMB may be involved in the modulation of the overall stress response, including anxiety and fear responses; 2) GRP may be more selective in mediating fear-related responses, and 3) BLPs may mediate their effects, through distinct neurochemical processes; NMB acting through the 5-HT and GRP through GABAergic systems. Over the years, a large body of evidence has emerged linking stressful life events with increased vulnerability to pathological conditions such as depression and anxiety disorders.[381] For example, stressful life events often precede the onset of depression, generalized anxiety, posttraumatic stress disorder (PTSD) and even schizophrenia.[51, 210, 523] The presence of BLPs and their receptors in stress-sensitive brain regions, such as the hippocampus,

amygdala, hypothalamus, pituitary and prefrontal cortex, support their potential physiological involvement. Furthermore, results from our laboratory and those of others,[297, 407-410] support the notion that NMB and GRP may represent modulators of emotionally based behaviors and memory processes. Since one important aspect of PTSD is a strong and long-lasting reaction and fear memory of the trauma, it is possible that pharmacological manipulation of the NMB, and more importantly the GRP systems might present a potential therapeutic target for such disorders implicating fear-dependent responses. Additionally, since modification of BB_1 receptor subtype function (either by activation or blockade) appears to reduce anxiety-type behaviors, this BLP system should be considered as a candidate in anxiety-related disorders. Benzodiazepines are amongst the more widely used drugs in the treatment of stress-related disorders, particularly those associated with anxiety. However, benzodiazepines are fraught with side effects such as sedation, memory impairments, augmentation of the alcohol effects of, and most importantly their propensity of inducing physical dependence.[127, 129, 286] Further research with more diverse animal models for stress and anxiety-related disorders are needed in order to gain a better insight into the physiological function and potential therapeutic role(s) of NMB and GRP systems.

Future Directions

Most of the studies reported in this thesis have provided a strong basis for future research and it would therefore be interesting to expand on some aspects of the presented research. Future directions might include the following:

1) Studies should attempt to identify the possible co-localization of BLPs and other peptides and/or transmitters known to be important in stress-related physiology and behavior. Co-localization of BLPs with other neuropeptides like CRH or neurotramitters like 5-HT or GABA might give insight on possible important interactions through which these peptides exert their actions and modulate the stress response and associated behaviors.

2) Deliniation of neuronal circuits through which NMB and GRP might exert their effects on anxiety and fear processes, using, for example, tracing and labeling techniques and in situ hybridization, could help to clarify the underlying mechanism leading to the development of stress-related disorders. Our studies have shown that BLPs work through presynaptic mechanisms to elicit their effect or modulate the stresss response. However, investigations looking at the possibility of postsynaptic changes in receptor sensitivity through administration of different doses of highly specific agonists and antagonists for instance might also be involved in the modulatory role of BLPs (in addition to the known presynaptic changes in BLP release) in stress and the development of stress-related illnesses.

3) Our studies have mainly focused on 3rd ventricular drug administration. Therefore, additional studies are needed that will focus on specific brain regions that are anatomically positioned to influence stress, anxiety and fear with therapeutic targets directed towards not only these particular peptidergic systems but also those co-expressed with them. Stress-related disorders, such as depression, anxiety and PTSD are known to

affect many areas of the brain. Microinjections in specific stress-relevant brain sites would be important in order to better understand the mechanisms of action of these neuropeptides; these sites might include the various nuclei of the amygdala (fear and emotional memory), the hippocampus (involved in memory), and the prefrontal cortex (responsible for cognition) which are all known to be affected by stressor exposure, and also known to underly various symptoms of depression and other stress-related conditions.

4) Furthermore, the behavioral paradigms used in our experiments focus on the attenuation or reduction of an activated or elicited behavior. More studies should be conducted using a wider range of behavioral paradigms in order to further distinguish the differential roles of GRP and NMB in fear and/or anxiety, as well as to further investigate if this distinction between these two peptidergic systems hold up.

5) Finally, when considering the percentage of people that develop stress-related disorders in the general population, there definitely seem to have certain genetic components involved in their development. Studies looking at genetic predispositions to develop stress-related ailments should be designed using different rat strains known to be useful model for depression, anxiety disorders and PTSD, such as the Wistar-Kyoto rats.

References

1. Abraham, M.E. and Gogate, M.G. (1989). Effect of stress on behaviour in rats. *Indian Journal of Physiology & Pharmacology*, **33**, 84-88.

2. Abramov, U., Raud, S., Koks, S., Innos, J., Kurrikoff, K., Matsui, T. and Vasar, E. (2004). Targeted mutation of CCK(2) receptor gene antagonises behavioural changes induced by social isolation in female, but not in male mice. *Behavioural Brain Research*, **155**, 1-11.

3. Aguilera, G. (1998). Corticotropin releasing hormone, receptor regulation and the stress response. *Trends Endocrinol. Metab.*, **9**, 329-36.

4. Aguilera, G., Millan, M.A., Hauger, R.L., Catt and KJ. (1987). Corticotropin-releasing factor receptors: distribution and regulation in brain, pituitary, and peripheral tissues. *Annals of the New York Academy of Sciences*, **512**, 48-66.

5. Aguilera, G., Pham, Q. and Rabadan-Diehl, C. (1994). Regulation of pituitary vasopressin receptors during chronic stress: Relationship to corticotroph responsiveness. *Journal of Neuroendocrinology*, **6**, 299-304.

6. Akhtar, S. and Agrawal, S. (1997). In vivo studies with antisense oligonucleotides. *Trends in pharmacological sciences*, **18**, 12-18.

7. Alonso, G. (1981). Radioautographic studies on the neurohypophysial projections of the supraoptic and paraventricular nuclei in the rat. *Cell & Tissue Research*, **219**, 525-34.

8. Anagnostaras, S.G., Maren, S. and Fanselow, M.S. (1999). Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. *Journal of Neuroscience*, **19**, 1106-14.

9. Anastasi, A., Erspamer, V. and Bucci, M. (1971). Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the european amphibian bombina and alytes. *Experientia*, **27**, 166-67.

10. Anderson, S.M., Kant, G.J. and De Souza, E.B. (1993). Effects of chronic stress on anterior pituitary and brain corticotropin-releasing factor receptors. *Pharmacology, Biochemistry & Behavior*, **44**, 755-61.

11. Andrews, N., Davis, B., Gonzalez, M.I., Oles, R., Singh, L. and McKnight, A.T. (2000). Effect of gastrin-releasing peptide on rat hippocampal extracellular GABA levels and seizures in the audiogenic seizure-prone DBA/2 mouse. *Brain Research*, **859**, 386-89.

12. Andrews, N., Hogg, S., Gonzalez, L.E. and File, S.E. (1994). 5-HT_{1A} receptors in the median raphe nucleus and dorsal hippocampus may mediate anxiolytic and anxiogenic behaviours respectively. *European Jounral of Pharmacology*, **264**, 259-64.

13. Angrini, M., Leslie, J.C. and Shephard, R.A. (1998). Effects of propranolol, buspirone, pCPA, reserpine, and chlordiazepoxide on open-field behavior. *Pharmacology, Biochemistry & Behavior*, **59**, 387-97.

14. Anisman, H. and Merali, Z. (1999). Regulating the stress response. *Alcohol Research & Health*, **23**, 247.

15. Antoni, F.A. (1993). Vasopressinergic control of pituitary adrenocorticotropin sectretion comes of age. *Frontiers in Neuroendocrinology*, **14**, 76-122.

16. Ashwood, V., Brownhill, V., Higginbottom, M., Horwell, D.C., Hughes, J. et al. (1998). PD 176252--the first high affinity non-peptide gastrin-releasing peptide (BB2) receptor antagonist. *Bioorganic & Medicinal Chemistry Letters*, **8**, 2589-94.

17. Assenmacher, I., Barbanel, G., Gaillet, S., Givalois, L., Ixart, G., Malaval, F., Mekaouche, M., Siaud, P. and Szafarczyk, A. (1995). Central regulation of ACTH release in stress. *Annals of the New York Academy of Sciences*, **771**, 41-54.

18. Aston-Jones, G., Rajkowski, J., Kubiak, P., Valentino, R.J. and Shipley, M.T. (1996). Role of the locus coeruleus in emotional activation. *Progress in Brain Research*, **107**, 379-402.

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

20. Aubry, J. M., Bartanusz, V., Jezova, D., Belin, D. and Kiss, J. Z. (1999). Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity. *Journal of Neuroendocrinology, **11**(**5**)*, 377-384.

21. Avanzi, V. and Brandao, M.L. (2001). Activation of somatodendritic 5-HT(1A) autoreceptors in the median raphe nucleus disrupts the contextual conditioning in rats. *Behavioural Brain Research*, **126**, 175-84.

22. Bartanusz, V., Aubry, J.M., Jezova, D., Baffi, J. and Kiss, J.Z. (1993). Up-regulation of vasopressin mRNA in paraventricular hypophysiotrophic neurons after acute immobilization stress. *Neuroendocrinology*, **58**, 625-29.

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

24. Battey, J. and Wada, E. (1991). Two distinct receptor subtypes for mammalian bombesin-like peptides. *Trends in Neuroscience*, **14**, 524-28.

25. Berecek, K.H., Olpe, H.R., Jones, R.S., Hofbauer and KG. (1984). Microinjection of vasopressin into the locus coeruleus of conscious rats. *American Journal of Physiology*, **247**, H675-H681.

26. Bernardini, R., Chiarenza, A., Kamilaris, T. C., Renaud, N., Lempereur, L., Demitrack, M., et al. (1994). In vivo and in vitro effects of arginine-vasopressin receptor antagonists on the hypothalamic-pituitary-adrenal axis in the rat. *Neuroendocrinology, 60*(5), 503-508.

27. Bhatnagar, S., Viau, V., Chu, A., Soriano, L., Meijer, O.C. and Dallman, M.F. (2000). A cholecystokinin-mediated pathway to the paraventricular thalamus is recruited in chronically stressed rats and regulates hypothalamic-pituitary-adrenal function. *Journal of Neuroscience*, **20**, 5564-73.

28. Bilezikjian, L.M. and Vale, W.W. (1987). Regulation of ACTH secretion from corticotrophs: the interaction of vasopressin and CRF. *Annals of the New York Academy of Sciences*, **512**, 85-96.

29. Blair, H.T., Schafe, G.E., Bauer, E.P., Rodrigues, S.M. and LeDoux, J.E. (2001). Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learning & Memory*, **8**, 229-42.

30. Blair, H.T., Sotres-Bayon, F., Moita, M.A. and LeDoux, J.E. (2005). The lateral amygdala processes the value of conditioned and unconditioned aversive stimuli. *Neuroscience*, **133**, 561-69.

31. Bloom, S.R., Edwards, A.V. and Ghatei, M.A. (1983). Endocrine responses to exogenous bombesin and gastrin releasing peptide in conscious calves. *Journal of Physiology*, **344**, 37-48.

32. Bonsall,R.W., Emery,M.S.and Weiss,J.M. (1996.) Corticosterone levels in rat brain microdialysates following exposure to noise stress or lipopolysaccharide, *Soc.Neurosci.Abs.* (Abstr.)

33. Bradwejn, J. and Koszycki, D. (1994). The cholecystokinin hypothesis of anxiety and panic disorder. *Annals of the New York Academy of Sciences,***713**, 273-82.

34. Bradwejn, J., Koszycki, D., Couëtoux du Tertre, A., Van Megen, H., Den Boer, J., Westenberg, H. and Annable, L. (1994). The panicogenic effects of cholecystokinin-tetrapeptide are antagonized by L-365,260, a central cholecystokinin receptor antagonist, in patients with panic disorder. *Arch.Gen.Psychiatry*, **51**, 486-93.

35. Bradwejn, J., Koszycki, D.and Payeur, R. (1991). Cholecystokinin Tetrapeptide: a Provocation Agent for Research in Panic Disorder? In: Briley,M. and File,S.E.(Eds.), *New Concepts in Anxiety*, The Macmillan Press Ltd, Boca Raton, FL, 313-19.

36. Bradwejn, J., Koszycki, D., Payeur, R., Bourin, M. and Borthwick, H. (1992). Replication of action of cholecystokinin tetrapeptide in panic disorder: Clinical and behavioral findings. *American Journal of Psychiatry*, **149**, 962-64.

37. Branch, A.D. (1996). A hitchhiker's guide to antisense and nonantisense biochemical pathways. *Hepatology*, **24**, 1517-29.

38. Branch, A.D. (1998). A good antisense molecule is hard to find. *Trends in Biochemical Sciences*, **23**, 45-50.

39. Bremner, J.D., Krystal, J.H., Southwick, S.M. and Charney, D.S. (1996). Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse*, **23**, 28-38.

40. Britton, D.R., Hoffman, D.K., Lederis, K. and Rivier, J. (1984). A comparison of the behavioral effects of CRF, sauvagine and urotensin 1. *Brain Research*, **304**, 201-05.

41. Britton, D.R., Koob, G., Rivier, J. and Vale, W. (1982). Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. *Life Sciences,* **31**, 363-67.

42. Brown, G.W. and Harris, T.O. (1993). Aetiology of anxiety and depressive disorders in an inner-city population. 1. Early adversity. *Psychological Medicine*, **23**, 143-54.

43. Brown, M. (1981). Neuropeptides: central nervous system effects on nutrient metabolism. *Diabetologia*, **20**, 299-304.

44. Brown, M. and Tache, Y. (1981). Hypothalamic peptides: central nervous system control of visceral functions. *Federation Proceedings*, **40(11)**, 2565-69.

45. Brown, M., Tache, Y. and Fisher, D. (1979). Central nervous system action of bombesin: mechanism to induce hyperglycemia. *Endocrinology*, **105 (3)**, 660-65.

46. Brown, M.R., Carver, K. and Fisher, L.A. (1988). Bombesin: Central nervous system actions to affect the autonomic nervous system. *Annals of the New York Academy of Sciences*, **547**, 174-82.

47. Brown, M.R., Fisher, L.A., Spiess, J., Rivier, C., Rivier, J. and Vale, W. (1982). Corticotropin-releasing factor (CRF): actions on the sympathetic nervous system and metabolism. *Endocrinology*, **111**, 928-31.

48. Brown, M.R., Fisher, L.A., Spiess, J., Rivier, J. and Vale, W. (1982). Comparison of the biologic actions of corticotropin releasing factor and sauvagine. *Regulatory Peptides*, **4**, 107-14.

49. Brown, M.R., Gray, T.S. and Fisher, L.A. (1986). Corticotropin-releasing factor receptor antagonist: effects on the autonomic nervous system and cardiovascular function. *Regulatory Peptides*, **16**, 321-29.

50. Brown, M.R., Rivier, J. and Vale, W.W. (1977). Bombesin affects the central nervous system to produce hyperglycemia in rats. *Life Sci.*, **21**, 1729-34.

51. Brugha, T.S., Bebbington, P.E., Stretch, D.D., MacCarthy, B. and Wykes. (1997). Predicting the short-term outcome of first episodes and recurrences of clinical depression: a prospective study of life events, difficulties, and social support networks. *Journal of Clinical Psychiatry*, **58**, 298-306.

52. Brunelli, S.A., Hofer, M.A. and Weller, A. (2001). Selective breeding for infant vocal response: a role for postnatal maternal effects? *Developmental Psychobiology*, **38**, 221-28.

53. Buchan, A.M.J., Meloche, M. and Coy, D.H. (1990). Inhibition of bombesin-stimulated gastrin release from isolated human G cells by bombesin analogs. *Pharmacology*, **41**, 237-45.

54. Buchel, C., Morris, J., Dolan, R.J. and Friston, K.J. (1998). Brain systems mediating aversive conditioning: an event-related fMRI study. *Neuron*, **20**, 947-57.

55. Buhot, M.C. (1997). Serotonin receptors in cognitive behaviors. *Current Opinion in Neurobiology*, **7**, 243-54.

56. Burghardt, N.S., Sullivan, G.M., McEwen, B.S., Gorman, J.M. and LeDoux, J.E. (2004). The selective serotonin reuptake inhibitor citalopram increases fear after acute treatment but reduces fear with chronic treatment: a comparison with tianeptine. *Biological Psychiatry*, **55**, 1171-78.

57. Burrows, H.L., Nakajima, M., Lesh, J.S., Goosens, K.A., Samuelson, L.C., Inui, A., Camper, S.A. and Seasholtz, A.F. (1998). Excess corticotropin releasing hormone-binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice. *Journal of Clinical Investigation*, **101**, 1439-47.

58. Bussey, T.J., Everitt, B.J. and Robbins, T.W. (1997). Dissociable effects of cingulate and medial frontal cortex lesions on stimulus-reward learning using a novel Pavlovian autoshaping procedure for the rat: implications for the neurobiology of emotion. *Behavioral Neuroscience*, **111**, 908-19.

59. Cador, M., Ahmed, S.H., Koob, G.F., Le Moal, M. and Stinus, L. (1992). Corticotropin-releasing factor induces a place aversion independent of its neuroendocrine role. *Brain Research*, **597**, 304-09.

60. Campeau, S. and Davis, M. (1995). Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. *Journal of Neuroscience*, **15**, 2301-11.

61. Carli, M., Prontera, C. and Samanin, R. (1989). Effect of 5-HT1A agonists on stress-induced deficit in open field locomotor activity of rats: evidence that this model identifies anxiolytic-like activity. *Neuropharmacology*, **28**, 471-76.

63. Carli, M., Tatarczynska, E., Cervo, L. and Samanin, R. (1993). Stimulation of hippocampal 5-HT1A receptors causes amnesia and anxiolytic-like but not antidepressant-like effects in the rat. *European Journal of Pharmacology*, **234**, 215-21.

64. Cecchi, M., Khoshbouei, H., Javors, M. and Morilak, D.A. (2002). Modulatory effects of norepinephrine in the lateral bed nucleus of the stria terminalis on behavioral and neuroendocrine responses to acute stress. *Neuroscience*, **112**, 13-21.

65. Cecchi, M., Khoshbouei, H. and Morilak, D.A. (2002). Modulatory effects of norepinephrine, acting on alpha 1 receptors in the central nucleus of the amygdala, on behavioral and neuroendocrine responses to acute immobilization stress. *Neuropharmacology*, **43**, 1139-47.

66. Cenci, M.A. and Kalen, P. (2000). Serotonin release from mesencephalic raphe neurons grafted to the 5,7-dihydroxytryptamine-lesioned rat hippocampus: effects of behavioral activation and stress. *Experimental Neurology*, **164**, 351-61.

67. Chaki, S., Nakazato, A., Kennis, L., Nakamura, M., Mackie, C. et al. (2004). Anxiolytic- and antidepressant-like profile of a new CRF1 receptor antagonist, R278995/CRA0450. *European Journal of Pharmacology*, **485**, 145-58.

68. Chaouloff, F. (2000). Serotonin, stress and corticoids. *Journal of Psychopharmacology*, **14**, 139-51.

69. Chappell, P.B., Smith, M.A., Kilts, C.D., Bissette, G., Ritchie, J., Anderson, C. and Nemeroff, C.B. (1986). Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. *Journal of Neuroscience*, **6**, 2908-14.

70. Charney, D.S. (2003). Neuroanatomical circuits modulating fear and anxiety behaviors. *Acta Psychiatrica Scandinavica, Supplementum*, 38-50.

71. Charney, D.S. (2004). Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress. *American Journal of Psychiatry*, **161**, 195-216.

72. Chronwall, B.B., Pisano, J.J., Bishop, J.F., Moody, T.W. and O'Donohue, T.L. (1985). Biochemical and histochemical characterization of ranatensin immunoreactive peptides in rat brain: lack of coexistence with Bombesin/GRP. *Brain Research*, **338**, 97-113.

73. Chrousos, G.P. (1998). Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. *Annals of the New York Academy of Sciences*, **851**, 311-35.

74. Clark, M.S., Vincow, E.S., Sexton, T.J. and Neumaier, J.F. (2004). Increased expression of 5-HT1B receptor in dorsal raphe nucleus decreases fear-potentiated startle in a stress dependent manner. *Brain Research*, **1007**, 86-97.

75. Clement, Y. and Chapouthier, G. (1998). Biological bases of anxiety. *Neuroscience & Biobehavioral Reviews*, **22**, 623-33.

76. Clenet, F., Hascoet, M., Fillion, G., Galons, H. and Bourin, M. (2005). Role of GABA-ergic and serotonergic systems in the anxiolytic-like mechanism of action of a 5-HT-moduline antagonist in the mouse elevated plus maze. *Behavioural Brain Research*, **158**, 339-48.

77. Cole, B.J. and Koob, G.F. (1988). Propranolol antagonizes the enhanced conditioned fear produced by corticotropin releasing factor. *Journal of Pharmacology & Experimental Therapeutics*, **247**, 902-10.

78. Cole, B.J. and Robbins, T.W. (1987). Dissociable effects of lesions to the dorsal or ventral noradrenergic bundle on the acquisition, performance, and extinction of aversive conditioning. *Behavioral Neuroscience*, **101**, 476-88.

79. Cone, R.D. (2000). The corticotropin-releasing hormone system and feeding behavior--a complex web begins to unravel. *Endocrinology*, **141**, 2713-14.

80. Cooper, S.J. and Dourish, C.T. (1990). Multiple cholecystokinin (CCK) receptors and CCK-monoamine interactions are instrumental in the control of feeding. *Physiology and Behavior*, **48**, 849-57.

81. Coste, S.C., Kesterson, R.A., Heldwein, K.A., Stevens, S.L., Heard, A.D. et al. (2000). Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. *Nature Genetics*, **24**, 403-09.

82. Cowan, A. (1988). Behavioral effects of bombesin. *Annals of the New York Academy of Sciences*, **547**, 204-09.

83. Crespi, F., Garratt, J.C., Sleight, A.J. and Marsden, C.A. (1990). In vivo evidence that 5-hydroxytryptamine (5-HT) neuronal firing and release are not necessarily correlated with 5-HT metabolism. *Neuroscience*, **35**, 139-44.

84. Croiset, G., Nijsen, M.J. and Kamphuis, P.J. (2000). Role of corticotropin-releasing factor, vasopressin and the autonomic nervous system in learning and memory. *European Journal of Pharmacology*, **405**, 225-34.

85. Cunningham, E.T. and Sawchenko, P.E. (1988). Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *Journal of Comparative Neurology*, **274**, 60-76.

86. Curtis, A. L., Bello, N. T., Connolly, K. R., and Valentino, R. J. (2002). Corticotropin-releasing factor neurones of the central nucleus of the amygdala mediate locus coeruleus activation by cardiovascular stress. *Journal of Neuroendocrinology, **14**(**8**)*, 667-682.

87. Daftary, S.S., Boudaba, C. and Tasker, J.G. (2000). Noradrenergic regulation of parvocellular neurons in the rat hypothalamic paraventricular nucleus. *Neuroscience*, **96**, 743-51.

88. Dallman, M.F., Akana, S.F., Strack, A.M., Hanson, S. and Sebastian, R.J. (1995). The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at sites proximal to CRF neurons. *Annals of the New York Academy of Sciences*, **771**, 730-42.

89. Dauge, V., Samir, A., Cupo, A. and Roques, B.P. (1999). Peripheral stimulation of CCK-B receptors by BC264 induces a hyperexploration, dependent on the delta opioid system in the nucleus accumbens of rat. *Neuropharmacology*, **38**, 999-1007.

90. Davies, M.F., Tsui, J., Flannery, J.A., Li, X., DeLorey, T.M. and Hoffman, B.B. (2004). Activation of alpha2 adrenergic receptors suppresses fear conditioning: expression of c-Fos and phosphorylated CREB in mouse amygdala. *Neuropsychopharmacology*, **29**, 229-39.

91. Davis, H.P. and Squire, L.R. (1984). Protein synthesis and memory: a review. *Psychological Bulletin*, **96**, 518-59.

92. Davis, M. (1998). Anatomic and physiologic substrates of emotion in an animal model. *Journal of Clinical Neurophysiology*, **15**, 378-87.

93. Davis, M. (1998). Are different parts of the extended amygdala involved in fear versus anxiety? *Biological Psychiatry*, **44**, 1239-47.

94. Davis, M. and Shi, C. (1999). The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved

in fear versus anxiety? *Annals of the New York Academy of Sciences*, **877**, 281-91.

95. Davis, M., Walker, D.L. and Myers, K.M. (2003). Role of the amygdala in fear extinction measured with potentiated startle. *Annals of the New York Academy of Sciences*, **985**, 218-32.

96. Day, H.E., Campeau, S., Watson, S.J., Jr. and Akil, H. (1999). Expression of alpha(1b) adrenoceptor mRNA in corticotropin-releasing hormone-containing cells of the rat hypothalamus and its regulation by corticosterone. *Journal of Neuroscience*, **19**, 10098-106.

97. De Caro, G., Polidori, C., Menani, J.V. and Johnson, A.K. (1997). Bombesin affects the central nervous system to produce sodium intake inhibition in rats. *Physiology & Behavior*, **63**, 15-23.

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

99. De Goeij., D.C.E., Dijkstra, H. and Tilders, F.J. (1992). Chronic psychosocial stress enhances vasopressin, but not corticotropin-releasing factor, in the external zone of the median eminence of male rats: relationship to subordinate status. *Endocrinology*, **131**, 847-53.

100. De Goeij, D.C.E., Jezova, D. and Tilders, F.J.H. (1992). Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. *Brain Research*, **577**, 165-68.

101. De Goeij., D.C.E., Kvetnansky, R., Whitnall, M.H., Jezova, D., Berkenbosch, F. and Tilders, F.J. (1991). Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. *Neuroendocrinology*, **53**, 150-59.

102. De Jongh, R., Groenink, L., van Der, G.J. and Olivier, B. (2002). The light-enhanced startle paradigm as a putative animal model for anxiety: effects of chlordiazepoxide, flesinoxan and fluvoxamine. *Psychopharmacology*, **159**, 176-80.

103. De Jongh, R., Groenink, L., van Der, G.J. and Olivier, B. (2003). Light-enhanced and fear-potentiated startle: temporal characteristics and effects of alpha-helical corticotropin-releasing hormone. *Biological Psychiatry*, **54**, 1041-48.

104. De Montigny, C. (1989). Cholecystokinin Tetrapeptide Induces Panic-like Attacks in Healthy Volunteers. Archives General Psychiatry **46**, 511-17.

105. Deak, T., Nguyen, K.T., Ehrlich, A.L., Watkins, L.R., Spencer, R.L. et al. (1999). The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. *Endocrinology*, **140**, 79-86.

106. Dhabhar, F.S., McEwen, B.S. and Spencer, R.L. (1997). Adaptation to prolonged or repeated stress--comparison between rat strains showing intrinsic differences in reactivity to acute stress. *Neuroendocrinology*, **65**, 360-68.

107. Dockray, G.J., Vaillant, C. and Walsh, J.H. (1979). The neuronal origin of bombesin-like immunoreactivity in the gastrointestinal tract. *Neuroscience*, **4**, 1561-68.

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

109. Doronbekov, T.K., Zhou, Y., Spencer, S.J., Zwanzger, P., Chaouloff, F., Krishnan, K.R., Plotsky, P.M. and Widmaier, E.P. (2005). Neural basis of fear conditioning induced by video clip: positron emission tomography study. *Psychiatry & Clinical Neurosciences*, **59**, 155-62.

110. Dourish C.T. (1987). Brain 5-HT_{1A} receptors and anxiety. In: Dourish C.T., Ahlenius,S., and Hutson,P.H.(Eds.), *Brain 5-HT_{1A} receptors*, Ellis Horwood, Chichester, 261-77.

111. Dronjak, S., Jezova, D. and Kvetnansky, R. (2004). Different effects of novel stressors on sympathoadrenal system activation in rats exposed to long-term immobilization. *Annals of the New York Academy of Sciences*, **1018**, 113-23.

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

113. Dunn, A.J. and Swiergiel, A.H. (1999). Behavioral responses to stress are intact in CRF-deficient mice. *Brain Research*, **845**, 14-20.

114. El Ghundi, M., O'Dowd, B.F. and George, S.R. (2001). Prolonged fear responses in mice lacking dopamine D1 receptor. *Brain Research*, **892**, 86-93.

115. Elkabir, D.R., Wyatt, M.E., Vellucci, S.V. and Herbert, J. (1990). The effects of separate or combined infusions of corticotrophin-releasing factor and vasopressin either intraventricularly or into the amygdala on aggressive and investigative behaviour in the rat. *Regulatory Peptides*, **28**, 199-214.

116. Erspamer, V. and Melchiorri, P. (1973). Active polypeptides of the amphibian skin and their synthetic analogues. *Pure and Applied Chemistry*, **35**, 463-94.

117. Falzone, T.L., Gelman, D.M., Young, J.I., Grandy, D.K., Low, M.J. and Rubinstein, M. (2002). Absence of dopamine D4 receptors results in enhanced reactivity to unconditioned, but not conditioned, fear. *European Journal of Neuroscience*, **15**, 158-64.

118. Fanselow, M.S. (2000). Contextual fear, gestalt memories, and the hippocampus. *Behavioural Brain Research*, **110**, 73-81.

119. Farook, J.M., Zhu, Y.Z., Wang, Q., Moochhala, S.M., Lee, L. and Wong, P.T. (2004). Analysis of strain difference in behavior to Cholecystokinin (CCK) receptor mediated drugs in PVG hooded and Sprague-Dawley rats using elevated plus-maze test apparatus. *Neuroscience Letters*, **358**, 215-19.

120. Fathi, Z., Corjay, M.H., Shapira, H., Wada, E., Benya, R., Jensen, R., Viallet, J., Sausville, E.A. and Battey, J.F. (1993). BRS-3: A novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J.Biol.Chem.*, **268**, 5979-84.

121. Fekete, E., Vigh, J., Bagi, E.E. and Lenard, L. (2002). Gastrin-releasing peptide microinjected into the amygdala inhibits feeding. *Brain Research*, **955**, 55-63.

122. Fendt, M. and Fanselow, M.S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews*, **23**, 743-60.

123. Fernagut, P.O., Chalon, S., Diguet, E., Guilloteau, D., Tison, F. and Jaber, M. (2003). Motor behaviour deficits and their histopathological and functional correlates in the nigrostriatal system of dopamine transporter knockout mice. *Neuroscience*, **116**, 1123-30.

124. Fernandez, E.E. (2003). Prefrontocortical dopamine loss in rats delays long-term extinction of contextual conditioned fear, and reduces social interaction without affecting short-term social interaction memory. *Neuropsychopharmacology*, **28**, 490-98.

125. Ferre, P., Fernandez-Teruel, A., Escorihuela, R.M., Driscoll, P., Corda, M.G., Giorgi, O. and Tobena, A. (1995). Behavior of the Roman/Verh high- and low-avoidance rat lines in anxiety tests: relationship with defecation and self-grooming. *Physiology & Behavior*, **58**, 1209-13.

126. Ferri, G.L., Adrian, T.E., Ghatei, M.A., O'Shaughnessy, D.J., Probert, L., Lee, Y.C., Buchan, A.M., Polak, J.M. and Bloom, S.R. (1983). Tissue localization and relative distribution of regulatory peptides in separated layers from the human bowel. *Gastroenterology*, **84**, 777-86.

127. File, S.E. (1990). The history of benzodiazepine dependence: a review of animal studies. *Neuroscience and Biobehavioral Reviews*, **14**, 135-46.

128. File, S.E. (1980). The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. *J.Neurosci.Methods*, **2**, 219-38.

129. File, S.E. (1990). New strategies in the search for anxiolytics. *Drug Design & Delivery*, **5**, 195-201.

130. File, S.E., Kenny, P.J. and Cheeta, S. (2000). The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. *Pharmacology, Biochemistry & Behavior*, **66**, 65-72.

131. Fisher, L.A., Rivier, J., Rivier, C., Spiess, J., Vale, W. and Brown, M.R. (1982). Corticotropin-releasing factor (CRF): central effects on mean arterial pressure and heart rate in rats. *Endocrinology*, **110**, 2222-24.

132. Flood, J.F. and Morley, J.E. (1988). Effects of bombesin and gastrin-releasing peptide on memory processing. *Brain Research*, **460**, 314-22.

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

134. Flynn, F.W. (1994). Bombesin-like peptides in the regulation of ingestive behavior. *Ann.NY Acad.Sci.*, **739**, 120-34.

135. Fontana, D.J., McMiller, L.V., Jr. and Commissaris, R.L. (1999). Depletion of brain norepinephrine: differential influence on anxiolyic treatment effects. *Psychopharmacology*, **143**, 197-208.

136. Fujimiya, M. and Inui, A. (2000). Peptidergic regulation of gastrointestinal motility in rodents. *Peptides*, **21**, 1565-82.

137. Funk, D., and Stewart, J. (1996). Role of catecholamines in the frontal cortex in the modulation of basal and stress-induced autonomic output in rats. *Brain Research,* **741(1-2)**, 220-229.

138. Garrido, M.M., Fuentes, J.A. and Manzanares, J. (2002). Gastrin-releasing peptide mediated regulation of 5-HT neuronal activity in the hypothalamic paraventricular nucleus under basal and restraint stress conditions. *Life Sciences*, **70**, 2953-66.

139. Garrido, M.M., Manzanares, J. and Fuentes, J.A. (1999). Hypothalamus, anterior pituitary and adrenal gland involvement in the activation of adrenocorticotropin and corticosterone secretion by gastrin-releasing peptide. *Brain Research*, **828**, 20-26.

140. Garrido, M.M., Martin, S., Ambrosio, E., Fuentes, J.A. and Manzanares, J. (1998). Role of corticotropin-releasing hormone in gastrin-releasing peptide-mediated regulation of corticotropin and corticosterone secretion in male rats. *Neuroendocrinology*, **68**, 116-22.

141. Gibbs, J. (1985). Effect of bombesin on feeding behavior. *Life Sciences*, **37**, 147-53.

142. Gibbs, J., Fauser, D.J., Rowe, E.A., Rolls, B.J., Rolls, E.T. and Maddison, S.P. (1979). Bombesin suppresses feeding in rats. *Nature*, **282**, 208-10.

143. Gibbs, J. and Smith, G.P. (1988). The Actions of Bombesin-Like Peptides on Food Intake. *Annals of the New York Academy of Sciences*, **547**, 210-16.

144. Gibbs, J. and Smith, G.P. (2001). Satiety: the roles of peptides from the stomach and the intestine. *Federation Proceedings*, **45**, 1391-95.

145. Gillies, G.E., Linton, E.A. and Lowry, P.J. (1982). Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature*, **299**, 355-57.

146. Girard, F., Aube, C., St Pierre, S. and Jolicoeur, F.B. (1983). Structure-activity studies on neurobehavioral effects of bombesin (BB) and gastrin releasing peptide (GRP). *Neuropeptides*, **3**, 443-52.

147. Giraud, A.S., Smith, A.I. and Rundle, S.E. (1987). Distribution and molecular forms of immunoreactive bombesin in the ovine median eminence. *Molecular and Cellular Endocrinology*, **53**, 245-50.

148. Gmerek, D.E. and Cowan, A. (1983). Studies on bombesin-induced grooming in rats. *Peptides*, **4**, 907-13.

149. Gomez, F., Lahmame, A., de Kloet, E.R. and Armario, A. (1996). Hypothalamic-pituitary-adrenal responses to chronic stress in five inbred rat strains: differential responses are mainly located at the adrenocortical level. *Neuroendocrinology*, **63**, 327-37.

150. Gorbulev, V., Akhundova, A., Büchner, H. and Fahrenholz, F. (1992). Molecular cloning of a new bombesin receptor subtype expressed in uterus during pregnancy. *European Journal of Biochemistry*, **208**, 405-10.

151. Gordon, J.A. and Hen, R. (2004). The serotonergic system and anxiety. *NeuroMolecular Medicine*, **5**, 27-40.

152. Gosnell, B.A., Krahn, D.D. and Majchrzak, M.J. (1990). The effects of morphine on diet selection are dependent upon baseline diet preferences. *Pharmacology, Biochemistry & Behavior*, **37**, 207-12.

154. Graeff, F.G., Viana, M.B.and Mora, P.O. (1997). Dual role of 5-HT in defense and anxiety. *Neuroscience and Biobehavioral Reviews,* **21(6)**, 791-9.

154. Greba, Q., Gifkins, A. and Kokkinidis, L. (2001). Inhibition of amygdaloid dopamine D2 receptors impairs emotional learning measured with fear-potentiated startle. *Brain Research*, **899**, 218-26.

155. Greba, Q. and Kokkinidis, L. (2000). Peripheral and intraamygdalar administration of the dopamine D1 receptor antagonist SCH 23390 blocks fear-potentiated startle but not shock reactivity or the shock sensitization of acoustic startle. *Behavioral Neuroscience*, **114**, 262-72.

156. Griebel, G., Rodgers, R.J., Perrault, G. and Sanger, D.J. (2000). The effects of compounds varying in selectivity as 5-HT(1A) receptor antagonists in three rat models of anxiety. *Neuropharmacology*, **39**, 1848-57.

157. Griebel, G., Simiand, J., Serradeil-Le Gal, C., Wagnon, J., Pascal, M., Scatton, B., Maffrand, J.P. and Soubrie, P. (2002). Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 6370-75.

158. Grillon, C. (2002). Startle reactivity and anxiety disorders: aversive conditioning, context, and neurobiology. *Biological Psychiatry*, **52**, 958-75.

159. Groenink, L., van Bogaert, M. J., van der Gugten, J., Oosting, R. S., and Olivier, B. (2003). 5-HT1A receptor and 5-HT1B receptor knockout mice in stress and anxiety paradigms. *Behavioral Pharmacology, 14*(**5-6**), 369-383.

160. Gross, C., Santarelli, L., Brunner, D., Zhuang, X. and Hen, R. (2000). Altered fear circuits in 5-HT(1A) receptor KO mice. *Biological Psychiatry*, **48**, 1157-63.

161. Grosskreutz, C.L. and Brody, M.J. (1988). Regional hemodynamic responses to central administration of corticotropin-releasing factor (CRF). *Brain Research*, **442**, 363-67.

162. Guarraci, F.A., Frohardt, R.J., Falls, W.A. and Kapp, B.S. (2000). The effects of intra-amygdaloid infusions of a D2 dopamine receptor antagonist on Pavlovian fear conditioning. *Behavioral Neuroscience*, **114**, 647-51.

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

164. Gutzwiller, J.-P., Drewe, J., Hildebrand, P., Rossi, L., Lauper, J.Z. and Beglinger, C. (1994). Effect of intravenous human gastrin-releasing peptide on food intake in humans. *Gastroenterology*, **106**, 1168-73.

165. Halford, J.C., Cooper, G.D. and Dovey, T.M. (2004). The pharmacology of human appetite expression. *Current Drug Targets*, **5**, 221-40.

166. Handa, R.J., George, M., Gordon, B.H., Campbell, D.B. and Lorens, S.A. (1996). Responses to novelty stress in female F344 rats: effects of age and d-fenfluramine treatment. *Pharmacology, Biochemistry & Behavior*, **53**, 641-47.

167. Harris, J.A. and Westbrook, R.F. (1995). Effects of benzodiazepine microinjection into the amygdala or periaqueductal gray on the expression of conditioned fear and hypoalgesia in rats. *Behavioral Neuroscience*, **109**, 295-304.

168. Hashimoto, S., Inoue, T. and Koyama, T. (1999). Effects of conditioned fear stress on serotonin neurotransmission and freezing behavior in rats. *European Journal of Pharmacology*, **378**, 23-30.

169. Hauger, R.L. and Aguilera, G. (1993). Regulation of pituitary corticotropin releasing hormone (CRH) receptors by CRH: Interaction with vasopressin. *Endocrinology*, **133**, 1708-14.

170. Hauger, R.L., Millan, M.A., Lorang, M., Harwood, J.P. and Aguilera, G. (1988). Corticotropin-releasing factor receptors and pituitary adrenal responses during immobilization stress. *Endocrinology*, **123**, 396-405.

171. Hayley, S., Borowski, T., Merali, Z. and Anisman, H. (2001). Central monoamine activity in genetically distinct strains of mice following a psychogenic stressor: effects of predator exposure. *Brain Research*, **892**, 293-300.

172. Hearn, S.C., Jones, P.M., Ghatei, M.A., Byrne, J., Hill, S.F. and Bloom, S.R. (1992). The presence, characterisation and synthesis of neuromedin B in the human pituitary gland. *Neuroendocrinology*, **56**, 729-34.

173. Hebb, A.L., Zacharko, R.M., Bowie, J.P. and Drolet, G. (2003). Differential startle reactivity following central CCK-8S and systemic Boc CCK-4 administration in mice: antecedent stressor history and testing condition. *Behavioral Neuroscience*, **117**, 704-15.

174. Heim, C., Owens, M.J., Plotsky, P.M. and Nemeroff, C.B. (1997). Persistent changes in corticotropin-releasing factor systems due to early life stress: relationship to the pathophysiology of major depression and post-traumatic stress disorder. *Psychopharmacology bulletin*, **33**, 185-92.

175. Heinrichs, S.C., Britton, K.T. and Koob, G.F. (1991). Both conditioned taste preference and aversion induced by corticotropin-releasing factor. *Pharmacology, Biochemistry & Behavior*, **40**, 717-21.

176. Heinrichs, S.C., Cole, B.J., Pich, E.M., Menzaghi, F., Koob, G.F. and Hauger, R.L. (1992). Endogenous corticotropin-releasing factor modulates feeding induced by neuropeptide Y or a tail-pinch stressor. *Peptides*, **13**, 879-84.

177. Heinrichs, S.C., Min, H., Tamraz, S., Carmouche, M., Boehme, S.A. and Vale, W.W. (1997). Anti-sexual and anxiogenic behavioral consequences of corticotropin-releasing factor overexpression are centrally mediated. *Psychoneuroendocrinology*, **22**, 215-24.

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

179. Herman, J.P., Cullinan, W.E. and Watson, S.J. (1994). Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. *Journal of Neuroendocrinology*, **6**, 433-42.

180. Herold, C.L., Behm, D.J., Buckley, P.T., Foley, J.J., Wixted, W.E., Sarau, H.M. and Douglas, S.A. (2003). The neuromedin B receptor antagonist, BIM-23127, is a potent antagonist at human and rat urotensin-II receptors. *British Journal of Pharmacology*, **139**, 203-07.

181. Higgins, G.A., Bradbury, A.J., Jones, B.J. and Oakley, N.R. (1988). Behavioural and biochemical consequences following activation of 5HT1-like and GABA receptors in the dorsal raphe nucleus of the rat. *Neuropharmacology*, **27**, 993-1001.

182. Higgins, G.A., Jones, B.J. and Oakley, N.R. (1992). Effect of 5-HT1A receptor agonists in two models of anxiety after dorsal raphe injection. *Psychopharmacology*, **106**, 261-67.

183. Hitchcock, J.M. and Davis, M. (1987). Fear-potentiated startle using an auditory conditioned stimulus: effect of lesions of the amygdala. *Physiology & Behavior*, **39**, 403-08.

184. Ho, S.P., Takahashi, L.K., Livanov, V., Spencer, K., Lesher, T. et al. (2001). Attenuation of fear conditioning by antisense inhibition of brain corticotropin releasing factor-2 receptor. *Molecular Brain Research*, **89**, 29-40.

185. Hosotani, R., Inoue, K., Fujii, N., Yajima, H. and Tobe, T. (1985). Effect of synthetic neuromedin C, a decapeptide of gastrin-releasing peptide (GRP [18-27]), on blood flow and exocrine secretion of the pancreas in dogs. *Life Sciences*, **36**, 2429-34.

186. Hotta, M., Shibasaki, T., Arai, K. and Demura, H. (1999). Corticotropin-releasing factor receptor type 1 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats. *Brain Research*, **823**, 221-25.

187. Hotta, M., Shibasaki, T., Masuda, A., Imaki, T., Demura, H., Ling, N. and Shizume, K. (1986). The responses of plasma adrenocorticotropin and cortisol to corticotropin-releasing hormone (CRH) and cerebrospinal fluid

immunoreactive CRH in anorexia nervosa patients. *Journal of Clinical Endocrinology & Metabolism*, **62**, 319-24.

188. Houben, H., Andries, M. and Denef, C. (1994). Autoradiographic demonstration of 125I-Tyr4-bombesin binding sites on rat anterior pituitary cells. *Peptides*, **15**, 1289-95.

189. Houben, H., Vandenbroucke, A.-T., Verheyden, A.-M. and Denef, C. (1993). Expression of the genes encoding bombesin-related peptides and their receptors in anterior pituitary tissue. *Molecular and Cellular Endocrinology*, **97**, 159-64.

190. Imaki, T., Shibasaki, T., Masuda, A., Demura, H., Shizume, K. and Ling, N. (1987). Effects of adrenergic blockers on corticotropin-releasing factor-induced behavioral changes in rats. *Regulatory Peptides*, **19**, 243-51.

191. Imaki, T., Xiao-Quan, W., Shibasaki, T., Yamada, K., Harada, S., Chikada, N., Naruse, M. and Demura, H. (1995). Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *Journal of Clinical Investigation*, **96**, 231-38.

192. Inoue, T., Izumi, T., Maki, Y., Muraki, I. and Koyama, T. (2000). Effect of the dopamine D(1/5) antagonist SCH 23390 on the acquisition of conditioned fear. *Pharmacology, Biochemistry & Behavior*, **66**, 573-78.

193. Inoue, T., Tsuchiya, K. and Koyama, T. (1996). Serotonergic activation reduces defensive freezing in the conditioned fear paradigm. *Pharmacology, Biochemistry & Behavior*, **53**, 825-31.

194. Itoh, S., Takashima, A., Itoh, T. and Morimoto, T. (1994). Open-field behavior of rats following intracerebroventricular administration of neuromedin B, neuromedin C, and related amphibian peptides. *Japanese Jounral of Physiology*, **44**, 271-81.

195. Jacobs, B. L., and Fornal, C. A. (1999). Activity of serotonergic neurons in behaving animals. *Neuropsychopharmacology, 21*(**2 Suppl**), 9S-15S.

196. Jahkel, M., Oehler, J. and Schumacher, H.E. (1994). Influence of nootropic and antidepressive drugs on open field and running wheel behavior in spontaneously high and low active mice. *Pharmacology, Biochemistry & Behavior*, **49**, 263-69.

197. Johnson, E.O., Kamilaris, T.C., Chrousos, G.P. and Gold, P.W. (1992). Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neuroscience andBiobehavioral Reviews*, **16**, 115-30.

198. Johnston, S.A. and Merali, Z. (1988). Specific neuroanatomical and neurochemical correlates of locomotor and grooming effects of bombesin. *Peptides*, **9 (suppl.1)**, 245-56.

199. Jones, P.M., Withers, D.J., Gathei, M.A. and Bloom, S.R. (1992). Evidence for neuromedin-B synthesis in the rat anterior pituitary gland. *Endocrinology*, **130**, 1829-36.

200. Kalen, P., Strecker, R.E., Rosengren, E. and Bjorklund, A. (1989). Regulation of striatal serotonin release by the lateral habenula-dorsal raphe pathway in the rat as demonstrated by in vivo microdialysis: role of excitatory amino acids and GABA. *Brain Research*, **492**, 187-202.

201. Kalin, N.H., Sherman, J.E. and Takahashi, L.K. (1988). Antagonism of endogenous CRH systems attenuates stress-induced freezing behavior in rats. *Brain Research*, **457**, 130-35.

202. Kandel, E.R., Klein, M., Castellucci, V.F., Schacher, S. and Goelet, P. (1986). Some principles emerging from the study of short- and long-term memory. *Neuroscience Research*, **3**, 498-520.

203. Kant, G.J., Anderson, S.M., Dhillon, G.S., Mougey and EH. (1988). Neuroendocrine correlates of sustained stress: the activity-stress paradigm. *Brain Research Bulletin*, **20**, 407-14.

204. Karlsson, S., Sundler, F. and Ahren, B. (1998). CCK receptor subtype in insulin-producing cells: a combined functional and in situ hybridization study in rat islets and a rat insulinoma cell line. *Regulatory Peptides*, **78**, 95-103.

205. Karlsson, S., Sundler, F. and Ahren, B. (2001). Direct cytoplasmic CA(2+) responses to gastrin-releasing peptide in single beta cells. *Biochemical & Biophysical Research Communications*, **280**, 610-14.

206. Karolyi, I.J., Burrows, H.L., Ramesh, T.M., Nakajima, M., Lesh, J.S., Seong, E., Camper, S.A. and Seasholtz, A.F. (1999). Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11595-600.

207. Katoh, K., Asari, M., Ishiwata, H., Sasaki, Y. and Obara, Y. (2004). Saturated fatty acids suppress adrenocorticotropic hormone (ACTH) release from rat anterior pituitary cells in vitro. *Comparative Biochemistry & Physiology*, **137**, 357-364.

208. Kawai, K., Chiba, Y., Mukai, H., Munekata, E. and Yamashita, K. (1988). Effects of neuromedin B, gastrin-releasing peptide-10 and their fragment peptides on secretion of gastrointestinal and pancreatic hormones in dogs. *Acta Endocrinologica*, **117**, 205-13.

209. Kawai, K., Mukai, H., Chiba, Y., Ohmori, H., Suzuki, S., Munekata, E. and Yamashita, K. (1989). Effects of neuromedin B on insulin and glucagon release from the isolated perfused rat pancreas. *Endocrinologia Japonica*, **36**, 587-94.

210. Kendler, K.S., Karkowski, L.M. and Prescott, C.A. (1999). Causal relationship between stressful life events and the onset of major depression. *American Journal of Psychiatry*, **156**, 837-41.

211. Kent, P., Anisman, H. and Merali, Z. (1997). Are bombesin-like peptides involved in the mediation of stress response? *Life Sciences*, **62**, 103-14.

212. Kent, P., Anisman, H. and Merali, Z. (2001). Central bombesin activates the hypothalamic-pituitary-adrenal axis: Effects on regional levels and release of corticotropin-releasing hormone and arginine vasopressin. *Neuroendocrinology*, **73**, 203-14.

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

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

215. Kim, J.J. and Fanselow, M.S. (1992). Modality-specific retrograde amnesia of fear. *Science*, **256**, 675-77.

216. Kim, M., Campeau, S., Falls, W.A. and Davis, M. (1993). Infusion of the non-NMDA receptor antagonist CNQX into the amygdala blocks the expression of fear-potentiated startle. *Behavioral & Neural Biology*, **59**, 5-8.

217. Kimbrell, T.A., George, M.S., Parekh, P.I., Ketter, T.A., Podell, D.M. et al. (1999). Regional brain activity during transient self-induced anxiety and anger in healthy adults. *Biological Psychiatry*, **46**, 454-65.

218. Kirby, L.G., Rice, K.C. and Valentino, R.J. (2000). Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology*, **22**, 148-62.

219. Kiss, A. and Aguilera, G. (1993). Regulation of the hypothalamic pituitary adrenal axis during chronic stress: responses to repeated intraperitoneal hypertonic saline injection. *Brain Research*, **630**, 262-70.

220. Kittner, H., Hoffmann, E., Krugel, U. and Illes, P. (2004). P2 receptor-mediated effects on the open field behaviour of rats in comparison with behavioural responses induced by the stimulation of dopamine D2-like and by the blockade of ionotrophic glutamate receptors. *Behavioural Brain Research*, **149**, 197-208.

221. Knight, D.C., Smith, C.N., Stein, E.A. and Helmstetter, F.J. (1999). Functional MRI of human Pavlovian fear conditioning: patterns of activation as a function of learning. *Neuroreport*, **10**, 3665-70.

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

223. Kolik, L.G., Gudasheva, T.A. and Seredenin, S.B. (2003). Pharmacogenetic study of anxiolytic effects of new cholecystokinin receptor antagonists in animals with different levels of emotionality. *Bulletin of Experimental Biology & Medicine*, **135**, 440-44.

224. Koob, G.F. (1999). Corticotropin-releasing factor, norepinephrine, and stress. *Biological Psychiatry*, **46**, 1167-80.

225. Kopin, I.J. (1995). Definitions of stress and sympathetic neuronal responses. *Annals of the New York Academy of Sciences*, **771**, 19-30.

226. Kovacs, K.J. (1998). Functional neuroanatomy of the parvocellular vasopressinergic system: transcriptional responses to stress and glucocorticoid feedback. *Progress in Brain Research*, **119**, 31-43.

227. Krahn, D.D., Gosnell, B.A., Grace, M. and Levine, A.S. (1986). CRF antagonist partially reverses CRF- and stress-induced effects on feeding. *Brain Research Bulletin*, **17**, 285-89.

228. Krishnan, K.R. (1991). Role of serotonin in hypothalamo pituitary adrenal axis escape from dexamethasone suppression. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, **15**, 637-42.

229. Kroog, G.S., Jensen, R.T. and Battey, J.F. (1995). Mammalian bombesin receptors. *Medicinal Research Reviews*, **15**, 389-417.

230. Kulkosky, P.J., Gibbs, J. and Smith, G.P. (1982). Feeding suppression and grooming repeatedly elicited by intraventricular bombesin. *Brain Research*, **242**, 194-96.

231. Kulkosky, P.J., Gibbs, J. and Smith, G.P. (1982). Behavioral Effects of Bombesin Administration in Rats. *Physiology & Behavior*, **28**, 505-12.

232. Kyrkouli, S.E., Stanley, B.G. and Leibowitz, S.F. (1987). Bombesin-Induced Anorexia: Sites of Action in the Rat Brain. *Peptides*, **8**, 237-41.

233. LaBar, K.S., Gatenby, J.C., Gore, J.C., LeDoux, J.E. and Phelps, E.A. (1998). Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. *Neuron*, **20**, 937-45.

234. Ladenheim, E. and Ritter, R.C. (1993). Caudal hindbrain participation in the suppression of feeding by central and peripheral bombesin. *American Journal of Physiology*, **264**, R1229-R1234.

235. Ladenheim, E.E., Jensen, R.T., Mantey, S.A., McHugh, P.R. and Moran, T.H. (1990). Receptor heterogeneity for bombesin-like peptides in the rat central nervous system. *Brain Research*, **537**, 233-40.

236. Ladenheim, E.E., Jensen, R.T., Mantey, S.A. and Moran, T.H. (1992). Distinct distributions of two bombesin receptor subtypes in the rat central nervous system. *Brain Research*, **593**, 168-78.

237. Ladenheim, E.E., Jensen, R.T., Mantey, S.A., Taylor, J.E., Coy, D.H. and Moran, T.H. (1993). Bombesin receptor antagonists differentiate receptor subtypes in rat brain. *Euopean Jounral of Pharmacology*, **235**, 121-25.

238. Ladenheim, E.E. and Ritter, R.C. (1988). Low-dose fourth ventricular bombesin selectively suppresses food intake. *American Jounral of Physiology*, **255**, R988-R992.

239. Ladenheim, E.E., Wirth, K.E. and Moran, T.H. (1996). Receptor subtype mediation of feeding suppression by bombesin-like peptides. *Pharmacology, Biochemistry & Behavior*, **54**, 705-11.

240. Ladenheim, E.E., Wohn, A., White, W.O., Schwartz, G.J. and Moran, T.H. (1999). Inhibition of gastric emptying by bombesin-like peptides is dependent upon cholecystokinin-A receptor activation. *Regulatory Peptides*, **84**, 101-06.

241. Ladenheim, E.E., Taylor,J.E., Coy,D.H., Moore,K.A.and Moran,T.H. (1996.) Hindbrain GRP receptor blockade antagonizes feeding suppression by peripherally administered GRP, *American Journal of Physiology* 271 :R180 (Abstr.)

242. Ladenheim, E.E., Taylor,J.E., Coy,D.H.and Moran,T.H. (1994.) Blockade of feeding inhibition by neuromedin B using a selective receptor antagonist, *European Journal of Pharmacology* 271 :R7 (Abstr.)

242. Lahdesmaki, J., Sallinen, J., MacDonald, E., Kobilka, B.K., Fagerholm, V. and Scheinin, M. (2002). Behavioral and neurochemical characterization of alpha(2A)-adrenergic receptor knockout mice. *Neuroscience*, **113**, 289-99.

244. Lang, P.J., Davis, M. and Ohman, A. (2000). Fear and anxiety: animal models and human cognitive psychophysiology. *Journal of Affective Disorders*, **61**, 137-59.

245. Langhans, W., Delprete, E. and Scharrer, E. (1991). Mechanisms of vasopressin's anorectic effect. *Physiology & Behavior*, **49**, 169-76.

246. Lapiz, M.D., Mateo, Y., Parker, T. and Marsden, C. (2000). Effects of noradrenaline depletion in the brain on response on novelty in isolation-reared rats. *Psychopharmacology*, **152**, 312-20.

247. Layton, B. and Krikorian, R. (2002). Memory mechanisms in posttraumatic stress disorder. *Journal of Neuropsychiatry & Clinical Neurosciences*, **14**, 254-61.

248. LeDoux, J.E. (1994). Emotion, memory and the brain. [Review] [3 refs]. *Scientific American*, **270**, 50-57.

249. LeDoux, J.E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience*, **23**, 155-84.

250. LeDoux, J.E., Cicchetti, P., Xagoraris, A. and Romanski, L.M. (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *Journal of Neuroscience*, **10**, 1062-69.

251. LeDoux, J.E., Iwata, J., Cicchetti, P. and Reis, D.J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *Journal of Neuroscience*, **8**, 2517-29.

252. Lee, E.H., Tang, Y.P. and Chai, C.Y. (1987). Stress and corticotropin-releasing factor potentiate center region activity of mice in an open field. *Psychopharmacology*, **93**, 320-23.

253. Lee, E.H. and Tsai, M.J. (1989). The hippocampus and amygdala mediate the locomotor stimulating effects of corticotropin-releasing factor in mice. *Behavioral & Neural Biology*, **51**, 412-23.

254. Lee, K., Dixon, A.K., Gonzalez, I., Stevens, E.B., McNulty, S., Oles, R., Richardson, P.J., Pinnock, R.D. and Singh, L. (1999). Bombesin-like peptides depolarize rat hippocampal interneurones through interaction with subtype 2 bombesin receptors. *Journal of Physiology,* **518**, 791-802.

255. Lee, Y. and Davis, M. (1997). Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle response. *Journal of Neuroscience*, **17**, 6434-46.

256. Lee, Y., Lopez, D.E., Meloni, E.G. and Davis, M. (1996). A primary acoustic startle pathway: obligatory role of cochlear root neurons and the nucleus reticularis pontis caudalis. *Journal of Neuroscience*, **16**, 3775-89.

257. Lelas, S., Wong, H., Li, Y.W., Heman, K.L., Ward, K.A. et al. (2004). Anxiolytic-like effects of the corticotropin-releasing factor1 (CRF1) antagonist DMP904 [4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-a]-pyrimidine] administered acutely or chronically at doses occupying central CRF1 receptors in rats. *Journal of Pharmacology and Experimental Therapeutics*, **309**, 293-302.

258. Lenz, H.J., Forquignon, I., Drüge, G. and Greten, H. (1989). Effects of neuropeptides on gastric acid and duodenal bicarbonate secretions in freely moving rats. *Regulatroy Peptides*, **24**, 293-300.

259. Lenz, H.J., Klapdor, R., Hester, S.E., Webb, V.J., Galyean, R.F., Rivier, J.E. and Brown, M.R. (1986). Inhibition of gastric acid secretion by brain peptide in the dog. *Gastroenterology*, **91**, 905-12.

260. Lesch, K.P., Zeng, Y., Reif, A. and Gutknecht, L. (2003). Anxiety-related traits in mice with modified genes of the serotonergic pathway. *European Journal of Pharmacology*, **480**, 185-204.

261. Liao, J.F., Hung, W.Y. and Chen, C.F. (2003). Anxiolytic-like effects of baicalein and baicalin in the Vogel conflict test in mice. *European Journal of Pharmacology*, **464**, 141-46.

262. Liebsch, G., Landgraf, R., Engelmann, M., Lorscher, P. and Holsboer, F. (1999). Differential behavioural effects of chronic infusion of CRH 1 and CRH

2 receptor antisense oligonucleotides into the rat brain. *Journal of Psychiatric Research*, **33**, 153-63.

263. Linthorst, A.C., Penalva, R.G., Flachskamm, C., Holsboer, F. and Reul, J.M. (2002). Forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotropin-releasing hormone receptor-dependent mechanism. *European Jounral of .Neuroscience*, **16**, 2441-52.

264. Lovejoy, D.A., Aubry, J.M., Turnbull, A., Sutton, S., Potter, E., Yehling, J., Rivier, C. and Vale, W.W. (1998). Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain. *Journal of Neuroendocrinology*, **10**, 483-91.

265. Lowry, C.A. (2002). Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. [Review] [156 refs]. *Journal of Neuroendocrinology*, **14**, 911-23.

266. Lucki, I., Ward, H.R. and Frazer, A. (1989). Effect of 1-(m-chlorophenyl)piperazine and 1-(m-trifluoromethylphenyl)piperazine on locomotor activity. *Journal of Pharmacology & Experimental Therapeutics*, **249**, 155-64.

267. Lundkvist, J., Chai, Z., Teheranian, R., Hasanvan, H., Bartfai, T., Jenck, F., Widmer, U. and Moreau, J.L. (1996). A non peptidic corticotropin releasing factor receptor antagonist attenuates fever and exhibits anxiolytic-like activity. *European Journal of Pharmacology*, **309**, 195-200.

268. Ma, X.M. and Aguilera, G. (1999). Transcriptional responses of the vasopressin and corticotropin-releasing hormone genes to acute and repeated intraperitoneal hypertonic saline injection in rats. *Molecular Brain Research*, **68**, 129-40.

269. Ma, X.M., Levy, A. and Lightman, S.L. (1997). Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *Journal of Endocrinology*, **152**, 81-89.

270. MacDonald, J.F., Bartlett, M.C., Mody, I., Reynolds, J.N. and Salter, M.W. (1990). The PCP site of the NMDA receptor complex. *Advances in Experimental Medicine and Biology*, **268**, 27-34.

271. Maciag, C.M., Dent, G., Gilligan, P., He, L., Dowling, K., Ko, T., Levine, S. and Smith, M.A. (2002). Effects of a non-peptide CRF antagonist (DMP696) on the behavioral and endocrine sequelae of maternal separation. *Neuropsychopharmacology*, **26**, 574-82.

272. Maidment, N.T., Evans, C.J. (1991). Measurement of extracellular neuropeptides in the brain: microdialysis linked to solid-phase radioimmunoassays with sub-femptomole limits of detection. In: Robinson,T.E. and Justice,J.B.(Eds.), *Techniques in the Behavioral and Neural Sciences*, Elsevier, The Netherlands, 275-303.

273. Makino, S., Smith, M.A. and Gold, P.W. (1995). Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology*, **136**, 3299-309.

274. Malendowicz, L.K. (1998). Role of neuromedins in the regulation of adrenocortical function. *Hormone & Metabolic Research*, **30**, 374-83.

275. Malendowicz, L.K., Macchi, C., Nowak, M., Nussdorfer, G.G. and Majchrzak, M. (1995). Neuromedin-B enhances blood corticosterone concentration via an ACTH-independent mechanism and accelerates dexamethasone- induced adrenal atrophy in the rat. *Neuroendocrinol.Lett.*, **17**, 29-36.

276. Malendowicz, L.K., Macchi, C., Nussdorfer, G.G. and Nowak, M. (1996). The role of neuromedin B in the regulation of rat pituitary-adrenocortical function. *Histology & Histopathology*, **11**, 895-97.

277. Malendowicz, L.K. and Nussdorfer, G.G. (1995). Investigations on the acute effects of neuropeptides on the pituitary-adrenocortical function in normal and cold- stressed rats. I. Bombesin and neuromedin B. *Experimental and Toxicologic Pathology*, **47**, 31-34.

278. Malpas, S.C. (1994). Role of vasopressin in sympathetic response to paraventricular nucleus stimulation in anesthetized rats. *American Journal of Physiology*, **266**, R228-R236.

279. Maren, S. and Quirk, G.J. (2004). Neuronal signalling of fear memory. *Nature Reviews Neuroscience*, **5**, 844-52.

280. Marks, I.M. (1987). Behavioral aspects of panic disorder. *American Journal of Psychiatry*, **144**, 1160-65.

281. Marti, O., Gavalda, A., Gomez, F. and Armario, A. (1994). Direct evidence for chronic stress-induced facilitation of the adrenocorticotropin response to a novel acute stressor. *Neuroendocrinology*, **60**, 1-7.

282. Marti, O., Harbuz, M.S., Andres, R., Lightman, SL and Armario, A. (1999). Activation of the hypothalamic-pituitary axis in adrenalectomised rats: potentiation by chronic stress. *Brain Research*, **821**, 1-7.

283. Martin, D.S. and Haywood, J.R. (1992). Sympathetic nervous system activation by glutamate injections into the paraventricular nucleus. *Brain Research*, **577**, 261-67.

284. Masui, A., Kato, N., Itoshima, T., Tsunashima, K., Nakajima, T. and Yanaihara, N. (1993). Scratching behavior induced by bombesin-related peptides. Comparison of bombesin, gastrin-releasing peptide and phyllolitorins. *European Jounral of Pharmacology*, **238**, 297-301.

285. Matsumoto, Y., Abe, M., Watanabe, T., Adachi, Y., Yano, T. et al. (2004). Intracerebroventricular administration of urotensin II promotes anxiogenic-like behaviors in rodents. *Neuroscience Letters*, **358**, 99-102.

286. McCall, R. B., Patel, B. N., & Harris, L. T. (1987). Effects of serotonin1 and serotonin2 receptor agonists and antagonists on blood pressure, heart rate and sympathetic nerve activity. *Jounral of Pharmacology and Experimental Therapeurtics, 242*(**3**), 1152-1159.

287. McCoy, J.G. and Avery, D.D. (1990). Bombesin: Potential integrative peptide for feeding and satiety. *Peptides*, **11**, 595-607.

288. McDonald, T.J., Houghton, P., Challis, J.R. and Hramiak, I.M. (1988). The effect of gastrin-releasing peptide on the endocrine pancreas. *Annals of the New York Academy of Sciences*, **547**, 242-54.

289. McDonald, T.J., Jornvall, H., Nilsson, G., Vagne, M., Ghatei, M.A., Bloom, S.R. and Mutt, V. (1979). Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochem.Biophys.Res.Commun.*, **90**, 227-37.

290. McGaugh, J.L. (1989). Dissociating learning and performance: drug and hormone enhancement of memory storage. *Brain Research Bulletin*, **23**, 339-45.

291. McGaugh, J.L. (1989). Involvement of hormonal and neuromodulatory systems in the regulation of memory storage. *Annual Review of Neuroscience*, **12**, 255-87.

292. McNaughton, N. and Corr, P.J. (2004). A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. *Neuroscience & Biobehavioral Reviews*, **28**, 285-305.

293. McNaughton, N. and Gray, J.A. (2000). Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. *Journal of Affective Disorders*, **61**, 161-76.

294. Meert, T.F. (1997). Antagonism of meta-chlorophenylpiperazine-induced inhibition of exploratory activity in an emergence procedure, the open field test, in rats. *Behavioural Pharmacology*, **8**, 353-63.

295. Melik, E., Babar-Melik, E., Ozgunen, T. and Binokay, S. (2000). Median raphe nucleus mediates forming long-term but not short-term contextual fear conditioning in rats. *Behavioural Brain Research*, **112**, 145-50.

296. Merali, Z., Johnston, S. and Zalcman, S. (1983). Bombesin-induced behavioral changes: Antagonism by neuroleptics. *Peptides*, **4**, 693-97.

297. Merali, Z., Kent, P. and Anisman, H. (2002). Role of bombesin-related peptides in the mediation or integration of the stress response. *Cellular and Molecular Life Sciences*, **59**, 272-87.

298. Merali, Z., Kent, P., Michaud, D., McIntyre, D. and Anisman, H. (2001). Differential impact of predator or immobilization stressors on central corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rat. *Brain Research*, **906**, 60-73.

299. Merali, Z., Khan, S., Michaud, D.S., Shippy, S.A. and Anisman, H. (2004). Does amygdaloid corticotropin-releasing hormone (CRH) mediate anxiety-like behaviors? Dissociation of anxiogenic effects and CRH release. *European Journal of Neuroscience*, **20**, 229-39.

300. Merali, Z., McIntosh, J. and Anisman, H. (1999). Role of bombesin-related peptides in the control of food intake. *Neuropeptides*, **33**, 376-86.

301. Merali, Z., McIntosh, J., and Anisman, H. 2004. Anticipatory cues differentially provoke in vivo peptidergic and monoaminergic release at the medial prefrontal cortex. *Neuropsychopharmacology*. (Abstr.)

302. Merali, Z., McIntosh, J., Kent, P., Michaud, D. and Anisman, H. (1998). Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *Journal of Neuroscience*, **18**, 4758-66.

303. Merali, Z., Moody, T., Kateb, P. and Piggins, H. (1988). Antagonism of Satiety and Grooming Effects of Bombesin by Antiserum to Bombesin and by [Try^{4}, D-Phe^{12}}Bombesin: Central versus Peripheral Effects. *Annals of the New York Academy of Sciences*, **547**, 489-92.

304. Merali,Z., Plamondon,H., Kent,P.and Banks,K. (1994.) Corticotropin-releasing factor (CRF) antagonist blocks the autonomic, endocrine, ingestive and behavioral effects of central bombesin (BN), *Soc.Neurosci.Abs.* 528.1(24th Ann. Meeting) (Abstr.)

305. Mercer, J.G. and Speakman, J.R. (2001). Hypothalamic neuropeptide mechanims for regulating energy balance: from rodent models to human obeisity. *Neuroscience & Biobehavioral Reviews*, **25**, 101-16.

306. Michelson, D., Licinio, J.and Gold, P.W. (1995). Mediation of the stress response by the hypothalamic-pituitary-adrenal axis. In: Freidman,M.J. and Charney,D.S.(Eds.), *Neurobiological and clinical consequences of stress*, Lippincott-Raven Publishers, Philadelphia,NY, 225-38.

307. Millan, M.J. (2003). The neurobiology and control of anxious states. *Progress in Neurobiology*, **70**, 83-244.

308. Millan, M.J., Brocco, M., Gobert, A., Dorey, G., Casara, P. and Dekeyne, A. (2001). Anxiolytic properties of the selective, non-peptidergic CRF(1) antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology*, **25**, 585-600.

309. Millan, M. J., Brocco, M., Papp, M., Serres, F., La Rochelle, C. D. and Sharp, T. (2004). S32504, a novel naphtoxazine agonist at dopamine D3/D2 receptors: III. Actions in models of potential antidepressive and anxiolytic activity in comparison with ropinirole. *Journal of Pharmacology and Experimental Therapeutics, **309**(3)*, 936-950.

310. Millan, M. J., Maiofiss, L., Cussac, D., Audinot, V., Boutin, J. A., and Newman-Tancredi, A. (2002). Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I. A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics, **303**(2)*, 791-804.

311. Millan, M.J., Seguin, L., Gobert, A., Cussac, D. and Brocco, M. (2004). The role of dopamine D3 compared with D2 receptors in the control of locomotor activity: a combined behavioural and neurochemical analysis with novel, selective antagonists in rats. *Psychopharmacology*, **174**, 341-57.

312. Minamino, N., Kangawa, K. and Matsuo, H. (1983). Neuromedin B : a novel bombesin-like peptide identified in porcine spinal cord. *Biochem.Biophys.Res.Commun.*, **114(2)**, 541-48.

313. Minamino, N., Kangawa, K. and Matsuo, H. (1984). Neuromedin B is a major bombesin-like peptide in rat brain: regional distribution of neuromedin B and neuromedin C in rat brain, pituitary and spinal cord. *Biochem.Biophys.Res.Commun.*, **124(3)**, 925-32.

314. Minamino, N., Kangawa, K. and Matsuo, H. (1984). Neuromedin C: a bombesin-like peptide identified in porcine spinal cord. *Biochem.Biophys.Res.Commun.*, **119(1)**, 14-20.

315. Minamino, N., Kangawa, K. and Matsuo, H. (1988). Neuromedin B and neuromedin C: Two mammalian bombesin-like peptides identified in porcine spinal cord and brain. *Annals of the New York Academy of Sciences*, **547**, 373-90.

316. Minamino, N., Sudoh, T., Kangawa, K. and Matsuo, H. (1985). Neuromedins: novel smooth-muscle stimulating peptides identified in porcine spinal cord. *Peptides*, **6(suppl.3)**, 245-48.

317. Mintz, E.M., Gillespie, C.F., Marvel, C.L., Huhman, K.L. and Albers, H.E. (1997). Serotonergic regulation of circadian rhythms in Syrian hamsters. *Neuroscience*, **79**, 563-69.

318. Miyasaka, K., Yoshida, Y., Matsushita, S., Higuchi, S., Shirakawa, O., Shimokata, H. and Funakoshi, A. (2004). Association of cholecystokinin-A receptor gene polymorphisms and panic disorder. *American Journal of Medical Genetics*, **127**, 78-80.

319. Moghaddam, B., and Bunney, B. S. (1989). Ionic composition of microdialysis perfusing solution alters the pharmacological responsiveness and basal outflow of striatal dopamine. *Journal of Neurochemisrty, 53*(**2**), 652-654.

320. Moghimzadeh, E., Ekman, R., Hakanson, R., Yanaihara, N. and Sundler, F. (1983). Neuronal gastrin-releasing peptide in the mammalian gut and pancreas. *Neuroscience*, **10**, 553-63.

321. Molewijk, H.E., Hartog, K., van der Poel, A.M., Mos, J. and Olivier, B. (1996). Reduction of guinea pig pup isolation calls by anxiolytic and antidepressant drugs. *Psychopharmacology*, **128**, 31-38.

322. Molewijk, H.E., Hartog, K., van der Poel, A.M., Mos, J. and Olivier, B. (1996). Reduction of guinea pig pup isolation calls by anxiolytic and antidepressant drugs. *Psychopharmacology*, **128**, 31-38.

323. Moody, T.W., Getz, R., O'Donohue, T.L. and Rosenstein, J.M. (1988). Localization of receptors for bombesin-like peptides in the rat brain. *Annals of the New York Academy of Sciences*, **547**, 114-30.

324. Moody, T.W., Korman, L.Y. and O'Donohue, T.L. (1986). Neuromedin B-like peptides in rat brain: biochemical characterization, mechanism of release and localization in synaptosomes. *Peptides*, **7**, 815-20.

325. Moody, T.W. and Merali, Z. (2004). Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. *Peptides*, **25**, 511-20.

326. Moody, T.W., O'Donohue, T.L. and Jacobowitz, D.M. (1981). Biochemical localization and characterization of bombesin-like peptides in discrete regions of rat brain. *Peptides*, **2**, 75-80.

327. Morgan, M.A. and LeDoux, J.E. (1995). Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behavioral Neuroscience*, **109**, 681-88.

328. Morgan, M.A., Romanski, L.M. and LeDoux, J.E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience Letters*, **163**, 109-13.

329. Morilak, D.A. and Frazer, A. (2004). Antidepressants and brain monoaminergic systems: a dimensional approach to understanding their behavioural effects in depression and anxiety disorders. *International Journal of Neuropsychopharmacology*, **7**, 193-218.

330. Morley, J.E. and Levine, A.S. (1981). Bombesin inhibits stress-induced eating. *Pharmacology, Biochemistry and Behavior*, **14**, 149-51.

331. Morrow, B.A., Redmond, A.J., Roth, R.H. and Elsworth, J.D. (2000). The predator odor, TMT, displays a unique, stress-like pattern of dopaminergic and endocrinological activation in the rat. *Brain Research*, **864**, 146-51.

332. Muglia, L., Jacobson, L., Dikkes, P. and Majzoub, J.A. (1995). Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature*, **373**, 427-32.

333. Muglia, L.J., Jacobson, L., Weninger, S.C., Karalis, K.P., Jeong, K. and Majzoub, J.A. (2001). The physiology of corticotropin-releasing hormone deficiency in mice. *Peptides*, **22**, 725-31.

334. Nader, K. (2003). Memory traces unbound. *Trends in Neuroscence*, **26**, 65-72.

335. Nader, K., Schafe, G.E. and Le Doux, J.E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, **406**, 722-26.

336. Nagalla, S.R., Barry, B.J., Creswick, K.C., Eden, P., Taylor, J.T. and Spindel, E.R. (1995). Cloning of a receptor for amphibian [Phe^{13}]bombesin distinct from the receptor for gastrin-releasing peptide: Identification of a fourth bombesin receptor subtype (BB4). *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 6205-09.

337. Nakamura, T., Otsuki, M., Okabayashi, Y., Fujii, M., Tani, S., Oka, T., Fujisawa, T. and Baba, S. (1989). [Effects of neuromedin B and neuromedin C on insulin release from isolated perfused rat pancreas]. [Japanese]. *Nippon Naibunpi Gakkai Zasshi - Folia Endocrinologica Japonica*, **65**, 695-703.

338. Nakane, T., Audhya, T., Kanie, N. and Hollander, C.S. (1985). Evidence for a role of endogenous corticotropin-releasing factor in cold, ether, immobilization, and traumatic stress. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 1247-51.

339. Namba, M., Ghatei, M.A., Bishop, A.E., Gibson, S.J., Mann, D.J., Polak, J.M. and Bloom, S.R. (1985). Presence of neuromedin B-like immunoreactivity in the brain and gut of rat and guinea-pig. *Peptides*, **6**, 257-63.

340. Namba, M., Ghatei, M.A., Gibson, S.J., Polak, J.M. and Bloom, S.R. (1985). Distribution and localization of neuromedin B-like immunoreactivity in pig, cat and rat spinal cord. *Neuroscience*, **4**, 1217-26.

341. Netto, C.F. and Guimaraes, F.S. (2004). Anxiogenic effect of cholecystokinin in the dorsal periaqueductal gray. *Neuropsychopharmacology*, **29**, 101-07.

342. Ohata, H., Arai, K. and Shibasaki, T. (2002). Effect of chronic administration of a CRF(1) receptor antagonist, CRA1000, on locomotor activity and endocrine responses to stress. *European Journal of Pharmacology*, **457**, 201-06.

343. Ohki-Hamazaki, H. (2000). Neuromedin B. *Progress in Neurobiology*, **62**, 297-312.

344. Ohki-Hamazaki, H., Iwabuchi, M. and Maekawa, F. (2005). Development and function of bombesin-like peptides and their receptors. *International Journal of Developmental Biology*, **49**, 293-300.

345. Ohki-Hamazaki, H., Sakai, Y., Kamata, K., Ogura, H., Okuyama, S., Watase, K., Yamada, K. and Wada, K. (1999). Functional properties of two bombesin-like peptide receptors revealed by the analysis of mice lacking neuromedin B receptor. *Journal of Neuroscience*, **19**, 948-54.

346. Ohki-Hamazaki, H., Wada, E., Matsui, K. and Wada, K. (1997). Cloning and expression of the neuromedin B receptor and the third subtype of bombesin receptor genes in the mouse. *Brain Research*, **762**, 165-72.

347. Ohki-Hamazaki, H., Watase, K., Yamamoto, K., Ogura, H., Yamano, M. et al. (1997). Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature*, **390**, 165-69.

348. Olsen, L., Knigge, U. and Warberg, J. (1992). Gastrin-releasing peptide stimulation of corticotropin secretion in male rats. *Endocrinology*, **130**, 2710-16.

349. Ono, N., Samson, W.K., McDonald, J.K., Lumpkin, M.D., Bedran, d., Castro, J.C. and McCann, S.M. (1985). Effects of intravenous and intraventricular injection of antisera directed against corticotropin-releasing factor on the secretion of anterior pituitary hormones. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 7787-90.

350. Oshima, A., Flachskamm, C., Reul, J.M., Holsboer, F. and Linthorst, A.C. (2003). Altered serotonergic neurotransmission but normal hypothalamic-pituitary-adrenocortical axis activity in mice chronically treated with the corticotropin-releasing hormone receptor type 1 antagonist NBI 30775. *Neuropsychopharmacology*, **28**, 2148-59.

351. Otagiri, A., Wakabayashi, I. and Shibasaki, T. (2000). Selective corticotropin-releasing factor type 1 receptor antagonist blocks conditioned fear-induced release of noradrenaline in the hypothalamic paraventricular nucleus of rats. *Journal of Neuroendocrinology*, **12**, 1022-26.

352. Overstreet, D.H., Commissaris, R.C., De La, G.R., File, S.E., Knapp, D.J. and Seiden, L.S. (2003). Involvement of 5-HT1A receptors in animal tests of

anxiety and depression: evidence from genetic models. [Review] [70 refs]. *Stress*, **6**, 101-10.

353. Owens, M.J. and Nemeroff, C.B. (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacological Reviews*, **43**, 425-73.

354. Packard, M.G. and Teather, L.A. (1997). Double dissociation of hippocampal and dorsal-striatal memory systems by posttraining intracerebral injections of 2-amino-5-phosphonopentanoic acid. *Behavioral Neuroscience*, **111**, 543-51.

355. Packard, M.G. and Teather, L.A. (1997). Posttraining injections of MK-801 produce a time-dependent impairment of memory in two water maze tasks. *Neurobiology of Learning & Memory*, **68**, 42-50.

356. Panula, P., Yang, H.Y.T. and Costa, E. (1982). Neuronal location of the bombesin-like immunoreactivity in the central nervous system of the rat. *Regulatory Peptides*, **4**, 275-83.

357. Pape, H.C. and Stork, O. (2003). Genes and mechanisms in the amygdala involved in the formation of fear memory. *Annals of the New York Academy of Sciences*, **985**, 92-105.

358. Pardon, M.C., Gould, G.G., Garcia, A., Phillips, L., Cook, M.C., Miller, S.A., Mason, P.A. and Morilak, D.A. (2002). Stress reactivity of the brain noradrenergic system in three rat strains differing in their neuroendocrine and behavioral responses to stress: implications for susceptibility to stress-related neuropsychiatric disorders. *Neuroscience*, **115**, 229-42.

359. Park, Y.D., Cui, Z.Y. and Park, H.J. (2002). Effects of gamma-aminobutyric acid on action of gastrin-releasing peptidergic neurons in exocrine secretion of isolated, perfused rat pancreas. *Pancreas*, **25**, 308-13.

360. Pattij, T., Groenink, L., Hijzen, T. H., Oosting, R. S., Maes, R. A., van der Gugten, J., et al. (2002). Autonomic changes associated with enhanced anxiety in 5-HT(1A) receptor knockout mice. *Neuropsychopharmacology, 27*(**3**), 380-390.

361. Paxinos, G. and Watson, C. (1982). The rat brain in stereotaxic coordinates, Academic press, New York.

362. Paxinos, G. and Watson, C. (1986). The rat brain in stereotaxic coordinates, Academic Press, New York.

363. Pazos-Moura, C.C., Ortiga-Carvalho, T.M. and Gaspar, d.M. (2003). The autocrine/paracrine regulation of thyrotropin secretion. *Thyroid*, **13**, 167-75.

364. Pellow, S., Chopin, P., File, S.E. and Briley, M. (1985). Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*, **14**, 149-67.

365. Pellow, S. and File, S. (1986). Anxiolytic and Anxiogenic Drug Effects on Exploratory Activity in an Elevated Plus-Maze: a Novel Test of Anxiety in the Rat. *Pharmacology Biochemistry and Behavior*, **24**, 525-29.

366. Penalva, R.G., Flachskamm, C., Zimmermann, S., Wurst, W., Holsboer, F., Reul, J.M. and Linthorst, A.C. (2002). Corticotropin-releasing hormone receptor type 1-deficiency enhances hippocampal serotonergic neurotransmission: an in vivo microdialysis study in mutant mice. *Neuroscience*, **109**, 253-66.

367. Peng Ho, S., Livanov, V., Zhang, W., Li, J., and Lesher, T. (1998). Modification of phosphorothioate oligonucleotides yields potent analogs with minimal toxicity for antisense experiments in the CNS. *Molecular Brain Research, **62**(**1**)*, 1-11.

368. Persson, K., Gingerich, R.L., Nayak, S., Wada, K., Wada, E. and Ahren, B. (2000). Reduced GLP-1 and insulin responses and glucose intolerance after gastric glucose in GRP receptor-deleted mice. *American Journal of Physiology - Endocrinology & Metabolism*, **279**, E956-E962.

369. Pert, A., Moody, T.W., Pert, C.B., Dewald, L.A. and Rivier, J. (1980). Bombesin: receptor distribution in brain and effects on nociception and locomotor activity. *Brain Research*, **193**, 209-20.

370. Pezze, M.A., Bast, T. and Feldon, J. (2003). Significance of dopamine transmission in the rat medial prefrontal cortex for conditioned fear. *Cerebral Cortex*, **13**, 371-80.

371. Phillips, R.G. and LeDoux, J.E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience*, **106**, 274-85.

372. Piazza, P.V. and Le Moal, M. (1997). Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Research Reviews*, **25**, 359-72.

373. Pinnock, R.D., Reynolds, T. and Woodruff, G.N. (1994). Different types of bombesin receptors on neurons in the dorsal raphe nucleus and the rostral hypothalamus in rat brain slices in vitro. *Brain Research*, **653**, 119-24.

374. Pinnock, R.D. and Woodruff, G.N. (1991). Bombesin excites a subpopulation of 5-hydroxytryptamine-sensitive neurones in the rat dorsal raphe nucleus in vitro. *Journal of Physiology*, **440**, 55-65.

375. Pinnock, S.B. and Herbert, J. (2001). Corticosterone differentially modulates expression of corticotropin releasing factor and arginine vasopressin mRNA in the hypothalamic paraventricular nucleus following either acute or repeated restraint stress. *European Journal of Neuroscience*, **13**, 576-84.

376. Plamondon, H. and Merali, Z. (1993). Effects of central neuromedin B and related peptides on blood glucose. *Regulatory Peptides*, **47**, 133-40.

377. Plamondon, H. and Merali, Z. (1994). Push-pull perfusion reveals meal-dependent changes in the release of bombesin-like peptides in the rat paraventricular nucleus. *Brain Research*, **668**, 54-61.

378. Plamondon, H. and Merali, Z. (1997). Anorectic action of bombesin requires receptor for corticotropin-releasing factor but not for oxytocin. *European Journal of Pharmacology*, **340**, 99-109.

379. Plamondon, H. and Merali, Z. (1997). Regulation of ingestion by CRF and bombesin-like peptides: distinct meal-related peptide level changes. *American Journal of Physiology*, **272**, R268-R274.

380. Plotsky, P.M. (1989). Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrine Reviews*, **10**, 437-58.

381. Plotsky, P.M., Owens, M.J. and Nemeroff, C.B. (1998). Psychoneuroendocrinology of depression. *Psychoneuroendocrinology*, 293-307.

382. Porter, J.R. and Sander, L.D. (1981). The effect of cholecystokinin octapeptide on pituitary-adrenal hormone secretion. *Regulatory Peptides*, **2**, 245-52.

383. Posener, J.A., Schatzberg, A.F., Williams, G.H., Samson, J.A., McHale, N.L., Bessette, M.P. and Schildkraut, J.J. (1999). Hypothalamic-pituitary-adrenal axis effects on plasma homovanillic acid in man. *Biological Psychiatry*, **45**, 222-28.

384. Posener, J.A., Schildkraut, J.J., Williams, G.H., Gleason, R.E., Salomon, M.S., Mecheri, G. and Schatzberg, A.F. (1994). Acute and delayed effects of corticotropin-releasing hormone on dopamine activity in man. *Biological Psychiatry*, **36**, 616-21.

385. Poulos, C.X. and Hinson, R. (1982). Pavlovian conditional tolerance to haloperidol catalepsy: evidence of dynamic adaptation in the dopaminergic system. *Science*, **218**, 491-92.

386. Poulos, C.X., Spielewoy, C., Davies, M.F., Yamada, K., Rubino, T., Yamada, K., Meert, T.F. and Ohki-Hamazaki, H. (2004). Low-dose diazepam primes motivation for alcohol and alcohol-related semantic networks in problem drinkers. *Behavioural Pharmacology*, **15**, 503-12.

387. Pradhan, T.K., Katsuno, T., Taylor, J.E., Kim, S.H., Ryan, R.R. et al. (1998). Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. *European Journal of Pharmacology*, **343**, 275-87.

388. Price, M.L., Curtis, A.L., Kirby, L.G., Valentino, R.J. and Lucki, I. (1998). Effects of corticotropin-releasing factor on brain serotonergic activity. *Neuropsychopharmacology*, **18**, 492-502.

389. Price, M.L., Hoffer, B.J. and Granholm, A.C. (1996). Effects of GDNF on fetal septal forebrain transplants in oculo. *Experimental Neurology*, **141**, 181-89.

390. Price, M.L. and Lucki, I. (2001). Regulation of serotonin release in the lateral septum and striatum by corticotropin-releasing factor. *Journal of Neuroscience*, **21**, 2833-41.

391. Prut, L. and Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. [Review] [185 refs]. *European Journal of Pharmacology*, **463**, 3-33.

392. Purba, J.S., Hoogendijk, W.J., Hofman, M.A. and Swaab, D.F. (1996). Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. *Archives of General Psychiatry*, **53**, 137-43.

393. Quirk, G.J., Russo, G.K., Barron, J.L. and Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *Journal of Neuroscience*, **20**, 6225-31.

394. Rabadan-Diehl, C., Lolait, S.J. and Aguilera, G. (1995). Regulation of pituitary vasopressin V1b receptor mRNA during stress in the rat. *Journal of Neuroendocrinology*, **7**, 903-10.

395. Rashidy-Pour, A. and Razvani, M.E. (1998). Unilateral reversible inactivations of the nucleus tractus solitarius and amygdala attenuate the effects of bombesin on memory storage. *Brain Research*, **814**, 127-32.

396. Rex, A., Fink, H. and Marsden, C.A. (1994). Effects of BOC-CCK-4 and L 365.260 on cortical 5-HT release in guinea-pigs on exposure to the elevated plus maze. *Neuropharmacology*, **33**, 559-65.

397. Rex, A., Marsden, C.A. and Fink, H. (1993). Effect of diazepam on cortical 5-HT release and behaviour in the guinea-pig on exposure to the elevated plus maze. *Psychopharmacology*, **110**, 490-96.

398. Ricci, A., Marchal-Victorion, S., Bronzetti, E., Parini, A., Amenta, F., and Tayebati, S. K. (2002). Dopamine D4 receptor expression in rat kidney: evidence for pre- and postjunctional localization. *J Histochem Cytochem,* ***50*****(8)**, 1091-1096.

399. Richard, D. (1993). Involvement of Corticotropin-Releasing Factor in the Control of Food Intake and Energy Expenditure. *Annals of the New York Academy of Sciences*, **697**, 155-72.

400. Rivier, C., Rivier, J., Mormede, P. and Vale, W. (1984). Studies of the nature of the interaction between vasopressin and corticotropin-releasing factor on adrenocorticotropin release in the rat. *Endocrinology*, **115**, 882-86.

401. Rivier, C., Rivier, J. and Vale, W. (1982). Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. *Science*, **218**, 377-79.

402. Rivier, J., Rivier, C. and Vale, W. (1984). Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. *Science*, **224**, 889-91.

403. Rizzi, C.A., Prudentino, A. and Giraldo, E. (1993). Effects on general behaviour and neurotransmitter functions of a new 5-hydroxytryptamine$_3$ receptor antagonist with potential therapeutic relevance in central nervous system disturbances. *Arzneimittelforschung*, **43**, 1033-41.

404. Rodrigues, S.M., Schafe, G.E. and LeDoux, J.E. (2004). Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. *Neuron*, **44**, 75-91.

405. Rodriguiz, R.M., Chu, R., Caron, M.G. and Wetsel, W.C. (2004). Aberrant responses in social interaction of dopamine transporter knockout mice. *Behavioural Brain Research*, **148**, 185-98.

406. Roesler, R., Henriques, J.A. and Schwartsmann, G. (2004). Neuropeptides and anxiety disorders: bombesin receptors as novel therapeutic targets. *Trends in Pharmacological Sciences*, **25**, 241-42.

407. Roesler, R., Kopschina, M.I., Rosa, R.M., Henriques, J.A., Souza, D.O. and Schwartsmann, G. (2004). RC-3095, a bombesin/gastrin-releasing peptide receptor antagonist, impairs aversive but not recognition memory in rats. *European Journal of Pharmacology*, **486**, 35-41.

408. Roesler, R., Lessa, D., Venturella, R., Vianna, M.R., Luft, T., Henriques, J.A., Izquierdo, I. and Schwartsmann, G. (2004). Bombesin/gastrin-releasing peptide receptors in the basolateral amygdala regulate memory consolidation. *European Journal of Neuroscience*, **19**, 1041-45.

409. Roesler, R., Meller, C.A., Kopschina, M.I., Souza, D.O., Henriques, J.A. and Schwartsmann, G. (2003). Intrahippocampal infusion of the bombesin/gastrin-releasing peptide antagonist RC-3095 impairs inhibitory avoidance retention. *Peptides*, **24**, 1069-74.

410. Roesler, R., Henriques, J.A.P. and Schwartsmann, G. (2004). Neuropeptides and anxiety disorders: bombesin receptors as novel therapeutic targets. *Trends in pharmacological sciences*, **25**, 241-42.

411. Rohmeiss, P., Becker, H., Dietrich, R., Luft, F. and Unger, T. (1986). Vasopressin: mechanism of central cardiovascular action in conscious rats. *Journal of Cardiovascular Pharmacology*, **8**, 689-96.

412. Rogers, D. C., Costall, B., Domeney, A. M., Gerrard, P. A., Greener, M., Kelly, M. E., Hagan, J.J. and Hunter, A.J. (2000). Anxiolytic profile of ropinirole in the rat, mouse and common marmoset. *Psychopharmacology, **151**(**1**)*, 91-97.

413. Rogoz, Z., Skuza, G., and Klodzinska, A. (2003). Anxiolytic-like effects of preferential dopamine D3 receptor agonists in an animal model. *Polish Journal of Pharmacology, **55**(**3**)*, 449-454.

414. Rosario, L.A. and Abercrombie, E.D. (1999). Individual differences in behavioral reactivity: correlation with stress-induced norepinephrine efflux in the hippocampus of Sprague-Dawley rats. *Brain Research Bulletin*, **48**, 595-602.

415. Rosen, J.B. (2004). The neurobiology of conditioned and unconditioned fear: a neurobehavioral system analysis of the amygdala. *Behavioral & Cognitive Neuroscience Reviews*, **3**, 23-41.

416. Rosmond, R., Holm, G. and Bjorntorp, P. (2000). Food-induced cortisol secretion in relation to anthropometric, metabolic and haemodynamic variables in men. *International Journal of Obesity & Related Metabolic Disorders*, **24**, 416-22.

417. Roth, K.A. and Katz, R.J. (1979). Stress, behavioral arousal, and open field activity--a reexamination of emotionality in the rat. *Neuroscience & Biobehavioral Reviews*, **3**, 247-63.

418. Roth, K.A., Weber, E. and Barchas, J.D. (1982). Distribution of gastrin releasing peptide - bombesin-like immunostaining in rat brain. *Brain Research*, **251**, 277-82.

419. Rothschild, A.J., Langlais, P.J., Schatzberg, A.F., Miller, M.M., Saloman, M.S., Lerbinger, J.E., Cole, J.O. and Bird, E.D. (1985). The effects of a single acute dose of dexamethasone on monoamine and metabolite levels in rat brain. *Life Sciences*, **36**, 2491-501.

420. Rubino, T. (2000). In vivo characterization of the specific cannabinoid receptor antagonist, SR141716A: behavioral and cellular responses after acute and chronic treatments. *Synapse*, **35**, 8-14.

421. Rushing, P.A. (1997). Spontaneous and scheduled eating: microstructural and peptide effects. *Appetite*, **29**, 385.

422. Rushing, P.A., Gibbs, J. and Geary, N. (1996). Brief, meal-contingent infusions of gastrin-releasing peptide1-27 and neuromedin B-10 inhibit spontaneous feeding in rats. *Physiology & Behavior*, **60**, 1501-04.

423. Rushing, P.A., Gibbs, J. and Geary, N. (1996). Vena caval bombesin infusion decreases spontaneous meal size in undisturbed rats. *Physiology & Behavior*, **59**, 307-10.

424. Rushing, P. A., and Houpt, T. A. (1999). Gastrin-releasing peptide suppresses independent but not intraoral intake. *Peptides, **20**(**6**)*, 737-741.

425. Ryan, R.R., Weber, H.C., Hou, W., Sainz, E., Mantey, S.A., Battey, J.F., Coy, D.H. and Jensen, R.T. (1998). Ability of various bombesin receptor agonists and antagonists to alter intracellular signaling of the human orphan receptor BRS-3. *Journal of Biological Chemistry*, **273**, 13613-24.

426. Ryan, R.R., Weber, H.C., Mantey, S.A., Pradhan, T.K., Hou, W., Coy, D.H. and Jensen, R.T. (1998). Pharmacology and intracellular signaling of the human Bombesin Receptor Subtype 3. *Gastroenterology*, **114**, G4812.

427. Saito, T., Ukai, K., Masuda, T., Nakagawa, T., Kimura, K., Fujii, M., Wakatsuki, K., Saeki, M. and Kasai, H. (1997). General pharmacological profile

of the novel cholecystokinin-A antagonist loxiglumide. *Arzneimittel-Forschung*, **47**, 1375-82.

428. Sakamoto, A., Kitamura, K., Haraguchi, Y., Yoshida, T. and Tanaka, K. (1987). Immunoreactive neuromedin B and neuromedin C: distribution and molecular heterogeneity in rat and human tissue extracts. *American Journal of Gastroenterology*, **82**, 1035-41.

429. Salacinski, P.R., Hope, J., McLean, C., Clement-Jones, V.V., Sykes, J., Price, J. and Lowry, P.J. (1979). A new simple method which allows theoretical incorporation of radioiodine into proteins and peptides without damage. *Jounral of Endocrinology*, **81**, 131.

430. Sananes, C.B. and Davis, M. (1992). N-methyl-D-aspartate lesions of the lateral and basolateral nuclei of the amygdala block fear-potentiated startle and shock sensitization of startle. *Behavioral Neuroscience*, **106**, 72-80.

431. Sander, L.D. and Porter, J.R. (1982). Influence of cholecystokinin on hypothalamic-stalk median-eminence-extract stimulation of ACTH output from isolated pituitary cells. *Life Sciences*, **31**, 1103-10.

432. Sanders, M.J., Wiltgen, B.J. and Fanselow, M.S. (2003). The place of the hippocampus in fear conditioning. *European Journal of Pharmacology*, **463**, 217-23.

433. Santo-Yamada, Y., Yamada, K. and Wada, K. (2001). Posttraining administration of gastrin-releasing peptide improves memory loss in scopolamine- and hypoxia-induced amnesic mice. *Physiology & Behavior*, **74**, 139-43.

434. Sapolsky, R.M., Romero, L.M. and Munck, A.U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, **21**, 55-89.

435. Sartory, G., MacDonald, R. and Gray, J.A. (1990). Effects of diazepam on approach, self-reported fear and psychophysiological responses in snake phobics. *Behaviour Research & Therapy*, **28**, 273-82.

436. Schafe, G.E., Nader, K., Blair, H.T. and LeDoux, J.E. (2001). Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends in Neuroscience*, **24**, 540-46.

437. Schmidt, E.D., Binnekade, R., Janszen, A.W.J.W. and Tilders, F.J.H. (1996). Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats. *Jounral of Neuroendocrinology*, **8**, 703-12.

438. Schmidt, E.D., Janszen, A.W.J.W., Wouterlood, F.G. and Tilders, F.J.H. (1995). Interleukin-1-induced long-lasting changes in hypothalamic corticotropin-releasing hormone (CRH) - Neurons and hyperresponsiveness of the hypothalamus-pituitary-adrenal axis. *Journal of Neuroscience*, **15**, 7417-26.

439. Schulz, B., Fendt, M. and Schnitzler, H.U. (2002). Clonidine injections into the lateral nucleus of the amygdala block acquisition and expression of fear-potentiated startle. *European Journal of Neuroscience*, **15**, 151-57.

440. Schulz, D.W., Kalivas, P.W., Nemeroff, C.B. and Prange, A.J.Jr. (1984). Bombesin-induced locomotor hyperactivity: evaluation of the involvement of the mesolimbic dopamine system. *Brain Research*, **304**, 377-82.

441. Schulz, D.W., Mansbach, R.S., Sprouse, J., Braselton, J.P., Collins, J. et al. (1996). CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 10477-82.

442. Serradeil-Le Gal, C., Wagnon, J., III, Tonnerre, B., Roux, R., Garcia, G., Griebel, G. and Aulombard, A. (2005). An overview of SSR149415, a selective nonpeptide vasopressin V(1b) receptor antagonist for the treatment of stress-related disorders. *CNS Drug Reviews*, **11**, 53-68.

443. Sherman, J.E. and Kalin, N.H. (1988). ICV-CRH alters stress-induced freezing behavior without affecting pain sensitivity. *Pharmacology, Biochemistry & Behavior*, **30**, 801-07.

444. Shi, C.J. and Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear- potentiated startle: Lesion studies. *Journal of Neuroscience*, **19**, 420-30.

445. Shumyatsky, G.P., Tsvetkov, E., Malleret, G., Vronskaya, S., Hatton, M. et al. (2002). Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. *Cell*, **111**, 905-18.

446. Sibille, E., Pavlides, C., Benke, D. and Toth, M. (2000). Genetic inactivation of the Serotonin(1A) receptor in mice results in downregulation of major GABA(A) receptor alpha subunits, reduction of GABA(A) receptor binding, and benzodiazepine-resistant anxiety. *Journal of Neuroscience*, **20**, 2758-65.

447. Siemiatkowski, M., Rokicki, D., Czlonkowska, A.I., Sienkiewicz-Jarosz, H., Bidzinski, A. and Plaznik, A. (2000). Locomotor activity and a conditioned fear response: correlation with cortical and subcortical binding of the dopamine D1 receptor antagonist. *Neuroreport*, **11**, 3953-56.

448. Silva, R.C., Cruz, A.P., Avanzi, V., Landeira-Fernandez, J. and Brandao, M.L. (2002). Distinct contributions of median raphe nucleus to contextual fear conditioning and fear-potentiated startle. *Neural Plasticity*, **9**, 233-47.

449. Silva, R.C.B., Gargaro, A.C. and Brandao, M.L. (2004). Differential regulation of the expression of contextual freezing and fear-potentiated startle by 5-HT mechanisms of the median raphe nucleus. *Behavioural Brain Research*, **151**, 93-101.

450. Smagin, G.N., Heinrichs, S.C. and Dunn, A.J. (2001). The role of CRH in behavioral responses to stress. *Peptides*, **22**, 713-24.

451. Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M. et al. (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron*, **20**, 1093-102.

452. Smith, J., Perez, S., Rushing, P.A., Smith, G.P. and Gibbs, J. (1997). Gastrin-releasing peptide1-27, unlike bombesin, does not reduce sham feeding in rats. *Peptides*, **18**, 1465-67.

453. Sommer, W., Moller, C., Wiklund, L., Thorsell, A., Rimondini, R., Nissbrandt, H. and Heilig, M. (2001). Local 5,7-dihydroxytryptamine lesions of rat amygdala: release of punished drinking, unaffected plus-maze behavior and ethanol consumption. *Neuropsychopharmacology*, **24**, 430-40.

454. Spencer, S.J. (2004). Differential involvement of rat medial prefrontal cortex dopamine receptors in modulation of hypothalamic-pituitary-adrenal axis responses to different stressors. *European Journal of Neuroscience*, **20**, 1008-16.

455. Spielewoy, C., Roubert, C., Hamon, M., Nosten-Bertrand, M., Betancur, C. and Giros, B. (2000). Behavioural disturbances associated with hyperdopaminergia in dopamine-transporter knockout mice. *Behavioural Pharmacology*, **11**, 279-90.

456. Spina, M., Merlo-Pich, E., Chan, R.K., Basso, A.M., Rivier, J., Vale, W. and Koob, G.F. (1996). Appetite-suppressing effects of urocortin, a CRF-related neuropeptide. *Science*, **273**, 1561-64.

457. Spina, M.G., Merlo-Pich, E., Akwa, Y., Balducci, C., Basso, A., Zorrilla, E.P., Britton, K.T., Rivier, J., Vale, W.W. and Koob, G.F. (2002). Time-dependent induction of anxiogenic-like effects after central infusion of urocortin or corticotropin-releasing factor in the rat. *Psychopharmacology*, **160**, 113-21.

458. Spindel, E.R., Giladi, E., Brehm, P., Goodman, R.H. and Segerson, T.P. (1990). Cloning and functional characterization of a complementary DNA encoding the murine fibroblast bombesin/gastrin-releasing peptide receptor. *Molecular Endocrinology*, **4**, 1956-63.

459. Spruijt, B.M., Van Hooff, J.A.R.A.M. and Gispen, W.H. (1992). Ethology and neurobiology of grooming behavior. *Physiology Reviews*, **72**, 825-52.

460. Stefanski, R., Palejko, W., Kostowski, W. and Plaznik, A. (1992). The comparison of benzodiazepine derivatives and serotonergic agonists and antagonists in two animal models of anxiety. *Neuropharmacology*, **31**, 1251-58.

461. Stenzel-Poore, M.P., Cameron, V.A., Vaughan, J., Sawchenko, P.E. and Vale, W. (1992). Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. *Endocrinology*, **130**, 3378-86.

462. Stenzel-Poore, M.P., Heinrichs, S.C., Rivest, S., Koob, G.F. and Vale, W.W. (1994). Overproduction of corticotropin-releasing factor in transgenic mice: A genetic model of anxiogenic behavior. *Journal of Neuroscence*, **14**, 2579-84.

463. Stork, O. and Pape, H.C. (2002). Fear memory and the amygdala: insights from a molecular perspective. *Cell & Tissue Research*, **310**, 271-77.

464. Stratakis, C.A. and Chrousos, G.P. (1995). Neuroendocrinology and pathophysiology of the stress system. *Ann.NY Acad.Sci.*, **771**, 1-18.

465. Stratford, T.R., Gibbs, J. and Smith, G.P. (1996). Simultaneous Administration of Neuromedin B-10 and Gastrin-Releasing Peptide(1-27) Reproduces the Satiating and Microstructural Effects of Bombesin. *Peptides*, **17**, 107-10.

466. Sutton, R.E., Koob, G.F., Le Moal, M., Rivier, J. and Vale, W. (1982). Corticotropin releasing factor produces behavioral activation in rats. *Nature*, **297**, 331-33.

467. Swerdlow, N.R., Britton, K.T. and Koob, G.F. (1989). Potentiation of acoustic startle by corticotropin-releasing factor (CRF) and by fear are both reversed by alpha-helical CRF (9-41). *Neuropsychopharmacology*, **2**, 285-92.

468. Swiergiel, A.H. and Dunn, A.J. (1999). CRF-deficient mice respond like wild-type mice to hypophagic stimuli. *Pharmacology, Biochemistry & Behavior*, **64**, 59-64.

469. Tache, Y., Brown, M. and Collu, R. (1979). Effects of neuropeptides on adenohypophyseal hormone response to acute stress in male rats. *Endocrinology*, **105(1)**, 220-24.

470. Tache, Y., Marki, W., Rivier, J., Vale, W. and Brown, M. (1981). Central nervous system inhibition of gastric secretion in the rat by gastrin-releasing peptide, a mammalian bombesin. *Gastroenterology*, **81(2)**, 298-302.

471. Takahashi, L.K., Kalin, N.H., Vanden Burgt, J.A. and Sherman, J.E. (1989). Corticotropin-releasing factor modulates defensive-withdrawal and exploratory behavior in rats. *Behavioral Neuroscience*, **103**, 648-54.

472. Tallroth, G., Ryding, E., Ekman, R. and Agardh, C.D. (1992). The response of regulatory peptides to moderate hypoglycaemia of short duration in type 1

(insulin-dependent) diabetes mellitus and in normal man. *Diabetes Research*, **20**, 73-85.

473. Tanaka, M., Yoshida, M., Emoto, H. and Ishii, H. (2000). Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. *European Journal of Pharmacology*, **405**, 397-406.

474. Thaw, A.K., Smith, J.C. and Gibbs, J. (1998). Mammalian bombesin-like peptides extend the intermeal interval in freely feeding rats. *Physiology & Behavior*, **64**, 425-28.

475. Thaw, A. K., and Quinn, T. J. (2003). Effects of gastrin-releasing peptide 1-27 on taste responses in the rat. *Cheical Senses, 28*(**1**), 3-9.

476. Thomas, K.S., Nelesen, R.A., Ziegler, M.G., Bardwell, W.A. and Dimsdale, J.E. (2004). Job strain, ethnicity, and sympathetic nervous system activity. *Hypertension*, **44**, 891-96.

477. Tilders, F.J.H., Schmidt, E.D. and De Goeij, D.C.E. (1993). Phenotypic plasticity of CRF neurons during stress. *Annals of the New York Academy of Sciences*, **697**, 39-52.

478. Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M. et al. (1998). Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nature Genetics*, **19**, 162-66.

479. Tjurmina, O.A., Goldstein, D.S., Palkovits, M. and Kopin, I.J. (1999). Alpha2-adrenoceptor-mediated restraint of norepinephrine synthesis, release, and turnover during immobilization in rats. *Brain Research*, **826**, 243-52.

480. Trinh, J.V., Nehrenberg, D.L., Jacobsen, J.P., Caron, M.G. and Wetsel, W.C. (2003). Differential psychostimulant-induced activation of neural circuits in dopamine transporter knockout and wild type mice. *Neuroscience*, **118**, 297-310.

481. Tsigos, C. and Chrousos, G.P. (1994). Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. *Endocrinology & Metabolism Clinics of North America*, **23**, 451-66.

482. Tsigos, C. and Chrousos, G.P. (2002). Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research*, **53**, 865-71.

483. Tsushima, H., Mori, M., Fujiwara, N. and Moriyama, A. (2003). Pharmacological characteristics of bombesin receptor mediating hypothermia in the central nervous system of rats. *Brain Research*, **969**, 88-94.

484. Tuchscherer, M.M., Knox, C. and Seybold, V.S. (1987). Substance P and cholecystokinin-like immunoreactive varicosities in somatosensory and autonomic regions of the rat spinal cord: a quantitative study of coexistence. *Journal of Neuroscience*, **7**, 3984-95.

485. Tucker, J.C. and File, S.E. (1986). The effects of tricyclic and 'atypical' antidepressants on spontaneous locomotor activity in rodents. *Neuroscience & Biobehavioral Reviews*, **10**, 115-21.

486. Vale, W., Vaughan, J., Smith, M., Yamamoto, G., Rivier, J. and Rivier, C. (1983). Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. *Endocrinology*, **113**, 1121-31.

487. Valentino, R.J. (1992). Corticotropin-releasing factor innervation of the locus coeruleus region: distribution of fibers and sources of input. *Neuroscience*, **48**, 689-705.

488. Valentino, R.J., Liouterman, L. and Van Bockstaele, E.J. (2001). Evidence for regional heterogeneity in corticotropin-releasing factor interactions in the dorsal raphe nucleus. *Journal of Comparative Neurology*, **435**, 450-63.

489. Van Dijken, H.H., De Goeij, D.C.E., Sutanto, W., Mos, J., de Kloet, E.R. and Tilders, F.J.H. (1993). Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats. *Neuroendocrinology*, **58**, 57-64.

490. Van Gaalen, M.M., Stenzel-Poore, M.P., Holsboer, F. and Steckler, T. (2002). Effects of transgenic overproduction of CRH on anxiety-like behaviour. *Journal of Neuroscience*, **15**, 2007-15.

491. Van Wimersma Greidanus, T.B. and Maigret, C. (1991). Neuromedin-induced excessive grooming/scratching behavior is suppressed by naloxone, neurotensin and a dopamine D_1 receptor antagonist. *European Journal of Pharmacology*, **209**, 57-61.

492. Van Wimersma Greidanus, T.J.B., Donker, D.K., Van Zinnicq Bergmann, F.F.M., Bekenkamp, R., Maigret, C. and Spruijt, B. (1985). Comparison between excessive gromming induced by bombesin or by ACTH: the differential elements of grooming and development of tolerence. *Peptides*, **6**, 369-72.

493. Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K. et al. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature*, **378**, 287-92.

494. Venturella, R., Lessa, D., Luft, T., Roozendaal, B., Schwartsmann, G., and Roesler, R. (2005). Dexamethasone reverses the memory impairment induced by antagonism of hippocampal gastrin-releasing peptide receptors. *Peptides,* ***26*****(5)**, 821-825.

495. Voigt, J.P., Rex, A., Sohr, R. and Fink, H. (1999). Hippocampal 5-HT and NE release in the transgenic rat TGR(mREN2)27 related to behavior on the elevated plus maze. *European Neuropsychopharmacology*, **9**, 279-85.

496. Wada, E., Watase, K., Yamada, K., Ogura, H., Yamano, M. et al. (1997). Generation and characterization of mice lacking gastrin-releasing peptide receptor. *Biochemical & Biophysical Research Communications*, **239**, 28-33.

497. Wada, E., Way, J., Lebacq-Verheyden, A.M. and Battey, J.F. (1990). Neuromedin B and gastrin-releasing peptide mRNAs are differentially distributed in the rat nervous system. *Journal of Neuroscience*, **10**, 2917-30.

498. Wada, E., Way, J., Shapira, H., Kusano, K., Lebacq-Verheyden, A.-M., Coy, D., Jensen, R. and Battey, J. (1991). cDNA cloning, characterization and brain region-specific expression of neuromedin-B preferring bombesin receptor. *Neuron*, **6**, 421-30.

499. Wahl, M.A., Plehn, R.J., Landsbeck, E.A., Verspohl, E.J. and Ammon, H.P. (1991). Gastrin releasing peptide augments glucose mediated 45Ca2+ uptake, electrical activity, and insulin secretion of mouse pancreatic islets. *Endocrinology*, **128**, 3247-52.

500. Walker, D.L. and Davis, M. (1997). Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *Journal of Neuroscience*, **17**, 9375-83.

501. Walker, D.L., Toufexis, D.J. and Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology*, **463**, 199-216.

502. Weiger, W.A. (1997). Serotonergic modulation of behaviour: a phylogenetic overview. *Biological Reviews of the Cambridge Philosophical Society*, **72**, 61-95.

503. Weninger, S.C., Dunn, A.J., Muglia, L.J., Dikkes, P., Miczek, K.A., Swiergiel, A.H., Berridge, C.W. and Majzoub, J.A. (1999). Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 8283-88.

504. Wesolowska, A., Paluchowska, M. and Chojnacka-Wojcik, E. (2003). Involvement of presynaptic 5-HT(1A) and benzodiazepine receptors in the anticonflict activity of 5-HT(1A) receptor antagonists. *European Journal of Pharmacology*, **471**, 27-34.

505. Whitnall, M.H. (1985). Co-localization of corticotropin-releasing factor and vasopressin in median eminence neurosectretory vesicles. *Nature*, **317**, 248-50.

506. Whitnall, M.H., Anderson, K.A., Lane, C.A., Mougey, E.H., Neta, R. and Perlstein, R.S. (1994). Decreased vasopressin content in parvocellular CRH neurosecretory system of Lewis rats. *Neuroreport*, **5**, 1635-37.

507. Whitton, P. and Curzon, G. (1990). Anxiogenic-like effect of infusing 1-(3-chlorophenyl) piperazine (mCPP) into the hippocampus. *Psychopharmacology*, **100**, 138-40.

508. Wigger, A., Sanchez, M.M., Mathys, K.C., Ebner, K., Frank, E. et al. (2004). Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. *Neuropsychopharmacology*, **29**, 1-14.

509. Wolf, S.S. and Moody, T.W. (1985). Receptors for GRP/bombesin-like peptides in the rat forebrain. *Peptides*, **6**, 111-14.

510. Wolf, S.S., Moody, T.W., O'Donohue, T.L., Zarbin, M.A. and Kuhar, M.J. (1983). Autoradiographic visualization of rat brain binding sites for bombesin-like peptides. *Euopean Journal of Pharmacoogy.*, **87**, 163-64.

511. Wolkowitz, O., Sutton, M., Koulu, M., Labarca, R., Wilkinson, L., Doran, A., Hauger, R., Pickar, D. and Crawley, J. (1986). Chronic corticosterone administration in rats: behavioral and biochemical evidence of increased central dopaminergic activity. *European Journal of Pharmacology*, **122**, 329-38.

512. Wong, P.T. and Ong, Y.P. (2001). Acute antidepressant-like and antianxiety-like effects of tryptophan in mice. *Pharmacology*, **62**, 151-56.

513. Woodruff, G.N., Hall, M.D., Reynolds, T. and Pinnock, R.D. (1996). Bombesin receptors in the brain. *Annals of the New York Academy of Sciences*, 223-43.

514. Wright, I.K., Upton, N. and Marsden, C.A. (1992). Effect of established and putative anxiolytics on extracellular 5-HT and 5-HIAA in the ventral hippocampus of rats during behaviour on the elevated X-maze. *Psychopharmacology*, **109**, 338-46.

515. Wunderlich, G.R., Raymond, R., DeSousa, N.J., Nobrega, J.N. and Vaccarino, F.J. (2002). Decreased CCK(B) receptor binding in rat amygdala in animals demonstrating greater anxiety-like behavior. *Psychopharmacology*, **164**, 193-99.

516. Yamada, K. (2000). Differential effects of social isolation upon body weight, food consumption, and responsiveness to novel and social environment in bombesin receptor subtype-3 (BRS-3) deficient mice. *Physiology & Behavior*, **68**, 555-61.

517. Yamada, K., Santo-Yamada, Y., Wada, E. and Wada, K. (2002). Role of bombesin (BN)-like peptides/receptors in emotional behavior by comparison of

three strains of BN-like peptide receptor knockout mice. *Molecular Psychiatry*, **7**, 113-17.

518. Yamada, K., Santo-Yamada, Y. and Wada, K. (2002). Restraint stress impaired maternal behavior in female mice lacking the neuromedin B receptor (NMB-R) gene. *Neuroscience Letters*, **330**, 163-66.

519. Yamada, K., Santo-Yamada, Y. and Wada, K. (2003). Stress-induced impairment of inhibitory avoidance learning in female neuromedin B receptor-deficient mice. *Physiology & Behavior*, **78**, 303-09.

520. Yamada, K., Wada, E., Yamano, M., Sun, Y.J., Ohara-Imaizumi, M., Nagamatsu, S. and Wada, K. (2002). Decreased marble burying behavior in female mice lacking neuromedin-B receptor (NMB-R) implies the involvement of NMB/NMB-R in 5-HT neuron function. *Brain Research*, **942**, 71-78.

521. Yamamoto, Y., Akiyoshi, J., Kiyota, A., Katsuragi, S., Tsutsumi, T., Isogawa, K. and Nagayama, H. (2000). Increased anxiety behavior in OLETF rats without cholecystokinin-A receptor. *Brain Research Bulletin*, **53**, 789-92.

522. Yamano, M., Ogura, H., Okuyama, S. and Ohki-Hamazaki, H. (2002). Modulation of 5-HT system in mice with a targeted disruption of neuromedin B receptor. *Journal of Neuroscience Research*, **68**, 59-64.

523. Yehuda, R. (2000). Biology of posttraumatic stress disorder. *Journal of Clinical Psychiatry*, **61**, 14-21.

524. Yilmazer-Hanke, D.M., Faber-Zuschratter, H., Linke, R. and Schwegler, H. (2002). Contribution of amygdala neurons containing peptides and calcium-binding proteins to fear-potentiated startle and exploration-related anxiety in inbred Roman high- and low-avoidance rats. *European Journal of Neuroscience*, **15**, 1206-18.

525. Yilmazer-Hanke, D.M., Wigger, A., Linke, R., Landgraf, R. and Schwegler, H. (2004). Two Wistar rat lines selectively bred for anxiety-related behavior show opposite reactions in elevated plus maze and fear-sensitized acoustic startle tests. *Behavior Genetics*, **34**, 309-18.

526. Yoshioka, M., Matsumoto, M., Togashi, H. and Saito, H. (1995). Effects of conditioned fear stress on 5-HT release in the rat prefrontal cortex. *Pharmacology, Biochemistry and Behavior*, **51**, 515-19.

527. Zarbin, M.A., Kuhar, M.J., O'Donohue, T.L., Wolf, S.S. and Moody, T.W. (1985). Autoradiographic localization of (125I-tyr4)bombesin-binding sites in rat brain. *Journal of Neuroscience*, **5(2)**, 429-37.

528. Zerbe, R.L. and Feuerstein, G. (1985). Cardiovascular effects of centrally administered vasopressin in conscious and anesthetized rats. *Neuropeptides*, **6**, 471-83.

529. Zhou, Y. (2004). Effects of selective D1- or D2-like dopamine receptor antagonists with acute "binge" pattern cocaine on corticotropin-releasing hormone and proopiomelanocortin mRNA levels in the hypothalamus. *Molecular Brain Research*, **130**, 61-67.

530. Zorrilla, E.P., Valdez, G.R., Nozulak, J., Koob, G.F. and Markou, A. (2002). Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. *Brain Research*, **952**, 188-99.

531. Zwanzger, P. (2003). Plasma gamma-amino-butyric acid (GABA) levels in cholecystokinine-tetrapeptide (CCK-4) induced anxiety. *Journal of Neural Transmission*, **110**, 313-16.