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**Characterization of the Role of the Medial Prefrontal Cortex and the Amygdala in Fear and Anxiety:
A Focus on Bombesin-like Peptides and Corticotropin-Releasing Hormone**

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Characterization of the role of the medial prefrontal cortex and the amygdala in fear and
anxiety: a focus on bombesin-like peptides and corticotropin-releasing hormone

By

Christine Mountney

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements

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Dedication

This thesis is dedicated to:

My loving husband who stood by me so patiently through the final years of my dissertation and supported me through to the end.

And to my beautiful daughter who gave me inspiration and motivation and to whom I strive to model only the best.

Acknowledgments

I would like to thank my supervisor, Dr. Zul Merali, who gave me the opportunity to open my mind and to learn in new ways by thinking outside of the proverbial box. He has been a mentor and role model, always showing encouragement, patience and kindness, while at the same time, pushing me to greater extents to achieve more than I ever thought possible. Thank you Zul for always being there in times of need, I cannot thank you enough for your guidance and support.

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Statement of Contributions of Collaborators and/or Co-Authors

Chapter 1 – Study 1

Bédard, T., Mountney, C., Kent, P., Anisman, H., and Merali, Z. (2007). The roles of gastrin-releasing peptide and neuromedin-B in anxiety and fear-related behaviour. *Behavioural Brain Research*, 179(1), 133-140.

This study was a collaboration between myself and Dr. Tania Bedard. It was conceptualized by myself, Dr. Bedard and Dr. Merali. Animal surgeries were completed by myself, Dr. Bedard and a summer student, Katie Mennie. Behavioral testing was conducted by myself and Katie Mennie. The original manuscript was prepared by Dr. Pamela Kent and subsequent revisions and final approvals were given by all authors. Guidance and funding was provided by Dr. Merali.

Chapter 1 – Study 2

Merali, Z., Mountney, C., Kent, P. & Anisman, H. (2010). Effects of intracerebral ventricular administration of gastrin-releasing peptide and its receptor antagonist RC-3095 on learned fear responses in the rat. *Behavioural Brain Research*, in press, doi:10.1016/j.bbr.2010.08.027

This study was conceptualized by myself and Dr. Merali. Animal surgeries were completed by myself and a laboratory technician, Christian Cayer. Behavioral testing was conducted by myself and Christian Cayer. The original manuscript was prepared by myself in collaboration with Dr. Pamela Kent and Dr. Merali and subsequent revisions and final approvals were given by all authors. Guidance and funding was provided by Dr. Merali.

Chapter 2

This study was conceptualized and conducted by myself with guidance and funding provided by Dr. Merali. Technical assistance, training and experimental advice were kindly provided by Nathalie Lukenbill, a laboratory technician and Dr. Tania Bedard. This study is not published and is not anticipated to be published.

Chapter 3 – Study 1

Mountney, C., Sillberg, V., Kent, P., Anisman, H., and Merali, Z. (2006). The role of gastrin-releasing peptide on conditioned fear: differential cortical and amygdaloid responses in the rat. *Psychopharmacology*, 289(3), 287-296.

This study was conceptualized and conducted by myself with the assistance of Victoria Sillberg, an honour's undergraduate student as well as Jonathan James and Christian Cayer, laboratory

technicians. The original manuscript was prepared by myself and subsequent revisions and final approvals were given by all authors. Guidance and funding was provided by Dr. Merali.

Chapter 3 – Study 2

Mountney, C., Anisman, H., and Merali, Z. (2008). Effects of gastrin-releasing peptide agonist and antagonist administered to the basolateral nucleus of the amygdala on conditioned fear in the rat. *Psychopharmacology*, 200(1), 51-58.

This study was conceptualized and conducted by myself with the assistance of Jonathan James and Christian Cayer, laboratory technicians. The original manuscript was prepared by myself and subsequent revisions and final approvals were given by all authors. Guidance and funding was provided by Dr. Merali.

Chapter 4

Mountney, C., Kent, P., Anisman, H., and Merali, Z. (2010). In Vivo Levels of Corticotropin-Releasing Hormone and Gastrin-Releasing Peptide at the Basolateral Amygdala and Medial Prefrontal Cortex in Response to Conditioned Fear in the Rat. Accepted pending minor revisions in *Neuropharmacology*.

This study was conceptualized and conducted by myself with the assistance of Jonathan James, a laboratory technician. The original manuscript was prepared by myself and subsequent revisions and final approvals were given by all authors. Guidance and funding was provided by Dr. Merali.

Chapter 5

This study was conceptualized and conducted by myself with the assistance of Jonathan James, a laboratory technician. The paper was prepared by myself, however it has not yet been submitted for publication. Guidance and funding was provided by Dr. Merali.

In all studies, extensive revisions, technical and statistical advice were kindly provided by Dr. Pamela Kent.

Abstract

This dissertation aimed to more fully characterize the involvement of bombesin-like peptides (neuromedin-B [NMB] and gastrin-releasing peptide [GRP]) and corticotropin-releasing hormone (CRH) in fear and anxiety-related processes. To this end, ventricularly injected GRP and antagonists of the NMB receptor decreased fear and/or anxiety across several animal models of behavior. Whereas NMB appeared to be involved in both fear and anxiety-related processes, GRP affected fear-related processes. In order to determine where in the brain these effects might be primarily localized, several neurochemical alterations were assessed in brain areas known to be involved in fear. Endogenous alterations in peptide levels were seen both within the medial prefrontal cortex (mPFC) and amygdala in response to recall of a fear-inducing shock. To explore this finding further, GRP or its receptor antagonist were microinjected into specific nuclei in the aforementioned areas and it was found that GRP consistently reduced fear at these loci, while the receptor antagonist exhibited both agonistic (reduced freezing) effects as well as antagonistic effects (increased freezing). Further, the observed reductions in freezing appeared to be specific to contextual components of conditioned fear, with the exception of drug administration to the infralimbic (IL) cortex within the mPFC, where we saw reduced freezing to a conditioned tone as well. To determine the endogenous release of GRP and CRH in response to conditioned fear, we collected dialysates from the IL of the mPFC and basolateral amygdala (BLA) in response to a previously conditioned tone. GRP and CRH were elevated 24h after fear conditioning at the BLA, an effect that appears to be related to animal's levels of fear. This was not observed at the IL. Finally, we explored whether the mPFC and amygdala communicated with each other via GRP and/or CRH through a GRP-specific pathway(s). We found that there appears to be a functional pathway between the IL cortex and BLA that is specific to GRP but not to CRH, upon manipulation of IL GRP levels. These studies have further characterized the role of BLPs and CRH in conditioned fear, particularly at the level of the mPFC and BLA and have provided a unique and rich foundation for the physiological role of these peptides and future studies to be developed.

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Summary

With the high prevalence of mental illnesses such as depression, anxiety, or post-traumatic stress disorder (PTSD) and chronic illnesses such as heart disease, diabetes and immune-deficient diseases, it is not surprising that the study of stress has received so much attention. Indeed, stressor exposure is often a precipitating factor for the conditions listed above.

Posttraumatic stress disorder is an anxiety disorder that is precipitated by an event that poses a threat to the life of self or close other, and is associated with intense fear, horror, or helplessness. It involves a pathological response to a traumatic event, with behavioral, emotional, functional, and physiological components [87]. Symptomatology of PTSD includes re-experiencing the original trauma, avoidance of reminders of the trauma, and increased arousal (seen as an exaggerated startle response). Various stimuli can become conditioned reminders of the trauma, whether external (e.g., noise) or internal (e.g., a memory), and may precipitate a cascade of distressing re-experiencing and arousal symptoms. One striking feature of PTSD is that not all individuals who experience the same traumatic event will develop PTSD. Even those who do develop PTSD will exhibit marked differences in symptomatology, underscoring the heterogeneity of symptoms in this population [229]. Researchers have identified several risk factors for PTSD, including prior traumatic experiences, low social support and family history of mood disorders.

Since recent events such as Hurricane Katrina, the tsunami in the Philippines, the earthquake in Haiti and September 11th, the incidence of PTSD has been on the rise. Epidemiological studies estimate that 1 to 14% of the normal population will develop PTSD in response to a severe trauma, however, estimates of the incidence of PTSD after September 11th alone approached 35% [510]. Clearly these high prevalence rates underscore the need to further

understand the underpinnings of this disorder, and to determine factors contributing to susceptibility to this disorder.

While currently available pharmacological treatments for anxiety disorders may help to alleviate symptoms of PTSD they do not eliminate or prevent the development of disease. Further, the beneficial effects of these treatments are often obfuscated by incapacitating adverse side effects such as memory impairments, sedation, and dependence. Thus, more effective and selective anxiolytic compounds without the adverse side effects are warranted. For this reason it is crucial to understand the mechanisms underlying the response to various stressors, including those mitigating anxiety and fear-type responses.

To this end, PTSD is thought to involve learned-fear associations between the traumatic events and danger or threat to life. Consequently, many of the paradigms used to study this phenomenon in animals often involve fear conditioning (i.e., pairing a tone with an aversive shock).

One area of the brain thought to be involved in learned fear is the lateral amygdala [29, 326, 436]. Several studies have shown that the lateral amygdala is the area where learned fear associations are formed (for a review see [100] and [272]) and lesions to this area abolish learned fear associations [100, 272]. There is now convincing evidence that gastrin-releasing peptide (GRP) may in fact be part of a signaling network specific to the fear response [436] located within the lateral amygdala. Mutant mice deficient in the GRP (BB_2) receptor demonstrated increased fearfulness to tones previously paired with shock, enhanced long-term potentiation (thought to reflect a measure of memory) and greater, more persistent long-term fear memory. Further, a preponderance of GRP encoding genes was localized in the lateral amygdala as well as in areas which convey fearful auditory information to the lateral nucleus. Moreover, this study

found that GRP activates receptors located on gamma aminobutyric acid interneurons, increasing their inhibition of principal neurons within the amygdala, and thus, creating negative feedback regulation of fear. Taken together, these results suggest that a low concentration of GRP, or reduced availability of GRP signaling, leads to an increased fear response to a learned fear association [436].

Several other studies support a role for GRP in fear and memory for fearful events, however the results of these studies have been contradictory and thus, the mechanisms by which GRP exerts its influence on fear memory remains to be elucidated. For example, systemic administration of GRP following scopolamine- or hypoxia-induced amnesia improved subsequent memory performance in a one-trial passive-avoidance task (a task in which the mouse learns to refrain from stepping through a door to an apparently safer but previously punished dark compartment) [417]. Roesler and his colleagues [393] found that microinjection of a GRP receptor antagonist directly into the hippocampus immediately after training impairs long-term, as well as short-term, memory for a step-down latency task (which pairs stepping down on a grid with a footshock). Similarly, when administered systemically, the GRP receptor antagonist impaired memory for aversive stimuli (footshock), but did not impair neutral recognition memory [390]. These results were replicated in mice using an inhibitory avoidance task [416]. Taken together, this evidence supports the involvement of GRP in aversive memory formation and points to its potential involvement in fear-based learning.

Given the prevalence of GRP in the neurocircuitry involved in learned fear and stress-related responses, the overall objective of this thesis research was to elucidate the mechanisms underlying the formation and expression of fear-related behaviors. Specifically, the goal was to more fully characterize the role of bombesin-like peptides (BLP's - namely GRP and

neuromedin-B [NMB]) in learned fear. Summarized below are the specific objectives of this research project and their key outcomes:

1. The impact of manipulating the bombesin peptidergic system has only been assessed in a limited number of anxiety and fear paradigms. Given that different behavioral paradigms reflect different aspects of anxiety, the objective of this study was to assess the effects of GRP and NMB in paradigms thought to reflect fear and anxiety-related behaviors. To this end, the effects of i.c.v. administration of NMB-30, GRP, the BB₁ receptor antagonist, BIM 23127, the BB₂ receptor antagonist [Leu13-(CH₂NH)Leu14]-BN and a mixed BB₁/BB₂ receptor antagonist, PD176252 were assessed in the elevated plus maze (EPM) and in a fear-potentiated startle (FPS) paradigm (a model thought to reflect conditioned fear). The BB₁ receptor antagonist and the mixed BB₁/BB₂ receptor antagonist elicited anxiolytic effects in the EPM, whereas the BB₂ receptor antagonist was without effect. In the FPS paradigm, pretreatment with either the BB₁ receptor antagonist or the BB₂ receptor agonist attenuated the FPS response without affecting basal startle amplitude. These data suggest that NMB and GRP do affect the stress response. However, whereas NMB manipulations affected both anxiety and fear responses, GRP alterations selectively affected fear-related responses.

Given the findings that GRP seemed selectively involved in fear-related responses, the second study in this chapter sought to explore this relationship further with the use of a new animal model of fear, as well as a more current antagonist. As a dose response curve was not previously done with the new antagonist (D-Tpi6, Leu13 psi[CH₂NH]-Leu14) BB⁽⁶⁻¹⁴⁾ (RC-3095), this study repeated the dose response curve seen in the first study for GRP and studied 3 doses of the antagonist. To this end, the effects of intracerebroventricular (i.c.v.) administration

of GRP (0.062, 0.30, 3.0 nmoles) and RC-3095 (0.3, 3.0 and 9.0 nmoles) were assessed in the conditioned emotional response (CER) and again in the fear-potentiated startle paradigm. In the CER paradigm, i.c.v. administration of GRP dose dependently (all doses) attenuated the expression of both contextual and cued fear as reflected by a reduction in freezing behavior to both the context (cage where shock was received) and cue (tone paired with shock). In contrast, pretreatment with RC-3095 (high dose), blocked the reduction of contextual and cued fear normally observed over time. Alternatively, in the FPS paradigm, i.c.v. administration of GRP significantly attenuated the FPS response at medium and high doses without affecting basal startle amplitude. While pretreatment with RC-3095 at the highest dose (9.0 nmoles) significantly increased the basal startle amplitude without affecting fear-potential, suggesting elevated fear at the onset of testing. These data provide further evidence that GRP is involved in conditioned fear responses.

2. The previous chapter demonstrated altered fear behavior when BLP analogues and antagonists were administered to the third ventricle, however ventricular administration of drugs and/or receptor knock-out strategies (as in the case of much previous work done with this peptide), do not point to brain areas involved in the mediation of learned fear behaviors. Thus, the current pilot project sought to determine where in the brain this peptidergic system may be altered in response to learned fear. To this end, we measured concentrations of GRP in several stress- or fear-relevant regions of the brain in response to tone + shock exposure and/or tone alone exposure. In addition to GRP, we also measured CRH as previous evidence suggests a relationship between BLP and CRH release patterns in stress-relevant brain areas. The results revealed that three brain regions had significant differences in peptide release; the prelimbic

cortex, ventromedial hypothalamus and amygdala. As a preponderance of research exists supporting the involvement of the amygdala in conditioned fear and the medial prefrontal cortex (mPFC - which includes the prelimbic cortex) in the extinction of conditioned fear, the remainder of the dissertation focused solely on these two brain regions and sought to more fully understand the role of BLPs in these brain regions. Further, as the results from chapter 1 did not support a strong role for NMB in conditioned fear, the remainder of the dissertation assessed the role of GRP only in these regions.

3. Central administration of GRP and its receptor antagonist to the third ventricle resulted in profound alterations in fear behavior in both a fear-potentiated startle paradigm as well as in a conditioned freezing test. Our pilot research suggested that these effects may have arisen from drug influences at the level of the amygdala and/or mPFC. Thus, this chapter examined the effects of administration of GRP and blockade of its receptor at the level of the prelimbic (PrL) cortex, infralimbic (IL) cortex, central nucleus (CeA) or basolateral nucleus (BLA) of the amygdala on conditioned freezing using the CER paradigm. The effects of these compounds on both contextual and cued fear conditioning were assessed following direct bilateral infusions into each region. GRP microinjected into each of the four target nuclei significantly reduced freezing to contextual cues. Similarly, in the cued portion of the CER test, GRP administered to the IL cortex significantly reduced freezing, however, was without effect at either the PrL or amygdaloid nuclei. Administration of the receptor antagonists resulted in mixed results. At the IL cortex, freezing was reduced by low dose antagonist administration to both contextual and cued conditions and was without effect at the PrL. In the amygdala, biphasic effects were observed at the CeA whereby high doses of drug decreased contextual freezing but low doses

increased freezing. At the BLA, freezing was reduced during contextual testing, but was unaffected by the cued test. Interestingly, pretreatment with the receptor antagonist at both the IL and BLA attenuated the GRP-elicited decrease in freezing. These results illustrate that i) GRP system(s) can significantly affect the expression of learned fear, ii) some of the relevant brain sites mediating these effects include the PrL, IL, CeA and BLA., and iii) such effects may be dependent upon whether responses were evoked by environmental contextual fear cues or by specific auditory cues that were explicitly paired with an aversive stimulus.

4. Both corticotropin-releasing hormone (CRH) and GRP have been shown to modulate conditioned fear. Given the modulatory effect of exogenously administered CRH and GRP on conditioned fear, the present study sought to measure the fear-induced endogenous release of CRH and GRP at both the mPFC and the BLA using *in vivo* microdialysis. Rats were divided into 2 conditions; one group received tone + shock pairings, while the second group received the tone alone (no shock). Dialysates were collected the following day from animals in their home cage both prior to and after testing for recall of fear conditioning, as well as in the testing cage during recall testing. The collection of dialysates was not interrupted during the testing period. Analyses of dialysates revealed that both CRH and GRP release at the BLA increased across time and that this increase was significantly elevated in animals who had previously received shock from the outset of the sampling period. Moreover, the elevated GRP and CRH release were significantly correlated with freezing behavior such that levels of freezing (an indication of fear in the rat), accounted for a significant portion of the variability in release of GRP/CRH in the BLA. These effects were not observed in the mPFC. These data indicate that at the level of the BLA, GRP and CRH release are significantly correlated with levels of fear, suggesting that

these peptides may be an indicator of fear and/or involved in determining the emotional salience of an aversive event, and that both GRP and CRH are elevated at 24h post-stressor exposure, suggesting a sensitization of these two substances upon traumatic stress experience.

5. The BLA is a key structure involved in the formation of associations developed during the acquisition of the conditioned fear response. The IL cortex alternatively, appears important to extinction-related learning. In Chapter III we showed that GRP or its receptor antagonists can modulate the conditioned fear response when exogenously administered at these sites, and further, we showed an increase in the release of this peptide at the BLA in response to conditioned fear stress and recall of the training event. The present study thus sought to determine whether a functional pathway utilizing GRP exists between the IL cortex and BLA. We further sought to determine if this pathway also impacts CRH, as evidence suggests that a functional relationship exists between these two peptidergic systems. Results demonstrated that in response to administration of either GRP (300ng/.05µl) or RC-3095 (a GRP receptor antagonist; 500ng/.05µl dose) into the IL cortex, interstitial levels of GRP were significantly elevated at the BLA compared to animals who received vehicle alone (controls) or animals who received both GRP and RC-3095 concomitantly in the same vehicle (300ng/.05µl and 500ng/.05µl respectively). No effects of drug administration into the IL were observed on amygdalar CRH release. The current results provide additional evidence for the involvement of GRP in stress and anxiety-related disorders by virtue of its role in brain areas known to be involved in these disorders.

In summary, this extensive research project provides evidence that NMB is significantly involved in the modulation of anxiety and/or fear, whereas GRP appears to more selectively

modulate fear-related behaviors. Further, it appears that GRP may exert its influence at the level of both the mPFC and the amygdala, possibly by reducing fear through the exogenous administration of peptide at these sites; a promising finding for the development of more specific, and therefore more efficient, therapeutic agents for the prevention and treatment of fear-related disorders. What is unclear however, is the effect of receptor blockade at these sites. Conflicting results may be a consequence of intrinsic properties of the drugs used, and/or may be as a result of biological re-regulation of the system as a consequence of the drug insult. This research project provides further evidence of a role for GRP in a signaling pathway for fear and strongly suggests that modulation of this system may provide therapeutic benefits in fear-related disorders.

General Introduction

In recent years the world has witnessed a torrent of emotionally-laden events; from middle-eastern political unrest and terrorist threats to deadly viruses and natural disasters. Events such as these contribute to an underlying fear that erodes one's sense of safety and security. Fear is a physical reaction to a perceived threat and in its adaptive form, mobilizes energy resources and facilitates the appropriate response to the threatening stimulus. However, when perceived threats become too severe, or are perceived as uncontrollable, maladaptive responses may exhibit themselves in the form of learned helplessness, immobilization, disorganization and possibly mood and/or anxiety disorders. Posttraumatic stress disorder (PTSD), phobias, panic attacks and generalized anxiety all contain a component of fear. In many cases, this fear has been learned (or conditioned) during the experience of emotionally-laden events. For example, the fear of public speaking may result from a negative previous experience talking in large groups, while the fear of loud noises may have arisen from combat experience for a war veteran. When fear becomes conditioned, it can be present even in the absence of the threat. Thus the importance of studying the fear system is paramount to understanding mental illness, and developing strategies for the treatment and/or prevention of stress-related disorders.

The delineation between fear and anxiety is unclear, thus they are constructs often used interchangeably. Although they share a similar phenomenology, there is a growing consensus that these constructs vastly differ [18, 150]. Many agree that fear occurs as the result of perceived danger that is real, identifiable and immediate, prompting a targeted behavioral response [74]; whereas anxiety arises to a potential threat, unforeseeable, but looming in the distant future [38]. Whereas fear provides the individual with a source to direct their behavior towards, anxiety leaves the individual unable to determine whether, or how to act. This element

of uncertainty initiates a physiological reaction akin to fear, but to a lesser degree, suggesting that they may exist on a continuum. Together, fear and anxiety are manifested via a stress response, involving activation of the hypothalamic-pituitary-adrenal (HPA) axis, the adrenergic system within the central nervous system (CNS) and the sympathetic branch of the autonomic nervous system [452]. Thus, the inability to distinguish between fear, anxiety and stress, may reflect a similar phenomenological experience of arousal. For this reason, the remainder of this introduction will not attempt to distinguish between fear, anxiety and stress with respect to their neurobiology, but will instead focus on research primarily garnered through the study of conditioned fear, firstly because of its simplicity, but secondly because the primary focus of this dissertation is largely related to understanding the mechanisms of fear. Additionally, in support of the preclinical data, clinical research on the anxiety disorder PTSD will be presented as this mood disorder arises predominantly due to a maladaptive response to conditioned fear. PTSD is a subdivision of anxiety disorders characterized by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* [14] as a threat to the life of self or close other, associated with intense fear, horror, or helplessness. Conditioned reminders of the trauma, whether external (e.g., noise) or internal (e.g., a memory) may precipitate pathological responses such as re-experiencing the original trauma and emotional and physical arousal [87], thus underscoring the relationship of this mood disorder with the fear system. Presentation of findings from other mood disorders with a fear-related component is beyond the scope of this review, thus the findings from clinical research involving subjects with PTSD alone will be presented.

The Neurobiology of Fear

Studies aimed at understanding the neurobiology of fear often target Pavlovian conditioning processes, largely due to their amenability to experimental manipulation. These studies include fear-potentiated startle (FPS) and the conditioned emotional response (CER). In Pavlovian fear conditioning, an emotionally neutral stimulus (CS) such as a tone, bell, light or white noise, is presented in conjunction with an aversive stimulus (US; often a foot or tail shock in rodents, puff of air to the eye or emotionally-laden imagery in humans), such that after a number of repeated pairings, the CS alone acquires the capacity to elicit responses akin to fear. These responses include behavioral (increased freezing, startle and escape responses in rodents, increased startle in humans), neuroendocrine (hormone release from pituitary and adrenal glands) and autonomic responses (increased heart rate, blood pressure, skin conductance) that are innate, species typical and automatic. Consequently, these stereotypical responses are easy to establish, persistent and are easily quantifiable, making this method ideal as an approach to studying fear mechanisms.

The Neurocircuitry of Fear

Sensory information entering the body through the five sensory modalities is initially relayed through the dorsal thalamus to cortical areas such as primary visual (occipital), auditory (temporal), or tactile (postcentral gyrus) areas. These brain areas subsequently project to multiple sites, one of which is the amygdala. Information entering the amygdala is first processed by the lateral nucleus (LA) of the amygdala, where it then proceeds to the central nucleus of the amygdala (CeA), both directly and indirectly by way of the basal amygdala (BA; also referred to as the basolateral amygdala).

The CeA modulates fear expression via projections to the midbrain and hypothalamus, which control autonomic responses and somatic motor centers [207, 234]. These projection sites

include the locus coeruleus (LC – involved in arousal), parabrachial nucleus (pain pathways, increased respiration) [136, 307]; nucleus of the solitary tract (NTS - connected to the vagal system) [473], periaqueductal gray (freezing, vocalizations, startle, analgesia and cardiovascular changes) [31, 385]; the paraventricular nucleus (PVN) of the hypothalamus which exerts hormonal influences (such as glucocorticoid release) that modulate brainstem centers [207, 234], and the lateral hypothalamus (heart rate and blood pressure). These projections are thought to be involved in the hyperarousal symptoms of PTSD [355, 482] such as increased startle response (a measure of autonomic arousal) and fear-related freezing seen in animals [156, 355, 436].

The basal nuclei of the amygdala (basal - BA and accessory basal - AB) sit just ventral to the LA and project to both the CeA and the LA [421]. In addition, these nuclei share extensive reciprocal projections with the orbital and medial prefrontal cortex (mPFC) [13, 333], thus, they modulate neuronal activity in both the LA and PFC [132, 333].

The bed nucleus of the stria terminalis (BNST) sits just ventral to the CeA and sends projections to several autonomic regulatory brain stem areas including the NTS [151, 186], caudal ventrolateral medulla [143], and dorsal nucleus of the vagus [151, 187], supporting the role of the BNST in the regulation of autonomic responses. Further, both the CeA and BNST have strong projections to ascending monoaminergic and cholinergic neuron groups; these include the noradrenergic LC, the dopaminergic substantia nigra and ventral tegmental area, the serotonergic raphé, and the cholinergic nucleus basalis [13, 105, 364]. Thus, these projections potentially modulate information processing in large regions of the forebrain and temporal lobe memory systems as well as providing inputs to the amygdaloid complex.

Projections carrying US information (i.e., from a foot-shock) arise from both the posterior parietal insula and the posterior intralaminar nuclei (PIN) of the thalamus [431, 432].

Finally, information regarding spatial and temporal aspects of events is processed by the hippocampus, a region which shares extensive reciprocal connections amongst both the mPFC nuclei and each of the aforementioned amygdalar nuclei [352, 464].

In the sections that follow, the amygdala and its extended nuclei as well as the mPFC and hippocampus will be detailed further by presenting both clinical and preclinical evidence to support their role in conditioned fear.

The Role of the Amygdala in Conditioned Fear

Preclinical Studies

The amygdala is an area thought to mediate emotional experiences. It is believed that the amygdala may provide the emotional valence associated with memories and thus, may enhance the connection with emotionally arousing stimuli [156]. For example, Ledoux and colleagues [232] found that disruption of either the lateral or central nuclei of the amygdala interferes with animals' ability to acquire new fears. Preclinical research has allowed investigators to explore each nuclei within the amygdala to a greater extent, thus, the sections that follow will present this preclinical evidence in detail and will delineate the role that each nucleus of the amygdala plays in the conditioned fear process. This review will begin with the lateral nucleus of the amygdala, the entry point at which CS-US associations are thought to be made.

Lateral Nucleus of the Amygdala

Sensory inputs to the amygdala terminate primarily in the lateral nucleus of the amygdala [13, 232, 274, 280, 397, 468, 469], thus the LA is thought to be the interface where fear conditioning develops [233]. Distinct amygdala afferents are recruited during fear conditioning

which are dependent on the CS modality utilized. Whereas auditory CS information enters the LA via projections from both the auditory cortex and medial geniculate nucleus (MGm) of the thalamus [67, 235, 396], visual CS information enters via afferents from the perirhinal cortex [67, 401, 431]. Lesions to these areas reliably disrupt conditioning to both auditory and visual CS information, however some evidence suggests that the perirhinal area may in fact function as a multimodal (both auditory and visual) sensory relay to the amygdala and/or be involved in general memory functions such as information storage or retrieval [67]. Projections carrying US information (i.e., from a foot-shock) arise from both the posterior parietal insula and the PIN of the thalamus [431, 432]. Lesions of these two areas in combination (but not separately), disrupt fear conditioning [395, 431, 432].

Lesions to the LA block acquisition and expression of fear conditioning [67, 68, 232, 265], while pharmacological inactivation of this nucleus with muscimol, a gamma-aminobutyric acid (GABA)_A agonist, disrupts fear conditioning if applied immediately before conditioning or during testing, but not when applied immediately after conditioning [173, 323, 495]. This suggests that acquisition and expression, but not consolidation of conditioned fear are affected by inhibition of the LA. Indeed, small lesions of either the LA or MGm/PIN prevent fear conditioning, whereas large lesions of the auditory cortex do not [232, 396], indicating that thalamo-amygdala inputs are sufficient for conditioned fear responses.

Recording studies showed that cells in the LA are responsive to both nociceptive and auditory inputs [395]. Romanski et al., [395] found that single neurons within the LA respond to both auditory and somatosensory information during fear conditioning suggesting that these neurons may serve as the locus for CS-US convergence. Indeed, the firing properties of cells in the LA become modified during fear conditioning [89, 262, 366, 372, 381] such that during

aversive conditioning, firing changes can be seen in the LA, the auditory thalamo-amygdala pathway and the cortico-amygdala pathway [366]. Conditioned responses in the thalamo-amygdala pathway precede changes observed in the cortico-amygdala pathway. Most of the conditioned increases in spike firing occurred within 15 ms of tone onset, corresponding to the latency of thalamic (12 ms) rather than cortical (>20 ms) activation of LA neurons [243]. This suggests that direct thalamo-amygdala transmission accounts primarily for the LA responses observed. In turn, these firing responses occur prior to firing changes observed in other amygdala nuclei. This supports the contention that the LA appears to be the initial point of sensory processing and the initial site of plasticity within the amygdala. It further supports the findings that thalamo-amygdala inputs are sufficient for conditioned fear responses [271].

Central Nucleus of the Amygdala

Downstream targets of the LA include projections to the CeA, both directly and indirectly by way of the BA (sometimes referred to as the basolateral amygdala). It is thought that the CeA constitutes the amygdala's interface to fear response systems [263]. For example, lesions of the CeA produce profound deficits in the expression of fear conditioned responses using either a visual or auditory CS [67, 148, 178, 179, 214, 518], while stimulation of the CeA produces a constellation of conditioned fear responses even in the absence of prior fear conditioning [198, 207]. Because the CeA receives inputs from the LA and BA [353], it is ideally situated to mediate conditioned fear expression elicited by both acoustic and contextual CSs.

The CeA modulates fear expression via projections to the midbrain, which controls autonomic responses, and the hypothalamus which exerts hormonal influences that modulate brainstem centers [207, 234]. Whereas lesions to the CeA interfere with conditioned fear expression overall [138, 178, 199, 206, 472], damage to CeA projection sites selectively

interrupts the expression of individual responses. For example, lateral hypothalamus lesions affect blood pressure but not freezing responses, while damage to the periaqueductal gray disrupts freezing but does not affect blood pressure responses. Similarly, damage to the BNST disrupts the conditioned release of pituitary-adrenal stress hormones [472] but no effect is observed on either blood pressure or freezing responses [234].

Other midbrain and hypothalamic projection sites from the CeA include the LC, parabrachial nucleus, NTS, and the PVN [325]. The LC sends norepinephrine projections to higher brain regions (such as the amygdala, hippocampus, striatum and PFC) while the hypothalamus sends excitatory transmitters to lower brain regions, specifically to regions in the brain stem and the cerebellum which activate the sympathetic branch of the autonomic nervous system. This in turn results in activation of the body's fight or flight response (via the deactivation of non-essential body systems such as digestion, and the enhancement of essential systems such as respiration). Projections to the brainstem include the periaqueductal gray (vocalizations, startle, analgesia and cardiovascular changes) [31, 385]; the parabrachial nucleus, (pain pathways) [136, 307]; and the NTS (connected to the vagal system) [473]. In addition to these output pathways, the CeA is also implicated in the modulation of attention, arousal and vigilance during conditioning [105, 184], effects which are mediated via CeA-striatal and basal forebrain connections [70, 163, 182, 183].

The Basolateral Complex

The basolateral complex (BLA for the purposes of this dissertation) comprises the LA, the BA (but in some journals solely referred to as the BLA), and the AB (which is also known as the basomedial nucleus). As these three nuclei form a distinct region (complex) within the amygdala, they are often studied together as a whole. Lesions of the BLA block both acquisition

and expression of fear conditioning [67, 232, 265], while functional inactivation of this region using muscimol impairs fear conditioning only when applied immediately before conditioning or during testing, but not when applied immediately after conditioning [173, 323, 495].

The BA and AB sit just ventral to the LA and project to both the CeA and the LA [421]. In addition, these nuclei share extensive reciprocal projections with the orbital and mPFC [333], thus, they modulate neuronal activity in both the LA and PFC [132, 333]. Functional inactivation of the LA and BA nuclei prior to fear conditioning impairs acquisition of learning, whereas inactivation immediately following conditioning has no effect on consolidation of fear memory [495].

Considerable evidence suggests that the BLA is also essential for the expression of fear responses. Freezing, [93, 260, 265, 488] tachycardia and hypertension [232] and fear-potentiated startle [68, 239, 412] all appear to be affected by lesions to the BLA. This effect has been seen even when lesions were made more than a month after fear conditioning [239, 265]. Further, unconditioned fear responses also appear to be affected by BLA lesions. For example, inactivation of BLA neurons with glutamate receptor antagonists attenuate the increase in the acoustic startle reflex produced by bright light [486], however, in a predator odor paradigm, unconditioned freezing remained intact when conditioned freezing was abolished by BLA lesions [488]. Interestingly, conditioned freezing could be developed in BLA-lesioned rats, but only when over-training was involved (i.e., if given 50 or 75 training trials), and only if lesions were given prior to training; posttraining lesions abolished freezing, despite extensive overtraining prior to surgery [261]. Further, this effect appeared to be specific to contextual cues and not to a discrete cue such as a tone. Thus, it seems that posttraining lesions disrupt the memory for fear conditioning and that the BLA is not essential for the performance of freezing

behavior, but rather, plays a critical role in associative processes underlying pavlovian fear conditioning [264].

The Bed Nucleus of the Stria Terminalis

The involvement of the BNST in fear remains unclear. It is ideally situated to substantially impact fear processes, but evidence to support its role in fear is sparse. Projections exist from the BNST to several autonomic regulatory brain stem areas including the NTS [151, 186], caudal ventrolateral medulla [143], and dorsal nucleus of the vagus [151, 187], supporting the role of the BNST in the regulation of autonomic responses. Further, afferents project from the perifornical area of the hypothalamus to the BNST [12]. Stimulation of this hypothalamic area mimics stress-induced neuroendocrine and autonomic responses, i.e. elevation of blood pressure, heart rate, plasma norepinephrine, and epinephrine [12, 447, 448]. The BNST also shares extensive reciprocal connections with the CeA and PVN and moreover, both CeA and BNST have strong projections to ascending monoaminergic and cholinergic neuron groups. These include the noradrenergic LC, the dopaminergic substantia nigra and ventral tegmental area, the serotonergic raphé, and the cholinergic nucleus basalis [13, 105, 364]. Thus, these projections potentially modulate information processing in large regions of the forebrain and temporal lobe memory systems as well as providing inputs to the amygdaloid complex.

In studies investigating fear, several reports have found support for the BNST's involvement in unconditioned or unlearned fear. For example, BNST lesions disrupt a rat's spontaneous preference for a dark over a light chamber, a behavior generally believed to reflect unconditioned fear [486]. Other studies have found that unconditioned freezing responses to aversive tones [426] or to trimethylthiazoline [120], a component of fox feces odor, are also affected by damage to the BNST. Furthermore, excitotoxic lesions of the BNST completely

blocked corticotropin-releasing hormone (CRH)-enhanced startle, as did intra-BNST infusions of the CRH antagonist, alpha-helical CRH. Infusions of CRH directly into the BNST increased startle amplitude however, neither BNST lesions nor intra-BNST alpha-helical CRH infusions disrupted fear-potentiated startle. Similarly, light-enhanced startle, like CRH-enhanced startle, appears dependent on the BNST [486].

In studies investigating the role of the BNST in conditioned fear, conflicting evidence has emerged. For example, in two lesion studies, obliteration of the BNST did not block fear-potentiated startle or conditioned freezing [234]. However, a third lesion study showed that the BNST appeared to be involved only in the contextual component of conditioned fear responses (and not to an explicit cue). This study revealed that the BNST was not involved in either the autonomic or freezing responses elicited by a tone CS, however freezing and corticosterone responses were intact for contextual cues [451]. The authors suggest that the involvement of the BNST with the contextual component of fear is likely due to its connections with the hippocampus [451], a structure essential to contextual fear conditioning [216, 348] as will be outlined below. It has been suggested that due to the similarities between the CeA and the BNST, that the CeA is more involved in quick responses to fear activated by relatively short stimuli which are predictable and stimulus-specific fear responses (i.e., to specific threat cues). The BNST however, mediates responses to relatively long cues under conditions in which the perceived danger is unpredictable (as in the case of contextual fear conditioning), and thus appears to have a more sluggish and sustained response to fear, more akin to anxiety [102]. It is thus believed that the CeA and BNST may reflect parallel systems in the fear process [487].

Clinical Studies

Clinical studies support the animal literature in linking the amygdala to learned fear. For example, using different forms of imaging, several researchers have reported increased regional cerebral blood flow (rCBF) in the amygdala (relative to control conditions) during symptom provocation (fear-inducing) tasks using PTSD subjects [378, 379, 428]. In one positron emission tomography ($[^{15}\text{O-CO}_2]$ - PET) study, script-driven imagery of autobiographical accounts of trauma revealed that in a mixed gender cohort, increases in rCBF were found in the right amygdala relative to neutral script-driven imagery in control individuals [378]. Similarly, a $[^{15}\text{O-CO}_2]$ - PET study of male combat veterans showed increases in rCBF in the right amygdala when subjects were asked to generate combat images [433]. Left rCBF changes were observed in a single-photon emission tomography (HMPAO-SPECT) study involving Vietnam veterans [246], while two other studies utilizing functional magnetic resonance techniques (fMRI) also demonstrated this exaggerated amygdala response [379, 428].

Imaging studies using human subjects with PTSD have also explored conditioned fear processes. For example, in one fMRI study using a discrimination task involving a visual cue predicting shock (CS+) compared to a visual cue presented alone (CS-), LaBar and colleagues found a significant activation of the amygdala during both the acquisition and extinction phases of fear conditioning [226]. Using event-related fMRI to study the classical conditioning of faces paired with aversive tones in healthy subjects, Buchel and colleagues [62, 63] also found activation in the anterior cingulate cortex (ACC) as well as the amygdala, however slight decrements in amygdala responses were observed suggesting rapid habituation of this region [62, 63]. These data support an earlier study showing that bilateral damage to the amygdala in humans results in impairments in long-term declarative memory for emotionally arousing

material [7]. Taken together, these studies provide evidence for the role of the amygdala and its extended nuclei in emotional learning and fear conditioning.

The Role of the Hippocampus in Conditioned Fear

Preclinical Studies

The hippocampus is thought to mediate the recall of facts and events (e.g., declarative memory function) and to assign significance for events within space and time [443]. This ability is critical to effectively build memory traces related to a potential threat in order to prevent, defend against, or avoid threats in the future. For this reason, the hippocampus plays a pivotal role in conditioned fear. Lesions of the hippocampus produce impairments in the acquisition of contextual-based conditioned fear (i.e., to the place in which a fearful incident occurred), suggesting that it bears an impact on memory for the emotional context of a stressor [216, 217, 266, 270, 348, 349]. This effect however, has not been consistently observed. More recent reports indicate that axon-sparing neurotoxic lesions of the dorsal hippocampus (DH) do not yield contextual conditioning deficits when made prior to training [81, 130, 145, 266]. Posttraining neurotoxic lesions of this area however, produce massive deficits in contextual fear conditioning [266]. These differential effects could be the result of alternate strategies for acquiring contextual fear [266].

Some researchers have argued that the disruption of freezing performance to hippocampal lesions may also be the result of locomotor hyperactivity which typically accompanies hippocampal damage [142, 147]. For example, hippocampal lesions disrupt not only conditioned freezing responses, but also unconditioned responses, such as those elicited by

a rat when exposed to a cat [142, 145, 384]. Further, Davis and colleagues using fear-potentiated startle (as opposed to freezing as a behavioral measure of fear), found that hippocampal lesions do not affect either contextual fear conditioning [287] or contextual blocking (a procedure whereby prior contextual fear conditioning retards subsequent cue conditioning) [288]. Thus, it is possible that locomotor hyperactivity may account for the lack of freezing observed in contextual fear paradigms; however, this argument appears to be overly simplistic. For example, lesions to the hippocampus do not disrupt freezing to an explicit cue (although the opposite has been found in at least one instance) [266]. Moreover, Anagnostaras et al., [16] reported that rats with DH lesions exhibit impaired freezing when tested in a context that had been paired with shock 1 day before the surgery, but had normal and/or high levels of freezing when tested in a context that had been paired with shock 28 days preoperatively. These findings suggest that the lack of freezing behavior to one context but not to the other cannot be explained in terms of a freezing-performance deficit.

Clinical Studies

Human literature supports a role for the hippocampus in fear processes. Imaging studies using patients with PTSD have found hippocampal alterations, particularly in volume and in function [44, 50, 51, 52, 158, 424, 445], and further, chronic or severe PTSD may play a larger role in the observed hippocampal volume reductions than acute or early onset PTSD [356].

It is believed that the abnormal hippocampal structure and function observed in patients with PTSD might be as a result of “enhanced negative feedback” of cortisol in an attempt to shut down the HPA response in these individuals [509]. HPA axis dysfunction is well-documented in PTSD sufferers [467] and involves increased levels of CRH even though cortisol levels appear to

be decreased. This implies that the brain is attempting to stimulate an HPA axis response [509]. Cortisol acts as a negative feedback mechanism to turn off the HPA response and the hippocampus is a major site of action for this mechanism as it is rich in glucocorticoid receptors.

Research based on animal studies has shown that stress is associated with damage to the hippocampus [282, 418], and further, high levels of glucocorticoids and excitatory amino acids may mediate this effect [43]. Under conditions of repeated or steady exposure to stress, atrophy of CA3 neurons in the region of Ammon's horn have been observed. Moreover, repeated administration of glucocorticoids (for 21 days in rodents) damaged CA3 region pyramidal neurons [492, 503]. It remains unclear however, whether reduced hippocampal volumes are a consequence of the traumatic exposure or reflect pre-trauma vulnerability to develop PTSD [356]. Two authors suggest that the development of clinical symptoms and/or a neuroendocrine imbalance appears to precede the decreased hippocampal volume [49, 419], as hippocampal atrophy has been directly shown to be caused by high levels of these stress related hormones [49]. Furthermore, a recent neurobiological model proposed by Villarreal and King [482] suggests that the hippocampal atrophy common to PTSD sufferers may be a downstream effect of amygdala-mediated hyperarousal symptoms.

To further support hippocampal dysfunction in PTSD, researchers using imaging techniques have tried to correlate memory components to their findings. Studies have shown that patients with PTSD have reduced long-term potentiation (LTP) and deficits in declarative memory and new learning [42]. It is thought that the hippocampal damage typical to PTSD sufferers may underlie distortions and fragmentations of traumatic memories as well [51]. For example, Bremner and colleagues [51] found a significant correlation between deficits in verbal declarative memory and hippocampal volume in a structural MRI design involving Vietnam

combat veterans, however, no correlation was found between impairments in declarative memory and hippocampal volume in other studies [158, 445]. It is important to note however, that Bremner's study [51] did not correct for overall brain volume in its analysis and therefore, the results could be wrongly interpreted.

The Role of the Prefrontal Cortex in Conditioned Fear

Preclinical Studies

Integral to the experience of a potential fear-eliciting event, is the ability to process that event, determine its salience and to shut down the fear response after the threat has passed. It is believed that the medial and orbital prefrontal cortices participate in interpreting the higher-order significance of experiential stimuli, modifying behavior according to the anticipated social outcome of behavioral responses to emotional events. Extensive reciprocal connections exist between the amygdala and PFC [13] and it is thought that PFC neurons inhibit or “gate” amygdalar activity [435, 482] as these prefrontal areas have inhibitory connections to the amygdala [13, 316, 317].

Lesions of the mPFC in animal studies are associated with a failure of extinction of fear [317, 373]. Indeed, this area is thought to be involved in the consolidation and/or storage of extinction memory [369]. Damage specifically to the ventral medial prefrontal cortex (infralimbic and prelimbic areas) of rats did not impair the acquisition of conditioned freezing nor did it impact extinction learning when testing was done on the same day (short-term consolidation of learning), however when tested the following day (long-term recall of extinction learning), freezing responses spontaneously recovered to maximal levels as if rats had never received extinction training. Consistent with this, Milad and Quirk [304] showed that neurons in

the infralimbic region (IL) of the mPFC are directly involved in the extinction of a tone/shock conditioning procedure. When rats were tested for recall of extinction learning the day following extinction training, neurons in the IL region showed robust tone responses suggesting that these neurons signal memory for extinction. This effect was not observed during either fear conditioning training or during extinction learning. According to these authors, the greater the neuronal activation in this region during the presentation of tone upon recall testing, the less rats froze. These authors suggest that extinction learning potentiates infralimbic neuronal activity, which in turn, inhibits fear during subsequent encounters with fear stimuli.

In support of this line of research, Barrett and colleagues [27] demonstrated through metabolic mapping that the highest level of neuronal activity following extinction training occurred in the mPFC, more specifically in the IL. These authors used uptake of fluorodeoxyglucose (FDG – a radiolabeled glucose analog used to assess energy metabolism) to examine brain regions involved in fear conditioning and extinction. Metabolic responses to a test tone were compared in groups of mice that received fear conditioning, a pseudorandom treatment (unpaired tones and shocks), or conditioning followed by extinction. The largest area of activity after extinction was observed in the mPFC, with the IL (but not prelimbic) region exhibiting greater activity than controls. Significant metabolic increases were also observed in dorsal, medial and lateral frontal cortex, suggesting that multiple frontal areas play a role in fear extinction. Further to this, a significant correlation was found with extinction behavior and FDG labeling in the dorsal mPFC, IL cortex and in dorsal and lateral frontal cortex areas, supporting the hypothesis that mPFC acts in an inhibitory capacity. Moreover, a strong negative correlation between prefrontal areas and regions thought to be involved in expression of conditioned fear, such as the ventral tegmental area, mediodorsal thalamus and the entire auditory system

(brainstem, thalamic, and cortical levels) was also observed [27]. These findings suggest that extinction training may act to both inhibit the conditioned response after extinction and to preserve some of the original CS-US associative effects from acquisition [374].

Clinical Studies

In line with the preclinical data, imaging studies using subjects with PTSD have now shown decreased blood flow in the mPFC, and in several instances, a failure of activation of anterior cingulate and medial orbitofrontal cortices [44, 51, 53, 428, 434]. Based on these findings, several authors [435, 482] posit that an underactive mPFC and anterior cingulate gyrus may be responsible for the failure to inhibit a hyperresponsive amygdala, thus leading to the inadequate extinction of conditioned fear and hyperarousal symptoms seen in PTSD sufferers. Furthermore, Hamner and colleagues [162] argue that this failure of normal activation may lead to increased fearfulness that is not appropriate for the context, a behavioral response that is highly characteristic of patients with PTSD [162].

Several studies have provided evidence for a failure to activate anterior cingulate and/or medial frontal cortex during symptom provocation in PTSD patients [44, 51, 53, 428, 434]. Shin and colleagues [434] examined 16 female survivors of childhood sexual assault (CSA): 8 with PTSD, 8 without, using script-driven guided mental imagery and [^{15}O -CO $_2$]- PET scanning. The subjects were asked to recall and imagine neutral and autobiographical abuse events. Only the comparison group exhibited rCBF increases in the anterior cingulate gyrus. Both groups exhibited greater increases in rCBF during the trauma condition compared to neutral condition in the orbitofrontal cortex, however, the PTSD group showed a significantly higher increase than the comparison group. In Bremner and colleagues' 1999 [^{15}O -CO $_2$]- PET studies looking at

female survivors of CSA and Vietnam veterans, subjects were asked to listen to scripts describing neutral and abusive events, or in the case of the combat veterans, were presented combat-related or neutral slides and sounds. These studies both found decreased activation of the mPFC and a failure to activate the cingulate gyrus [50, 53].

In a cognitive activation paradigm, Shin and colleagues [435] used the Emotional Counting Stroop task and fMRI to demonstrate a failure of anterior cingulate activation in combat-related PTSD. This task involves presenting a series of trauma-related and neutral words to subjects while they are asked to name the color in which each word is printed [435]. This study showed blood-oxygen level dependent (BOLD) signal increases in rostral anterior cingulate cortex in Vietnam veterans without PTSD ($n = 8$), however, an increase was not seen in veterans with PTSD ($n = 8$).

To further validate these findings, one study using proton magnetic resonance spectroscopy (^1H -MRS) to measure the ratio of n-acetylaspartate to creatine (NAA/CRE) in these areas, found deficits in anterior cingulate and mPFC in a childhood trauma population (11 children with maltreatment-related PTSD compared to 11 control individuals) [108]. These results are suggestive of neuronal loss in the anterior cingulate and mPFC, however, caution should be exercised with this interpretation, as these results are from a small sample involving children, and may reflect the global neuronal loss seen in previous work by this group [107]. These results need to be replicated in adult populations to make more definitive inferences on PTSD-related pathology.

As with other cerebral areas, imaging results have not been consistent in regards to the PFC. For example in symptom provocation studies, one study found an increased rCBF in the ventral anterior cingulate gyrus in Vietnam combat veterans [433], while a second study found

increased rCBF in the rostral anterior cingulate gyrus in PTSD secondary to various traumas [378]. Both of these studies used [^{15}O -CO $_2$]-PET imaging, however, as no control group was used in the second study for comparison, these results are questionable. Furthermore, two additional studies using HMPAO-SPECT and the presentation of combat sounds found no group differences in rCBF in the ACC of Vietnam veterans with PTSD compared to healthy combat-matched controls and/or normal controls [246, 525].

In summary, the development of the fear response involves an intricate web of brain pathways intertwined to form a constellation of networks designed to precipitate the response to fear. The major players in this network have been outlined above, namely the amygdala, BNST, PFC and hippocampus, although the projection sites that these areas recruit are equally important to eliciting a fear response. In conjunction with knowledge of the brain loci involved in the fear response, it is equally important to understand the neuroendocrine and neurochemical mechanisms that are involved in the fear response. To this end, the sections that follow are aimed at reviewing the literature on these mechanisms in an attempt to better understand their involvement.

Neuroendocrine Responses to Fear

Exposure to a potentially harmful or life-threatening event triggers a cascade of physiological and behavioral effects. The primary effect is activation of the HPA axis and the sympathetic nervous system. Afferent projections from the CeA, BNST or ventral striatum [422] stimulate the lateral nucleus of the hypothalamus which in turn activates the sympathetic branch of the autonomic nervous system leading to increases in blood pressure and heart rate, sweating, piloerection, and papillary dilation. In turn, release of CRH from the paraventricular nucleus (PVN) of the hypothalamus increases peripheral adrenocorticotrophic hormone (ACTH) levels,

which stimulate the adrenal glands to secrete glucocorticoids - either cortisol (in humans) or corticosterone (in animals). Other hypothalamic afferent projections include the ACC, anterior insula, and posterior orbital cortex which modulate cardiovascular and endocrine responses to threat and stress [131, 231, 333].

Just as the HPA axis provides the organism with a mechanism to react and adapt to stress, so to is it equally necessary to have a mechanism in place to shut down this response once the threat has passed. 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 terminating further synthesis and release of CRH and adrenocorticotrophic hormone (ACTH) [303]. Protracted activation of this system can be detrimental or maladaptive to an organism, and in fact, could play a major role in the hippocampal atrophy observed in subjects with PTSD as described earlier.

This maladaptive response in PTSD sufferers involves primarily increased levels of CRH even though cortisol levels appear to be decreased. This implies that the brain in PTSD sufferers is attempting to stimulate an HPA axis response, and since CRH levels are increased, it follows that the HPA axis is not underactive [509]. Thus, Yehuda and colleagues put forth the theory of an “enhanced negative feedback” system involving inhibition of cortisol to explain the above paradox [509]. Cortisol acts as a negative feedback mechanism to turn off the HPA response. The hippocampus is a major site of action for this mechanism and is rich in cortisol-binding glucocorticoid receptors. In Yehuda’s model, an increased number and sensitivity of glucocorticoid receptors in the hippocampus leads to deleterious effects on the CA2 and CA3 regions of this structure. A series of studies in rats have now shown that this damage occurs through a cascade of events eventually leading to abnormally high levels of intracellular calcium

(a consequence of inhibited glutamate reuptake and increased activation of N-methyl-D-aspartate [NMDA] receptors). This abnormally high level of calcium eventually leads to cell death.

While in a separate series of events, increased glucocorticoid activation inhibits glucose transport, thereby depriving the hippocampus of the energy to reverse the events that have compromised its neuronal integrity [509]. Thus, understanding the underlying mechanisms of the HPA axis in fear and/or stress is imperative to understanding PTSD, and by association, developing therapies and/or pharmacological treatments for this disorder.

Neurochemical Responses to Fear

Several neurotransmitters and neuropeptides are involved in the response to threat or fear. These neurochemical systems subserve important adaptive functions such as increasing vigilance, modulating memory, mobilizing energy stores, and elevating cardiovascular output. Nevertheless, these biological responses to threat and stress can become maladaptive if they are chronically or inappropriately activated.

Neuropeptides are small protein-like signaling molecules released by neurons which allow neurons to communicate with each other. They can function as hormones by traveling through the blood stream to act upon distant organs and tissues; they can act as neuromodulators when released into the extracellular fluid in the brain, thus influencing neurons; or they can function as neurotransmitters when released from axon terminals to act on receptors within close vicinity [209].

The following sections will attempt to cover some of the key neurochemicals that have been explored in an attempt to understand the fear system. Although the focal interest of this dissertation is the role of neuropeptides in the mediation of the fear response, of which the primary peptides of interest, namely CRH and bombesin-like peptides (BLPs), in this work are

covered below, it is important to keep in mind that several classical neurotransmitters (that are affected by stressors) may also influence peptidergic functioning. Thus, neurotransmitters which may affect the action of CRH and BLPs will also be discussed. It should also be recognized that while the focus of this discussion is restricted to the role of the neuropeptides/neurotransmitters in fear and/or stressor-related responses, as a rule rather than an exception, peptides/neurotransmitters have multifunctional roles that go well beyond the response to stress and/or fear.

Neurotransmitters in Conditioned Fear

Norepinephrine

Preclinical Studies

One neurotransmitter system given extensive attention in fear-related research is the noradrenergic system. Central noradrenergic cell bodies are predominantly located in the LC, a nucleus located in the hindbrain. Fear-related activation of the LC results in increased release of norepinephrine (NE) in multiple brain regions that are involved in perceiving, evaluating and responding to potentially threatening stimuli; regions such as the amygdala, hippocampus, striatum and PFC.

Norepinephrine is thought to be involved in an organism's overall state of arousal [1, 386]. For example, across several animal species, an increase in LC neuronal firing is associated with alertness, whereas a decrease in firing is associated with drowsiness [34, 128, 200]. Further, activation of the LC by direct electrical stimulation or by pharmacologic agents such as yohimbine or piperoxan, both noradrenergic antagonists which cause an increase in LC firing,

elicits alerting and fear responses in primates. In contrast, such behaviors are reduced by pharmacologic agents that decrease LC firing [380]. Interestingly however, Cain and colleagues [65] demonstrated that yohimbine accelerates extinction learning in mice by blocking α_2 -autoreceptors, but blocking β noradrenergic receptors with propranolol did not have a significant effect on the rate of extinction. This suggests that each receptor subtype may subserve a different function within the fear-system.

It is believed that NE may also play a role in encoding memories for fearful events. For example, several studies have shown that an intact NE system is necessary for the acquisition of fear-conditioned responses [78, 88, 377] and further, that consolidation of memory for a fear-eliciting event can be enhanced by posttraining administration of NE. Moreover, NE has been shown to enhance memory retrieval when administered at the time of memory testing. It has been suggested that traumatic events stimulate the release of NE which in turn causes an over consolidation of memory for the stressful event. The resultant outcome is a deeply engraved traumatic memory that is clinically expressed in the form of intrusive recollections, flashbacks, repetitive nightmares and conditioned emotional responses.

NE is also thought to play a role in selective attention and vigilance [22]. For example, in freely moving cats when confronted with a dog or other aggressive cat, marked increases in LC firing are observed, however the same was not found when cats were exposed to non-threatening stimuli such as a mouse. These data suggest that the meaning and intensity of the stimuli are important factors in LC responding [200].

When an organism experiences chronic uncontrollable stress, two outcomes are possible. The first, an adaptive response, results in increased responsivity of LC neurons [437], which may result in an exaggerated NE response to future stressors. This enhanced catecholamine synthesis

and release may help to protect the organism from depletion of neurotransmitter stores and allow the organism to respond more rapidly and robustly to future stressors. The second effect of chronic uncontrollable stress is maladaptive. For example, animals exposed to repeated severe stressors from which they cannot escape often develop a behavioral pattern termed *learned helplessness*, which is associated with NE depletion, a point at which NE synthesis cannot keep pace with NE release [46, 47]. NE depletion in turn, impairs neuroendocrine responses to fear [332].

Clinical Studies

In patients with PTSD, a heightened peripheral sympathetic arousal is thought to correlate with excessive noradrenergic function. This is supported by patients who report that alcohol, benzodiazepines and opiates, agents known to decrease LC neuronal firing activity, ease their hyperarousal symptoms and intrusive memories, whereas cocaine, which increases LC neuronal firing, exacerbates these symptoms. Further, several studies have consistently reported elevated NE activity in patients with PTSD. For example, women with PTSD suffering from childhood sexual abuse showed elevated 24-hour urinary excretion of catecholamines and cortisol [241]. In another study, men with PTSD resulting from a motor vehicle accident (MVA) exhibited elevated urinary levels of epinephrine, NE, and cortisol 1 month after the accident with lingering elevated epinephrine levels 5 months later [168]. This effect was not observed in female MVA survivors with PTSD. Children with PTSD have also exhibited increased 24-hour urinary dopamine (DA), NE, and cortisol, with the urinary catecholamine and cortisol output positively correlated with the duration of PTSD trauma and the severity of PTSD symptoms [106]. This same phenomenon was observed in combat vets with PTSD. Exposure to traumatic

reminders (e.g., combat films or sounds) produced greater increases in plasma epinephrine, NE, and cortisol in patients with PTSD than in control subjects [37, 168, 283], although baseline concentrations of catecholamines are not consistently altered in combat-related PTSD [440, 441]. Finally, elevated cerebrospinal fluid (CSF) NE concentrations have been found in subjects with PTSD [139]. In summary, a hypersensitive NE system seems to be a major facilitator of many symptoms in PTSD patients, symptoms such as an exaggerated startle response, hypervigilance, and hyperarousal.

Serotonin

Preclinical Studies

A second neurotransmitter extensively studied in fear-related research is serotonin (5-HT). It has been suggested that 5-HT and NE may play complementary roles in an organism's response to arousing stimuli. For example, during the orienting response, NE's neuronal firing rate increases substantially, whereas the firing rate of 5-HT neurons decreases or even ceases momentarily. A decrease in 5-HT neuronal firing is associated with disinhibition of sensory processing, a decrease in vegetative activities, and suppression of gross motor movement.

Studies investigating the role of 5-HT in conditioned fear have found an increased metabolism of 5-HT, especially prominent in the mPFC, in response to exposure to an environment previously paired with foot shock [192]. Further, extracellular 5-HT concentrations in the mPFC increased during freezing behavior and returned to normal when animals were returned to their home cages [165, 516]. This increase in 5-HT appears particularly sensitive to the severity of stress, increasing as the aversiveness of the US and the magnitude of the

conditioned fear behavioral response increases [194]. The rise in 5-HT during freezing behavior can be blocked by pretreatment of diazepam and tropisetron, a 5-HT₃ receptor antagonist. Moreover, diazepam, ipsapirone, a 5-HT_{1A} receptor agonist and the selective 5-HT reuptake inhibitor citalopram also reduce freezing behavior [165, 195]. In a later study, Hashimoto et al., [165] found that the conditioned fear induced increase in extracellular 5-HT levels at the mPFC resulted in a resolution in freezing behavior, while increasing 5-HT levels via injection of citalopram also resulted in reduced freezing behavior. Taken together, these results suggest that facilitation of brain 5-HT neurotransmission results in decreased anxiety or fear.

In studies involving acoustic startle reflex (ASR), researchers have found that direct administration of 5-HT into the ventricles of rodents depresses the acoustic startle reflex. In addition, reducing 5-HT using receptor antagonists enhances the ASR while drugs that result in increased levels of 5-HT decrease the ASR, similar to that found with conditioned fear. Furthermore, animals that are genetically deficient of 5-HT₂ receptors show a reduced ASR.

Studies investigating chronic inescapable stress have found depletion of 5-HT in the mPFC with accompanying behavioral deficits that have been described as learned helplessness [344, 346]. Pretreatment with benzodiazepines or tricyclic antidepressants can prevent this decrease in 5-HT and accompanying behavioral deficits [344, 430]. Moreover infusion of 5-HT into the frontal cortex after stress exposure reverses learned-helplessness behavior. Finally, administration of 5-HT-receptor antagonists produce behavioral deficits resembling those of the learned helplessness seen after inescapable shock during animal stress models in which learned helplessness is not ordinarily reported [345].

Clinical Studies

In clinical studies, alterations in 5-HT have also been reported in patients with PTSD. For example, platelet paroxetine binding was decreased in subjects with combat-related PTSD relative to controls, a finding suggesting alterations in the 5-HT transporter [21]. Additionally, the prolactin response to the 5-HT-releasing and uptake inhibitor d-fenfloramine and the behavioral effects of m-chloro-phenyl-piperazine (MCPP), a 5-HT agonist, were examined in combat veterans with PTSD. Davis et al., [98] found that the prolactin response to d-fenfloramine was blunted in PTSD subjects compared to controls, and that the symptoms of re-experiencing and aggression were inversely correlated with changes in prolactin. In terms of MCPP findings, 5 of 14 subjects with PTSD experience panic anxiety and “flashbacks” after the MCPP challenge [440], suggesting that a subgroup of patients with PTSD may have increased reactivity to serotonergic provocation.

Taken together, the above preclinical and clinical studies suggest that 5-HT may indeed have a role in conditioned fear and that traumatic stress can lead to alterations in central 5-HT functions and stress-related behavioral changes.

Dopamine

Preclinical Studies

Effective coping with environmental stimuli that might present a threat to one's survival requires that an organism is first able to identify the stimulus as being potentially harmful, and then eliminating or minimizing exposure to it. It is believed that the dopaminergic system may play an important role in the neural processes involved in the attribution of emotional salience to various types of stimuli [35, 36]. Neuronal cell bodies synthesizing DA appear to be

predominantly localized at the ventral tegmental area (VTA). DA neurons project extensively from these loci to innervate diverse target nuclei throughout the brain including frontal and limbic areas, which are involved in the genesis of cognitive and behavioral responses to stressors, as well as to areas involved in mobility (i.e., the substantia nigra) [125, 315, 465].

Several lines of evidence have demonstrated a role for dopaminergic neurotransmission in the acquisition and expression of conditional fear [157, 324]. For example, impairment of both acquisition and expression of the fear-potentiated startle response was observed following intraamygdalar injection of raclopride (a D₂ receptor antagonist) [153, 154]. Similarly, administration of a D₁ receptor antagonist (SCH 23390) prevented the acquisition of fear-potentiated startle in animals [154]. Studies conducted using conditioned fear stress have also found a role for DA in fear however this role appears to be limited to expression and extinction, it does not appear to be implicated in the acquisition phase of fear memory [121, 316, 317, 373]. For example, lesions of the mPFC, resulting in DA loss, produced longer extinction periods in both contextual and cued fear conditioning, without affecting the acquisition of fear conditioning [121, 316, 317, 373].

Further evidence for the involvement of dopaminergic neurotransmission in the expression of conditioned fear comes from measuring extracellular fluctuations of DA during the testing phases of conditioned fear. In a microdialysis study designed to examine extracellular concentrations of DA in the mPFC during conditioned fear testing, DA was increased by conditioned fear stress. This increased DA returned to pretreatment levels when rats were returned to their home cages. Moreover, diazepam suppressed both the conditioned freezing and extracellular DA levels at the mPFC [517].

In addition, pharmacological studies using dopaminergic agonists and antagonists have supported a role for DA in conditioned fear. Intra amygdaloid and/or intra-mPFC 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 [157, 347]. Moreover, several recent studies have shown that extinction learning in rats can be accelerated and strengthened with systemically applied drugs, including the dopamine D₂ receptor antagonist raclopride [360].

Finally, mice lacking the D₁ receptor showed no differences in their capacity to learn fear conditioning, but displayed delays in the extinction of conditioned fear [116], while deletion of the D₄ receptors failed to alter both acquisition and expression of learned fear [118]. Taken together these studies support the contention that DA plays a role in conditioned fear.

Clinical Studies

In human research, DA may be involved in memory encoding, subsequent failure of extinction or unsuccessful coping in patients with PTSD [444]; however, evidence to support these claims is still very limited. Patients presenting with co-morbid PTSD and depression have higher levels of homovanillic acid, a metabolite of DA in lumbar CSF than depressed patients without PTSD or healthy controls [429]. These concentrations correlate with DA concentration in the dorsolateral PFC, but not in other brain regions [111]. Further, there is some evidence that antipsychotics which partially act by blocking DA receptors in mesolimbic or mesocortical target areas appear to reduce symptoms in PTSD patients with vivid flashbacks and nightmares, or with comorbid psychosis [11]. Functional imaging studies assessing central dopaminergic function would be highly beneficial to further understand the role DA plays in PTSD symptomatology.

Glutamate

Preclinical Studies

The role of glutamate in conditioned fear has been extensively studied via one of its receptor ligands, the NMDA receptor. Research on the NMDA receptor linked LTP to Pavlovian fear-conditioning, and thus, much of what we know about learning and memory processes comes from its study. For example, NMDA receptors are necessary for the induction of LTP in both the hippocampus [39, 268] and the amygdala [190, 269].

Studies involving conditioned fear have found that blocking the NMDA receptor using antagonists such as APV (2-amino-5-phosphonopentanoic acid) infused into the amygdala, prevents the acquisition of conditioned fear. This result has been replicated in both the FPS paradigm, and in conditioned freezing to a tone or contextual CS [69, 119, 141, 215, 236, 267, 519]. Unlike in the fear-potentiated startle paradigm however, conditioned freezing *expression* was also ablated when APV was administered to the amygdala [236, 267], a phenomenon which may be due to the influence of NMDA receptor antagonists on evoked potentials in the amygdala [242, 269].

Stressor exposure has been shown to increase the outflow of glutamate in the PFC and hippocampus of rats [341], while adrenalectomy attenuated this effect. Further, Moghaddam et al., [309] demonstrated that the stressor-induced increase in glutamate release could be abolished by glucocorticoid replacement, suggesting that the glutamate increase is mediated by glucocorticoids [309]. Finally, blockade of NMDA receptors can acutely increase glutamate release by reducing excitation of inhibitory GABA neurons [308].

Clinical Studies

In human literature, drugs that increase or decrease neuronal excitation have also been studied in PTSD populations. For example, drugs that inhibit glutamate and enhance GABA neurotransmission, decrease PTSD symptoms [32, 33]. These drugs include topiramate [32, 33], lamotrigine [176], carbamazepine [499], valproate [84, 122] and gabapentin [161], each of which either decrease glutamate neurotransmission by inhibiting Na^+ -currents and/or kainate and AMPA (a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) glutamate receptors, and thus neuronal excitation, and/or enhance GABA-ergic inhibitory neurotransmission. It is important to note though, that many studies utilizing receptor ligands are open-label trials and are lacking proper control groups, and further, that many of these drugs have multifactorial effects. Thus, additional double blind, placebo-controlled studies with glutamatergic/GABA-ergic ligands are warranted.

GABA

Preclinical Studies

Recent studies suggest that the failure to extinguish fear may result from a dysfunctional interaction between the PFC and the amygdala. It is believed that a failure of the mPFC to inhibit emotions through its connections with the amygdala [117] may indeed be a factor in the etiology of PTSD.

Glutamatergic efferents arise in the mPFC and synapse on GABAergic neurons in the amygdala, providing an inhibitory mechanism within the amygdala. Of particular interest is the pathway from the infralimbic region of the mPFC to the intercalated cell mass of the capsular

subdivision of the CeA, comprised mainly of GABAergic interneurons. This cell mass exhibits powerful inhibitory control over the CeA [337, 403]. It is believed it is through this pathway that the IL inhibits the expression of conditioned fear [338].

GABA also exerts an influence on conditioned fear via neurotransmission within the BLA [456, 490, 491]. The BLA contains numerous benzodiazepine/GABA_A receptors [328], and infusion of benzodiazepines or GABA_A agonists into the BLA reduces fear conditioning and anxiety [201, 323, 343, 423], while blockade of these receptors attenuates the anxiolytic influence of systemic benzodiazepines [414]. Inactivation of BLA neurons with muscimol, a GABA_A receptor agonist, prevents both the acquisition and expression of fear conditioning [173, 323, 495]. In addition, muscimol only blocks conditioning when it is infused prior to training, immediate post-training infusions of muscimol do not affect the acquisition of fear conditioning [495].

GABA is also involved in extinction. For example, attenuating the effects of GABA at its receptor using the benzodiazepine inverse agonist FG7142, retards extinction of conditioned fear [164, 450]. Similarly, benzodiazepine agonists administered following training significantly facilitated extinction during a food-reinforced lever-press procedure [278]. This same effect observed with the benzodiazepine agonist chlordiazepoxide when administered prior to extinction training in a food-reinforced paradigm [497], was seen with the GABA_A agonist muscimol when infused into the IL before extinction training [10]. In contrast, the GABA_A antagonist picrotoxin enhanced extinction learning when administered after extinction training in an inhibitory avoidance paradigm [285], while diazepam impaired extinction retention when administered before extinction in a shuttle avoidance task. Administration of muscimol to the IL

after extinction training had no effect on extinction, however when administered to the BLA, facilitation of extinction was observed for at least 48 h post-drug infusion [10].

Taken together, it appears that the GABAergic system may exhibit differential effects on conditioned fear depending on whether manipulations are administered pre- or post extinction training or before a retention test. The behavioral paradigm used also appears to be important to the behavioral outcomes observed. Further, it is hypothesized that GABA_A neurotransmission in the IL facilitates the onset of fear extinction and its maintenance, whereas in the BLA, GABA_A neurotransmission facilitates extinction consolidation [9].

Clinical Studies

Ligand studies are also used in the study of PTSD. Drugs which increase the availability of GABA and/or activate GABA receptors tend to improve PTSD symptoms, particularly those associated with hyperarousal and avoidance [113, 461]. In line with this research, Bremner et al., [45] found reduced baseline binding at the benzodiazepine site of the GABA_A receptor within the mPFC of PTSD patients compared to healthy controls. This was supported by a more recent study which found reduced binding of flumazenil, a GABA_A-benzodiazepine antagonist, in the frontal cortex of veterans with PTSD compared to combat veterans without PTSD, suggesting that changes in GABA receptor function are associated with PTSD development, rather than trauma exposure alone [140].

All in all, receptor-binding and fear conditioning studies indicate that the GABA system is important to fear-related processes, particularly where reduction of fear is concerned; while studies in patients with PTSD suggest that the GABA system in the brain may be downregulated in PTSD, thus making it difficult for these individuals to attenuate fear.

Neuropeptides in Conditioned Fear

Corticotropin-releasing hormone

Preclinical Studies

A multitude of literature suggests a role for CRH in fear and anxiety [26, 457, 524]. Converging lines of evidence show that CRH and its family of peptides (including the urocortins) are important mediators of the body's response to stress. In instances of threat, activation of a stress response is critical for an animal's survival, just as rapid down-regulation of this system is required to return the animal to homeostasis upon threat termination. As a major modulator of the HPA axis [470], CRH regulates behavioral, neuroendocrine, autonomic and immunologic responses to stressor exposure [26, 97, 166, 342, 470]. The CRH family of peptides and its receptors (CRH₁ and CRH₂) are widely distributed throughout the central nervous system including limbic sites [26, 66, 152, 410, 454], making it an ideal candidate for mediation of stress-related behaviors.

Administration of CRH to the CeA elicits increases in locomotor activity in a familiar environment (indicative of stress) [462], and decreased exploratory behavior in the open field and open arms of an elevated plus maze [171, 244]. This effect could be reversed by administering the CRH antagonist α -helical CRH₉₋₄₁ [171]. Further, microdialysis studies have revealed that stress induces increases in the release of CRH from the CeA [91, 297, 301, 350], and this effect could be observed even when the challenge was appetitive (i.e., during consumption of a palatable food snack). Similarly, studies investigating messenger ribonucleic

acid (mRNA) showed an increase in CRH mRNA in the CeA following both restraint stress [189], and cat exposure [123], however this effect has not been found consistently [172, 175]. Finally, chronic delivery of an antisense oligodeoxynucleotide directed against the CRH₁ mRNA into the CeA reduced anxiety-like behavior immediately after exposure to social defeat [247].

Administration of CRH or urocortin 1 (a CRH agonist) into the BLA led to a dose-dependent increase in social interaction (SI) time in the SI test, while chronic injection of an otherwise inert dose of urocortin 1 over 5 days resulted in long-lasting anxiety-like responses that persisted for weeks [137, 407, 408]. CRH has also been implicated in the consolidation of emotional memory. In general, administration of CRH exacerbates fear memory, while antagonists for CRH receptors and receptor knockdown strategies attenuate these emotions, thus eliciting anxiolytic effects [457]. For example, peripheral administration of a 20mg/kg dose of the nonpeptide CRH₁ antagonist antalarmin prior to delivery of foot shocks reduced contextual freezing when rats were tested the next day [110]. Another study using primates found that antalarmin reduced fear and anxiety responses including teeth gnashing, body tremors, urination and defecation in a primate psychosocial stress model. In contrast, responses associated with reduced stress such as sexual and exploratory behavior were promoted by antalarmin [159]. Further, under high stress conditions, antalarmin was reported to blunt endocrine and autonomic responses to stress, however was ineffective at reducing either ACTH or corticosterone secretion [110]. Administration of CP-154,526, a CRH₁ antagonist chemically related to antalarmin, reduced freezing behavior in rats in a shock induced stress paradigm [494] while reducing both CRH-induced startle and fear-potentiated startle [251, 427]. Further, CP-154,526 improved escape latency scores 1 day after exposure to inescapable shocks [258]. Similarly, the CRH₁

antagonist DPC904 [180] dose-dependently reduced conditioned freezing in rats when re-exposed to the environment in which they received footshocks.

Studies have also supported a role for CRH₂ receptors in fear. For example, antisense oligonucleotide studies that target the CRH₂ receptor have found attenuated freezing in rats during the immediate post-shock period as well as in a subsequent conditioned testing situation [180]. However, antagonists of the CRH₂ receptor have demonstrated mixed results. For example, one study using mice found that anti-sauvagine-30 (anti-svg-30), a CRH₂ specific antagonist, had no effect on freezing in a conditioned fear paradigm when the antagonist was administered to the hippocampus. However, increases in CRH-induced freezing were observed when anti-svg-30 was infused into the lateral septum [177]. In contrast, reduced freezing was observed in rats injected i.c.v. with anti-svg-30, while similar anxiolytic behaviors were observed in the elevated-plus maze and defensive-withdrawal tests in this same study [458].

Clinical Studies

In studies using humans, abnormalities in HPA and/or CRH function are commonly linked with PTSD. For example, several studies have found that basal plasma or 24-hour urinary cortisol concentrations were elevated in subjects with PTSD relative to healthy or trauma-matched controls [106, 241, 245, 252]. However, an equal number of studies found no effect [354, 513] or in fact the opposite to be true [275, 511, 512]. It is likely that the discrepancies observed in these studies are a result of gender, age of illness onset (childhood versus adult-onset), trauma type and duration, or physiologic variation relative to illness phase (i.e., 1 month versus 6 months post traumatic experience).

Studies examining the central release of CRH from cerebrospinal fluid found significantly increased levels of CRH release in subjects suffering from chronic combat-related PTSD compared to matched controls [24, 48], consistent with several studies demonstrating that basal cortisol secretion and excretion are abnormally increased in PTSD [106, 241, 245, 252]. Changes in plasma cortisol and ACTH concentrations did not differ between PTSD subjects with combat experience and matched controls upon symptom provocation. While dexamethasone administration (a synthetic glucocorticoid agonist) resulted in either enhanced suppression of cortisol [446, 512, 514] or no changes [223] supporting the notion of glucocorticoid dysregulation in PTSD.

It is not clear whether elevated levels of CRH relates to PTSD status or to trauma exposure, nor to what degree it contributes to behavioral abnormalities or HPA axis dysfunction, but, as outlined above, it is hypothesized that CRH hypersecretion leads to a blunted pituitary response in PTSD patients. It is unclear, however, to what degree CSF concentrations are representative of levels of CRH in the PVN or in the bloodstream. More direct measures of CRH release and pituitary receptor binding are needed to shed light on these issues.

Bombesin

Another peptide beginning to gain recognition as having a role in stress, and further, to be involved in the fear system is bombesin. Bombesin (BB) is an amphibian-expressed tetradecapeptide originally isolated in 1971 [17]. Two mammalian counterparts to the amphibian BB have been discovered, namely gastrin releasing peptide (GRP) and neuromedin B (NMB) [76]. Neurons expressing BLP's are extensively distributed within the CNS [210, 467]. The fact that these peptides are present within various stress-relevant regions, including the PVN, medial

preoptic area, amygdaloid nuclei, arcuate nucleus, median eminence, pituitary, NTS, hippocampus, LC, and olfactory bulbs, suggests their potential involvement in the mediation of stress-related responses [58, 59, 92, 359]. Further, molecular mapping studies have shown that mRNA for GRP synthesizing cell bodies are dominant in the parvocellular layer of the PVN, suprachiasmatic and supraoptic nuclei, BNST, amygdaloid nuclei, and hippocampus while, NMB mRNA is predominantly expressed at the anterior pituitary gland with traces in other brain regions such as the dorsal raphé nucleus [188].

Receptors for this family of peptides include BB₁, BB₂, BB₃ and BB₄ receptors [149, 327, 362, 442, 485]. Neuromedin B and GRP exert their effects by activating BB₁ and BB₂ receptor subtypes respectively. The BB₃ and BB₄ receptors are designated as orphan receptors, as their natural ligands remain unidentified to date [259, 494]. Although BB₃ exists in both amphibians and mammals, the BB₄ receptor subtype has only been identified in the amphibian brain [327]. The BB₁ and BB₂ receptors have been localized within the PVN, median eminence, and pituitary (anatomical loci constituting the HPA axis), as well as extra-hypothalamic brain regions such as the LC, NTS, BNST, hippocampus and amygdala [501]. BB₁ and BB₂ receptor distribution appears to match the relative preponderance of NMB and GRP respectively, and taken together, suggests that endogenous BLPs may give rise to some of the endocrine, autonomic, and behavioral changes that occur during exposure to threat or stress [501].

It is believed that BLPs may in fact belong to a class of so called “stress peptides” [210, 211, 212, 301]. Several studies have shown that stressor exposure can affect changes in release patterns and tissue levels of BLPs as well as in the density of their receptors, particularly in stress relevant brain regions [210, 213, 296, 301, 467]. For example, exposure to acute immobilization stress is associated with increased immunoreactive BLP (ir-BLP) levels at the hypothalamus and

medulla, as well as increased density of related receptors within the PVN and NTS [210]. Further, exposure to an acute stressor (i.e., restraint or air-puff stress) resulted in increased interstitial levels of both NMB and GRP at the anterior pituitary [295] and the CeA [301] respectively.

Additional evidence to support the role of BLP's in the response to stress is shown by BB's effect on the autonomic and endocrine systems. Centrally applied BB activates the sympathetic nervous system (as reflected by elevated plasma epinephrine, norepinephrine, glucose, changes in blood pressure and heart rate), in a dose-dependent manner [55, 56, 57, 60, 61, 72, 210, 212, 281, 357, 359]. This BB-induced sympathetic activation appears particularly prominent when the NTS is stimulated [72]. The NTS sends projections to the dorsal vagal complex (where BLP terminals arising from the parvocellular PVN have been localized) [92].

Ample evidence also exists suggesting that BLPs activate the HPA axis. For example, centrally administered BLPs stimulate the release of ACTH and corticosterone from the anterior pituitary gland and adrenal cortex respectively [135, 211, 212], while systemic administration of NMB increases circulating levels of ACTH and corticosterone [255, 256]. In contrast, although one study showed that GRP significantly altered both ACTH and corticosterone levels whether it was delivered intravenously or intraperitoneally [413], two other studies failed to see a stimulatory effect of GRP on plasma levels of ACTH or corticosterone when administered intravenously [135, 331]. Further, *in vitro* analysis showed increased ACTH and corticosterone concentrations in response to GRP administration [135]. These discrepant results may be explained by methodological variations including drug dosages, method of delivery and species of animal utilized.

Interestingly, BLPs appear to mediate their effects, at least in part, through CRH neurons [211, 212]. For example, BB's ability to dose-dependently activate the HPA axis and sympathetic nervous system is attenuated in animals pretreated with a-helical CRH₉₋₄₁ [210]. Similarly, a-helical CRH₉₋₄₁ pretreatment blocks the GRP-induced increases in plasma ACTH and corticosterone [135]. Further, it was reported that intracerebroventricular GRP pretreatment potentiated CRH-induced ACTH secretion [331], an effect which was blocked by pretreatment with BB₂ receptor antagonist or with CRH antiserum [23, 135, 331]. Finally, microinfusion of BB into the 3rd ventricle evoked an increase in the availability of CRH at the median eminence and the anterior pituitary gland as detected by push-pull perfusion [211].

Taken together, these results support the notion that BLPs may be important in the genesis of the endocrine and autonomic responses to stressors. Furthermore, BLPs evoke behavioral changes reminiscent of those observed following stressor exposure. For example, BLPs are implicated in the suppression of food intake, increased grooming, increased exploration in a familiar environment, and decreased exploration in a novel environment [197, 202, 225].

In terms of its role in fear, research involving BLPs is still in its infancy. Studies utilizing tasks to understand the role of BLPs in aversive learning and emotional memory processes are just beginning to surface. In one study utilizing a one-trial passive avoidance task, systemic administration of GRP immediately following training significantly improved scopolamine-induced deficits in performance in mice, an effect seen only when relatively low doses of scopolamine were administered. This result is supported by previous research showing that high doses of GRP produced an amnesic effect in a similar foot-shock avoidance paradigm, however moderate doses attenuated the amnesia produced by pre-training injections of scopolamine [126]. In studies using BB₂ antagonists, performance in emotional memory tasks

(i.e., inhibitory avoidance) was impaired [390, 391, 393]. This effect was prominent whether the BB₂ receptor antagonist (RC-3095) was administered systemically or centrally, as well as before or after the training session. Using micropunch technology, Adamec and colleagues [2] found that BB/GRP was significantly elevated in both the cingulate cortex and CeA of the limbic system, as well as the ventromedial and paraventricular nuclei of the hypothalamus in response to cat exposure (compared to handled counterparts). Another study using single-prolonged stress (SPS), a putative rat model for PTSD, found that exposure of SPS to male rats decreases both the local content and axonal distribution of GRP in the lower lumbar spinal cord [409]. While prenatal chronic stress caused increased fearfulness in a defensive withdrawal test but had no effect on the expression of GRP receptors in the amygdala [365]. Interestingly, exposure to an enriched environment postnatally can dramatically increase the expression of BB₂ receptors in the amygdala and reduces fearfulness in the defensive withdrawal test [365]. But perhaps the most convincing evidence that GRP may be involved in fear comes from a 2002 study by Shumyatsky and colleagues [436]. This study showed that mice deficient in the BB₂ receptor exhibited higher levels of freezing behavior in a conditioned fear paradigm when presented the CS (a tone), or returned to the context where conditioning occurred. Further, they showed that BB₂ receptors were prominent on GABA interneurons located within the LA, thus establishing a possible pathway through which GRP may exert its effects on fear.

Evidence for a role of NMB in fear is sparse, however, one study has demonstrated the possible participation of the NMB/BB₁ receptor subtype system in an aversive memory-related task [506]. When subjected to a 30 minute restraint stress prior to conditioning, BB₁ receptor deficient mice presented longer step-through latencies in the passive avoidance test compared to their wild-type counterparts. This impairment in aversive memory performance/conditioning in

BB₁ receptor deficient mice suggests that this system may serve a function in aversive memory processes. Another study using a combined BB₁/BB₂ receptor antagonist (but has a stronger affinity to BB₁ receptors - PD176252), found a reduced fear-potentiated startle, both when the drug was administered systemically and when administered into the third ventricle [292]. Further studies, however, are needed in order to establish stronger evidence for the involvement of the NMB system in fear and fear memory.

To summarize, in addition to playing a role in a wide variety of physiological functions, both within the CNS and peripheral nervous system, it is clear that BLPs are involved in orchestrating the endocrine, behavioral and autonomic aspects of the stress and/or fear response. No clinical studies examining BLPs and their receptors have been conducted to date.

Overall Thesis Objectives

The importance of studying the fear system is paramount to understanding mental illness, and developing strategies for the treatment and/or prevention of stressor-related disorders. As outlined in the general introduction, BLPs appear to be involved in the neurobiology of fear and have been located in several stress-relevant regions of the brain [134, 202, 301, 313, 485, 500]. Their involvement in the mediation of the stress response points to their importance in mental illness, and given recent research suggesting that a lowered availability of GRP or its receptor may contribute to an increased fear response to a learned fear association [436], it is important to further elucidate the role of BLPs in fear.

Given the prevalence of GRP in the neurocircuitry involved in learned fear and stress-related responses, the overall objective of this thesis research is to elucidate the mechanisms underlying the formation and expression of fear-related behaviors. Five major objectives formed the framework for this dissertation, which ultimately represents an effort to more fully understand the role of BLPs in learned fear.

1. Does GRP or NMB play a role in fear and anxiety? Specifically, does central administration of GRP and NMB produce anxiolytic effects as measured in the elevated plus maze, fear-potentiated startle and conditioned fear paradigms? Further, does administration of their respective receptor antagonists produce anxiogenic effects in these same animal models?
2. What are some of the central neuroanatomical nodes in the fear circuitry where these peptides mediate their effects, specifically, which areas of the brain show pronounced

alterations in GRP activity in response to an aversive footshock, and how do these changes compare to those of another stress-relevant peptide, namely CRH?

3. Are these peptidergic effects on anxiety and fear-type responses site-specific?
Specifically this chapter will assess the effects of localized microinjections of GRP and its receptor antagonists at selected nuclei on behavior expressed in the conditioned emotional response paradigm.
4. Given that the administration of GRP and/or blockage of BB₂ receptors affect the expression of fear-type behaviors in a fear-conditioning paradigm, is endogenous GRP released at these sites during the recall of fear-conditioning? If so, or alternatively, does the local availability of GRP functionally correlate with the expression of fear?
5. Are the amygdala and mPFC functionally and anatomically interconnected via GRP signaling pathways? Specifically, are the mPFC and amygdala architectonically connected in such a way that manipulation of the upstream nucleus (mPFC) will result in modifications to the downstream nucleus (amygdala)? For example, what impact will the injection of GRP into the mPFC have on the release of CRH and/or the up- or down-regulation of GRP synthesis at the downstream target nucleus (amygdala)?

Preface to Chapter 1

As ample evidence has demonstrated the involvement of NMB and GRP in the response to stress, and further, that new evidence suggests that these peptides may be involved in the response to fear, the aim of this set of experiments was to further elucidate the role(s) of NMB, GRP and their respective receptors in anxiety and fear-related responses. Specifically, we assessed the behavioral effects of centrally administered BB₁ and BB₂ receptor agonists and antagonists on rodent paradigms thought to reflect anxiety and/or fear; namely the elevated plus maze, the fear-potentiated startle and conditioned emotional response (conditioned freezing) paradigms.

Chapter 1 – Part I

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

Abstract

Bombesin (BB)-like peptides have been implicated in the mediation and/or modulation of the stress response. However, the impact of manipulating this peptidergic system has only been assessed in a limited number of anxiety and fear paradigms. Given that different behavioral paradigms reflect different aspects of anxiety, the objective of the present investigation was to assess the effects of two mammalian BB-related peptides, namely gastrin-releasing peptide (GRP) and neuromedin B (NMB), in paradigms thought to reflect fear and anxiety-related behaviors. To this end, the effects of central (3rd ventricular; i.c.v.) administration of GRP (0.30 nmol), GRP receptor (BB₂) antagonist, [Leu¹³-(CH₂NH)Leu¹⁴]-BN (1.26 nmol), NMB-30 (0.29 nmol), NMB (BB₁) receptor antagonist, BIM 23127 (1.70 nmol) and a mixed BB₁/BB₂ receptor antagonist, PD176252 (0.621 nmol) were assessed in the elevated plus maze (EPM) and in a fear-potentiated startle paradigm (a model thought to reflect conditioned fear). The BB₁ receptor antagonist and the mixed BB₁/BB₂ receptor antagonist elicited anxiolytic effects in the EPM, whereas, the BB₂ receptor antagonist was without effect. In the fear-potentiated startle paradigm, pretreatment with either the BB₁ receptor antagonist or the BB₂ receptor agonist attenuated the fear-potentiated startle response, without affecting basal startle amplitude. These data suggest that NMB and GRP do affect the stress response. However, whereas NMB manipulations affected both anxiety and fear responses, GRP alterations selectively affected fear-related 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 BB₁, BB₂, BB₃, and BB₄ [28, 149, 327, 362, 442, 485]. Whereas NMB has a greater affinity for the BB₁ receptor subtype, GRP has a greater affinity for the BB₂ receptor subtype [28, 228]. In contrast, NMB and GRP have only a weak affinity for the BB₃ and BB₄ receptor subtypes, suggesting that there may be other yet to be identified peptides, that are endogenous ligands for these receptors [329]. Although BB₁ and BB₂ receptors are located throughout the mammalian central nervous system, their distribution patterns are distinct [227, 312, 484]. Specifically, there is a preponderance of BB₂ receptors and its endogenous ligand (GRP) in forebrain structures, various hypothalamic and amygdaloid nuclei and in the hippocampal formation [28, 312, 484, 485]. In contrast, NMB peptide and its BB₁ receptors are more pervasive in the lateral septum, olfactory regions, several thalamic nuclei, and the dorsal raphe nucleus [28, 312, 329, 484, 485]. The heterogeneous distribution pattern of GRP, NMB, BB₁ and BB₂ receptors raises the possibility that these two peptidergic systems have distinct physiological and/or behavioral functions.

As BB and related peptides were found to be potent suppressants of food intake, they gained propriety as being satiety peptides [127, 279, 299]. However, these peptides were subsequently shown to have a wide variety of effects, including the expression of the stress response [296]. Indeed, like traditional stressors, central BB administration provokes the release of adrenocorticotrophic hormone (ACTH) and corticosterone, and evokes behaviors associated

with fear and/or distress, such as increased grooming and locomotor activity in a familiar environment, and decreased food intake and reduced locomotor activity in a novel (presumably stressful) environment [144, 146, 197, 202, 225]. It is of interest to note that not only does the exogenous administration of these peptides evoke stressor-like responses, but conversely, stressor-exposure also provoke the release of endogenous BB-like peptides (BLPs) from stress-relevant brain regions (including the central nucleus of the amygdala and anterior pituitary gland) [296, 301]. .

Even though these peptides appear to be involved in the mediation or modulation of the stress response, few specifics are available regarding the differential roles of these peptides. Central administration of GRP 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 BB₂ receptor antagonist [135, 212]. Although subcutaneous administration of NMB also elicits the release of ACTH and corticosterone, it is not clear if these effects are mediated centrally, or directly at the level of the pituitary gland.

Paralleling the neuroendocrine findings, BB has been found, albeit under specific conditions, to elicit anxiety-like behaviors. In this regard, both BB₁ and BB₂ receptor deficient mice do not appear to differ from their wild-type counterparts in the light-dark box and elevated plus maze tests [505, 506, 508]. Yet, decreased emotionality in other anxiety-related paradigms was observed in mice with BB₁ receptor deletion [507]. Furthermore, we have recently shown that pretreatment with PD 176252, a predominantly BB₁ receptor agonist (with modest effects on BB₂ receptors), produced anxiolytic effects in several animal models thought to reflect anxiety and/or fear [292]. Interestingly, although evidence is scant suggesting a role for GRP and related BB₂ receptors in anxiety-like behavior, there is more compelling evidence suggesting their

involvement in fear-related responses. Specifically, it was shown that BB₂ receptor deficient mice displayed greater and more persistent long-term memory of fear [436]. In contrast, however, Roesler et al., [391] reported that microinjection of a selective BB₂ receptor antagonist directly into the basolateral amygdala, impaired memory retention on an inhibitory avoidance task, suggesting that blockade of BB₂ receptors impairs aversive memory. It is likely that some of the apparent inconsistencies may be attributable to the different types of test paradigms utilized. Indeed, differential activation of the multiple stress pathways mediating effects of distinct stressor types (e.g., neurogenic vs. psychogenic; innate vs. learned), underlie such apparent discordant observations [169, 292, 319]. Moreover, there is evidence that fear and anxiety may involve peptidergic actions (corticotropin-releasing hormone) in different portions of the extended amygdala, namely the central nucleus of the amygdala and the bed nucleus of the stria terminalis [101, 104]. In light of these observations, 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 (elevated plus maze) and conditioned responses (fear-potentiated startle).

Materials and methods

Subjects

Male Sprague-Dawley rats (Charles River, St-Constant, Canada), weighing between 275 and 300 g were individually housed and maintained on a 12-h light/dark cycle (lights on at 07:00-h). Temperature and humidity were maintained at 23°C and 60%, respectively. Throughout the study, animals had free access to food and water. All experiments 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 22 gauge stainless steel guide canulae (Plastics One, Roanoke, VA) aimed at the 3rd ventricle. The placement coordinates [340] used were 4.4 mm posterior to bregma, 0 mm lateral, and 4.4 mm below the skull surface. The cannulae were 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 at least one week of post surgical recovery time prior to 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 BB₁ receptor antagonist, BIM 23127 (Bachem; 1.70 nmoles) and the BB₂ receptor antagonist, [Leu¹³-(CH₂NH)Leu¹⁴]-BN (Bachem; 1.26 nmoles), were dissolved in Krebs Ringer Buffered saline solution (KRB) consisting of (in nmoles): 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻, 0.05 ascorbate, pH 7.4 (Moghadden & Bunney, 1989). PD 176252 (Parke Davis, UK; 0.621 nmoles), a mixed BB₁/BB₂ receptor antagonist (with only modest effects on BB₂ receptors), was dissolved in a 30% dimethyl sulfoxide (DMSO) and dH₂O 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) and 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 drug diffusion.

Experiment 1: *Central Effects of BB₁ receptor agonist and antagonist on anxiety and/or fear.*

Elevated-plus maze (EPM)

The EPM, consisting of two open arms (planks) and two arms that are enclosed by 40cm high walls, is commonly used to assess anxiety-like behavior in laboratory rats [36]. The open arms are perpendicular to the closed arms, with four arms intersecting to form the shape of a plus sign. The EPM was elevated approximately 50 cm above the floor. Security is provided by the closed arms while the open arms offer exploratory value. A black curtain surrounded the chamber to limit the influence of spatial cues and other extraneous stimuli. A video camera mounted above the arena permitted remote monitoring and recording.

Rats (N= 9/group) were individually placed in the testing room for a 1-h acclimatization and then injected centrally (i.c.v.) with either vehicle (KRB solution), NMB-30 (0.29 nmol) or the BB₁ receptor antagonist (BIM 23127; 1.70 nmol) 15-min prior to testing. Each rat was then placed onto the open central platform of the EPM (facing a closed arm). The rats' behavior was monitored for 5-min and scored as follows: (1) frequency of entries in open arms (all four paws 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. Between tests, the EPM were cleaned with 70% ethanol.

In a second experiment, designed to examine the combined effects of BB₁ and BB₂ receptor antagonism, rats were centrally injected with either vehicle (30% DMSO solution; n = 8) or PD 176252 (0.621 nmol; n = 7) 15-min prior to testing in the EPM. Otherwise, the procedure was the same as that of the first EPM experiment.

Fear-Potentiated Startle

The startle apparatus (Coulbourn Instruments) consisted of a sound attenuated chamber containing two calibrated platforms (18 x 10 cm) designed to measure the animal's startle response [37]. Animals were placed in a Teflon cage (18.5 x 11 cm), positioned atop the platforms. The cage floor consisted of stainless steel rods (4 mm diameter spaced 1.8 cm apart) that were connected to shock generators (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 (75 kHz – Coulbourn Instruments).

The training and testing for fear-potentiated startle spanned 5 days. On Day 1, naïve rats (n=8-10/group) were placed inside the startle chamber and exposed to random bursts of white noise (95, 110, and 115 db) for acclimatization and establishment of individual baseline startle amplitudes. On Day 2, animals received their first conditioning session where a tone (conditioning stimulus; CS) is paired with a shock (unconditioned stimulus; US). Specifically a 0.6 mA, 0.5-s footshock (US) was administered during the last 500-ms of the CS (a 4-s tone; 75 kHz). There were 7 CS–US trials in total with an average of 1-min (randomized) intertrial interval (ITI). On Day 3, animals were tested for fear potentiation in the absence of any drug treatment, to distinguish those animals who demonstrated fear potentiation from non-potentiators. Twenty trials of 110 db white noise bursts (random 1-min ITI) were followed by 5 trials of tones paired with noise bursts, and finally, 5 noise alone trials. At the end of testing, animals were separated into drug treatment groups based on matched startle response (equally divided across all groups). Rats that did not exhibit fear-induced potentiation on Day 3 were excluded from 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 Day 5 following the administration (15-min prior to test) of vehicle, NMB-30 (0.29 nmol) or BIM 23127 (1.70 nmol). Cages were cleaned with 70% ethanol between testing of each animal.

Experiment 2: *Effects of central administration of BB₂ receptor agonist and antagonist on anxiety and/or fear responses.*

The design of this experiment was identical to that of Experiment 1 with the exception that rats (n=8/group) were injected centrally (i.c.v.) with either vehicle, GRP (0.30 nmoles) or a BB₂ receptor antagonist, [Leu¹³-(CH₂NH)Leu¹⁴]-BN (1.26 nmoles) 15- min prior to testing.

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. With the exception of 2 cannulae, all others were correctly positioned.

Statistics

Data obtained from the EPM test 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 BB₁ receptor agonist and antagonist on anxiety and/or fear responses.

Analyses of the EPM 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 ($F_{2,20} = 3.96, p < 0.05$), and the frequency of open arm entries ($F_{2,20} = 5.456, p < 0.05$) (see Figures 1a-c). The follow-up tests revealed that rats injected centrally with the BB₁ 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.

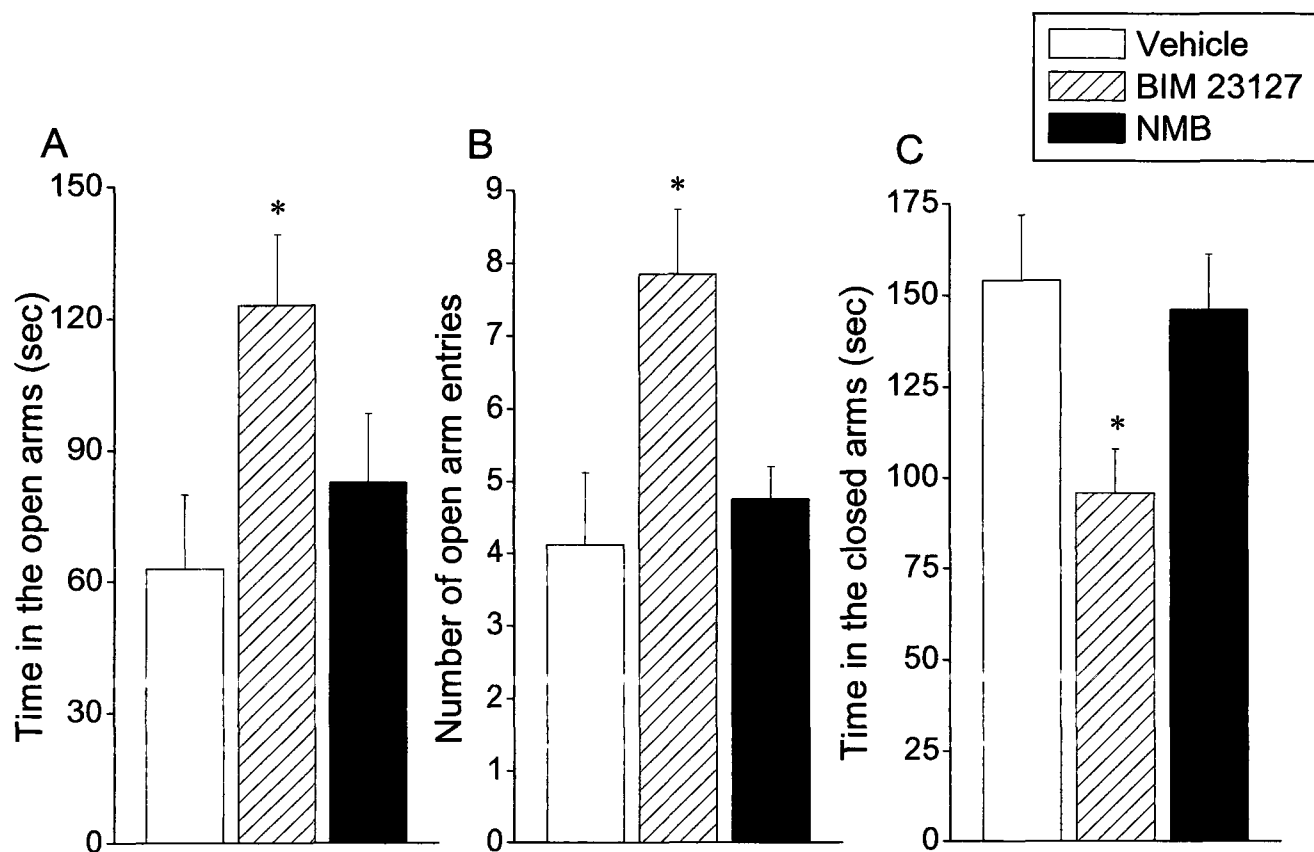


Figure 1. Mean $\pm$ SEM for each behavior on the EPM for animals treated with either vehicle (open columns), BB₁ receptor agonist (NMB-30; hatched columns) or BB₁ receptor antagonist (BIM 23127; solid columns). A) mean time (s) $\pm$ SEM) spent on the open arms of the EPM, B) mean number $\pm$ SEM of entries onto the open arms of the EPM and C) mean time (s) $\pm$ SEM spent on the closed arms of the EPM.

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

Fig. 2 depicts the results from the second EPM experiment assessing the effects of the mixed BB₁/BB₂ receptor antagonist, PD 176252. ANOVA revealed that time spent in the open arms of the EPM was increased by PD 176252 treatment ($F_{1,13} = 5.28, p < .03$). In addition, the frequency of open arm entries was increased somewhat in rats treated with PD 176252, although this effect was just shy of statistical significance ($p < .06$). None of the other EPM measures were significantly altered by the treatment.

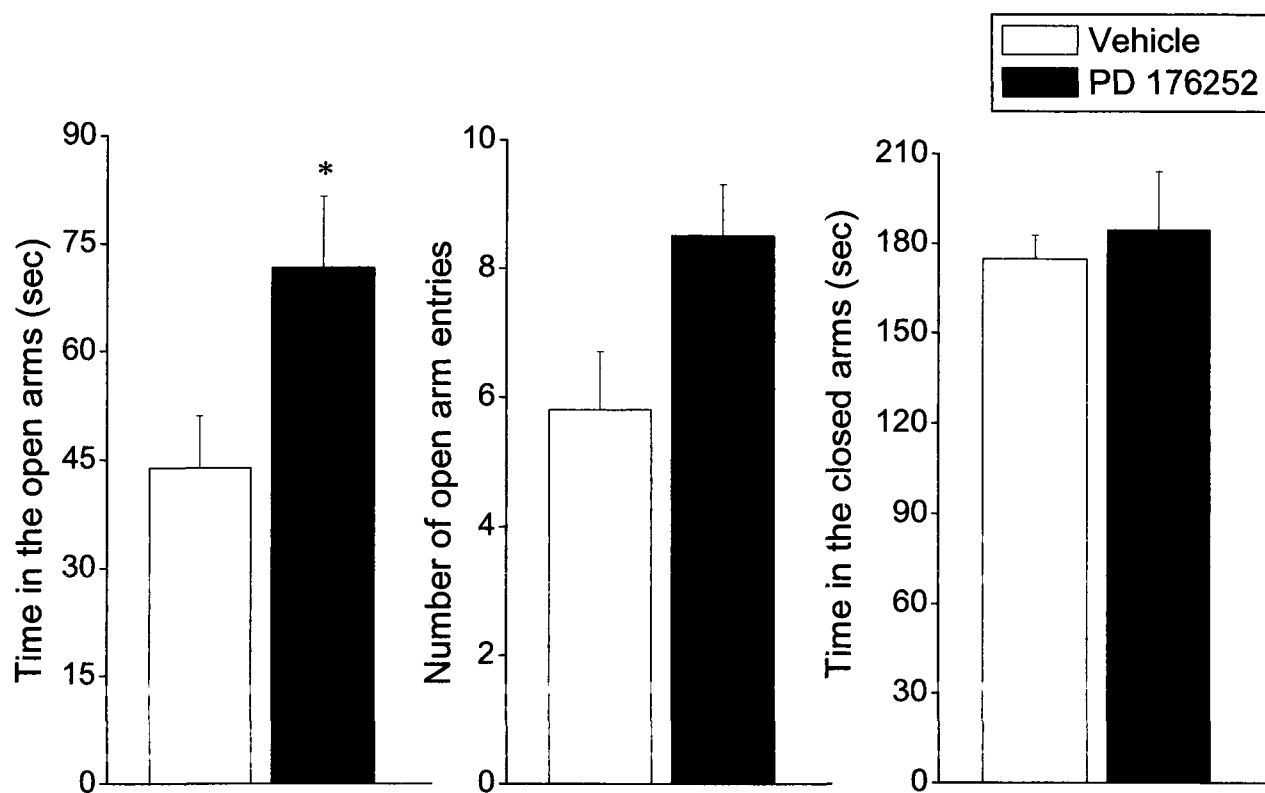


Figure 2. Mean $\pm$ SEM for each behavior on the EPM for rats treated with either vehicle (open columns) or the mixed BB₁/BB₂ receptor antagonist (PD 176252; solid columns). A) mean time (s) $\pm$ SEM spent on the open arms of the EPM, B) mean number $\pm$ SEM of entries onto the open arms of the EPM and C) mean time (s) $\pm$ SEM spent on the closed arms of the EPM.

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

Analysis of the startle amplitude scores revealed a significant Treatment x Trial interaction, ($F_{2,31} = 4.799, p < 0.01$; see Figure 3a). 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 (CS - 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.

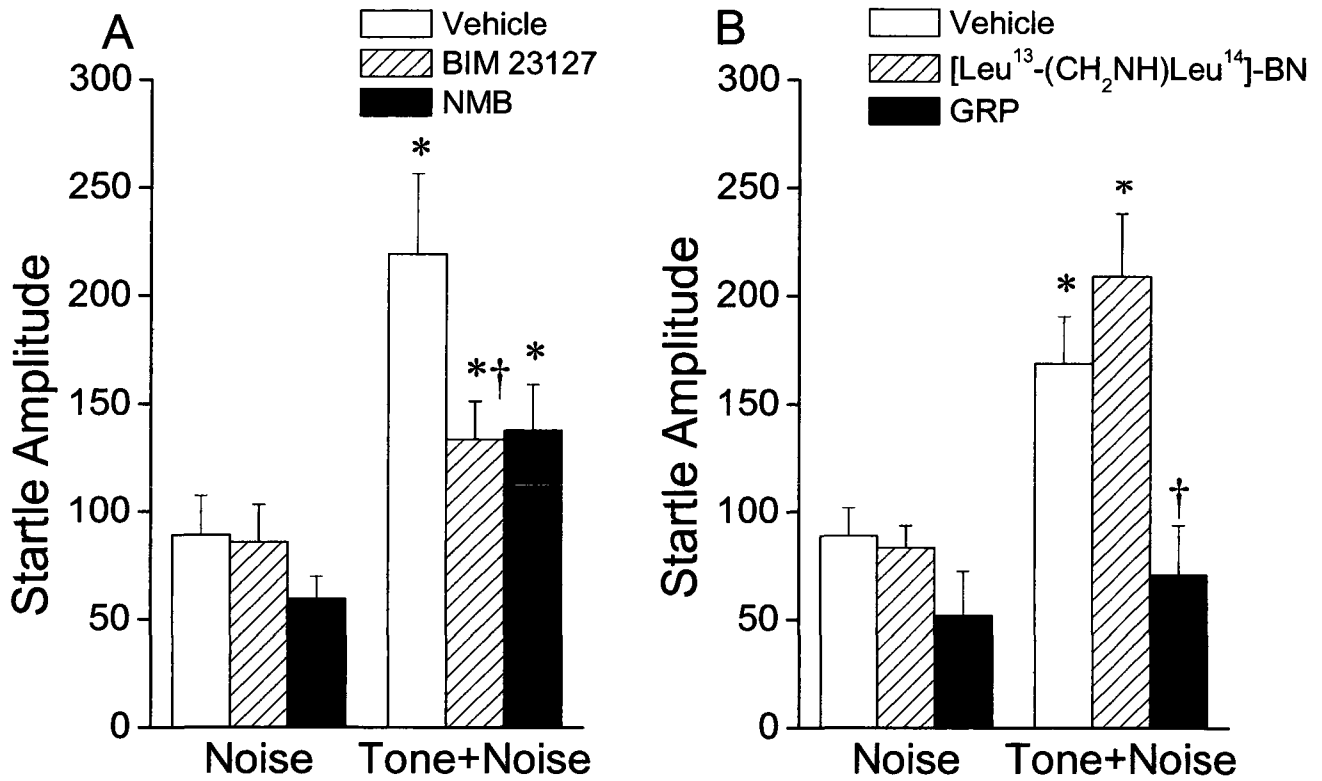


Figure 3. Mean startle amplitude (in arbitrary units) $\pm$ SEM for noise alone and tone + noise trials in rats treated with either A), vehicle (open columns), BIM 23127 (hatched columns) or NMB-30 (solid columns) or B), vehicle (open columns), [Leu¹³-(CH₂NH)Leu¹⁴]-BN (hatched columns) or GRP (solid columns).

* Significantly different from group-matched noise alone trials $p < 0.05$.

† Significantly different from trial-matched vehicle condition at $p < 0.05$.

Experiment 2: Effects of central administration of BB₂ receptor agonist and antagonists on anxiety and/or fear responses.

The BB₂ receptor manipulations failed to influence performance significantly in the elevated plus maze (see Figure 4a-c).

In the fear-potentiated startle paradigm, startle amplitude varied as a function of the Treatment x Trial interaction, ($F_{2,23} = 6.85, p < 0.004$; see Figure 3b). 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 during non-cued (noise alone) trials, the groups did not differ. Treatment with BB₂ receptor antagonist ([Leu¹³-(CH₂NH)Leu¹⁴]-BN) did not affect cued startle response, which was comparable to that of vehicle-treated animals.

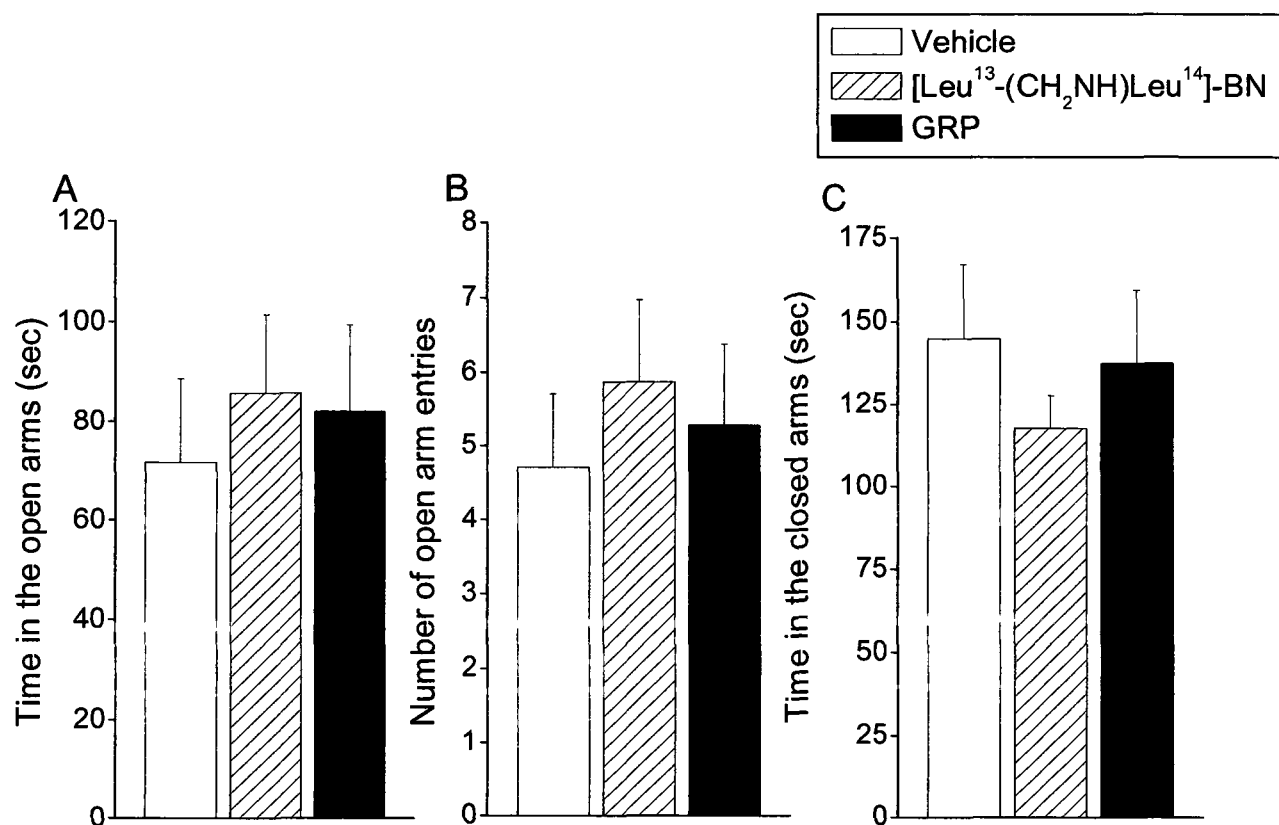


Figure 4. Mean $\pm$ SEM for each behavior on the EPM for animals treated with either vehicle (open columns), BB₂ receptor agonist (GRP; hatched columns) or BB₂ receptor antagonist ([Leu¹³-(CH₂NH)Leu¹⁴]-BN; solid columns). A) mean time (s) $\pm$ SEM animals spent on the open arms of the EPM, B) mean number $\pm$ SEM of entries onto the open arms of the EPM and C) mean time (s) $\pm$ SEM spent on the closed arms of the EPM.

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

Discussion

Despite the accumulating pharmacological and neurochemical evidence supporting involvement of BB-related peptides 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 BB₁ and BB₂ receptor agonists and antagonists in validated models of anxiety that involve unlearned (EPM exploration) and learned (fear-potentiated startle) responses. Consistent with the involvement of BB-like peptides in the stress response [134, 135, 257, 296, 331], both mammalian BB analogues, NMB and GRP, affected the expression of behavior in stressor-related situations. However, whereas NMB appeared to affect the expression of both anxiety and conditioned fear responses, GRP seemed to influence conditioned fear responses selectively. These findings raise the possibility that the two peptidergic systems may subserve distinct roles in stress-related responses, and call for more in-depth explorations using a wider array of test paradigms and drug doses. Indeed, we are currently investigating the dose-related effects of GRP and NMB as well as their respective receptor antagonists in other models of anxiety and/or fear including social interaction, novelty induced suppression of food intake, predator odor exposure and conditioned emotional response [291, 292].

In the EPM, treatment with the NMB or BB₁ receptor antagonist (BIM 23127) markedly reduced anxiogenic behavior, reflected by increased open arm entries, time spent in the open arms and decreased time spent in the closed arms. Consistent with the anxiolytic action of BIM 23127 in the EPM, this BB₁ receptor antagonist attenuated the fear-potentiated startle response. In addition, central administration of PD 176252, which is primarily a non-peptide BB₁ receptor

antagonist (with modest effects on BB₂ receptors), increased time spent on the open arms. Taken together, these findings implicate BB₁ receptor-related processes in anxiety and fear.

Consistent with the present report, we recently showed that administration of PD 176252 produced anti-anxiety effects in several other behavioral models reflecting anxiety and/or fear. Specifically, the antagonist increased social interaction, attenuated the number of vocalizations emitted by guinea pig pups separated from their mother, reduced the latency to approach a palatable snack in an anxiogenic (unfamiliar) environment, and reduced the fear-potentiated startle response [292]. Most other attempts to assess the role of the BB₁ receptor in anxiety-related behavior have been based primarily on behavioral analyses in mutant mice deficient of BB₁ receptors. Some of these studies failed to detect differences between BB₁ receptor deficient mice and their wild-type counterparts on either the EPM or light/dark box tests [505, 506, 508]. In contrast, using a marble burying test, Yamada et al., [507] found that mice deficient of BB₁ receptors displayed diminished anxiety. Given the procedural differences, as well as the differences in the species tested, the data from studies using knockout mice are difficult to reconcile with those of the present studies, which involved pharmacological blockade of receptors. However, it ought to be considered that life-long receptor deficiency in knockout models could potentially instigate compensatory mechanisms distinct from those observed in pharmacological models of receptor blockade [334].

It was intriguing to note that like the BB₁ antagonist, administration of the agonist, namely NMB-30, also reduced the fear-potentiated startle response. Although these findings were unanticipated, there are several factors that could account for the apparent inconsistencies. First, although the test-dose of NMB was chosen based on its proven efficacy in other studies [299, 357], the dose response function pertaining to anxiety and fear may be different.

Furthermore, some drugs display an inverted-U dose–response curve where specific drug doses are optimal while doses above or below this window are non-effective or less effective [284, 335, 391]. Ultimately, parametric studies will be necessary to assess these possibilities. Another consideration worthy of mention is that intracerebroventricular injection can lead to the activation of diverse brain sites through volume diffusion. It is possible that NMB has contrasting effects at distinct brain sites depending on the neurochemical circuit(s) affected by this peptide. In this regard, we have recently shown that microinjection of NMB into the dorsal raphe nucleus is anxiogenic (as assessed in the social interaction test), whereas injection of PD 176252 into this same region is anxiolytic [292]. Specific microinjections of NMB into targeted brain regions known to be involved in anxiety and/or fear related behavior may help clarify this matter.

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 not fully understood. One possibility involves interactions with the serotonergic system(s) as NMB has a stimulatory effect on 5-HT neurons at the dorsal raphe nucleus [351]. Indeed, anxiety has been linked to the over activity of central 5-HT system(s) [85, 86], and increased release of 5-HT has been reported in association with anxiety and/or fear [124, 483, 504, 516]. Conversely, drugs with anxiolytic properties tend to reduce endogenous levels of 5-HT [83, 382, 383]. Indeed, we demonstrated that 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. In parallel, the suppressed social interaction elicited by intra-DRN administration of NMB was attenuated by a systemically administered 5-HT_{2C} (but not 5-HT_{1A}) receptor antagonist [292]. Together, these findings suggest that NMB and its related

BB₁ receptors mediate their anxiety and/or fear-related effects via interactions with the serotonergic system.

There is evidence that in addition to its effects on BB₁ receptors, BIM 23127 may have antagonistic actions on urotensin receptors [174]. As i.c.v. administration of urotensin promotes anxiogenic behavior in rats [277], it is difficult to ascertain whether the anxiolytic and fear-reducing effects observed in the present investigation with BIM 23127 are purely a BB₁ effect or may in part have been attributable to its action on urotensin receptors. Importantly, however, similar results were observed in the EPM using a distinct antagonist, namely PD 176252, with no known affinity for urotensin receptors. Although PD 176252 did not reduce time spent in the closed arms (as observed with BIM 23127), the percentage of time spent on the open arms and the number of open arm entries are considered to be the best measures of anxiety [292]. Given that the selective BB₂ receptor antagonist was ineffective in the EPM, it is likely that the anxiolytic effect observed with PD 176252 was a result of its action on BB₁ receptors rather than urotensin receptors.

Unlike the effects of the BB₁ receptor antagonists, the BB₂ receptor agonists and antagonists, GRP or [Leu13- (CH₂NH)Leu14]-BN, were ineffective in modifying behavior reflecting anxiety in the EPM paradigm. Consistent with these findings, mutant mice studies indicated that a deficiency of BB₂ receptors was not associated with behavioral changes in either the light-dark box [505] or on the EPM [436, 505]. It is noteworthy that Martins et al., [273] reported that peripheral administration of the GRP antagonist RC-3095, increased time spent on the closed arms of the EPM. This finding, however, is equivocal, as the accompanying decreased in time spent in the open arms was not observed.

In contrast, administration of GRP reduced the fear-potentiated startle response, whereas administration of the BB₂ receptor antagonist, [Leu¹³-(CH₂NH)Leu¹⁴]-BN provoked a modest (but non-significant) enhancement of this response. Consistent with this finding, we recently reported that direct microinjection of GRP into the prelimbic and infralimbic cortices as well as into the central nucleus of the amygdala significantly reduced freezing to contextual cues in the conditioned emotional response paradigm [322]. These findings also parallel the enhanced conditioned emotional response noted in BB₂ receptor deficient mice [436] and support the view that the GRP peptidergic system may preferentially be 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 thought to measure learned (classically conditioned) fear responses [155, 400]. In contrast, the EPM measures behaviors reflecting unconditioned responses and thus innate anxiety [104, 155, 400, 515]. It seems likely that fear- and anxiety-type responses are mediated through distinct neuronal systems [101, 286, 400]. Although the neurocircuitry involved in anxiety responses associated with the EPM has not been well delineated, there are data regarding neuronal correlates of conditioned fear responses. For instance, lesions to the central nucleus of the amygdala (CeA) blocked the fear-potentiated startle response [68, 179] and this pattern of results was observed in other models of conditioned fear, including the conditioned emotional response [234, 400]. 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 [388, 436]. Shumyatsky et al., [436] reported that the gene which encodes GRP is highly expressed in the lateral amygdala, and GRP 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 BB₂ receptors showed decreased inhibition of principal neurons, increased long-term potentiation and enhanced long term memory of fear [436]. Although the present study only examined the effects of i.c.v. drug administration and hence does not speak to 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 BB₂ receptors at this site.

In summary, the present findings implicate mammalian BB-like peptides, GRP and NMB, in anxiety and/or fear responses. Whereas NMB appeared to play a 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.

Chapter 1 – Part II

Effects of intracerebral ventricular administration of gastrin-releasing peptide and its receptor analog RC-3095 on conditioned fear and fear-potentiated startle in the rat

Abstract

Several lines of evidence have implicated bombesin and its mammalian analogue, gastrin-releasing peptide (GRP), in the mediation and/or modulation of the stress response. However, the physiological role of GRP in mediating conditioned fear responses remains to be elucidated. The objective of the present study was to characterize the role(s) of GRP and its receptor antagonist (D-Tpi6, Leu13 psi[CH₂NH]-Leu14) BB⁽⁶⁻¹⁴⁾ (RC-3095) in fear-related responses using two animal models of conditioned fear. To this end, the effects of intracerebroventricular (i.c.v.) administration of GRP (0.062, 0.30, 3.0 nmoles) and RC-3095 (0.3, 3.0 and 9.0 nmoles) were assessed in the conditioned emotional response (CER) and the fear-potentiated startle (FPS) paradigms. In the CER paradigm, i.c.v. administration of GRP dose dependently (all doses) attenuated the expression of both contextual and cued fear as reflected by a reduction in freezing behavior to both the context (cage where shock was received) and cue (tone paired with shock). Conversely, pretreatment with RC-3095 (high dose), blocked the reduction of contextual and cued fear normally observed over time. Further, in the FPS paradigm, i.c.v. administration of GRP significantly attenuated the fear-potentiated startle response at medium and high doses without affecting basal startle amplitude. In contrast, pretreatment with RC 3095 at the highest dose (9.0 nmoles) significantly increased the basal startle amplitude without affecting fear-potentiation, suggesting elevated fear at the onset of testing. These data provide further evidence that GRP is involved in conditioned fear responses.

Introduction

Bombesin (BB)-like peptides (BLPs) initially received appreciable attention owing to their potential role in the regulation of food intake and satiety [127, 279, 299]. It now appears that BLPs also serve in the mediation and/or modulation of the stress response [296]. In this regard, central BB administration can elevate plasma levels of adrenocorticotrophin-releasing hormone (ACTH) and corticosterone (CORT) and induce behaviors that are commonly associated with fear and/or stress. These behaviors include increased grooming and locomotor activity in a familiar environment as well as decreased food intake and locomotor activity in a novel environment [144, 146, 197, 202, 225]. Moreover, we have shown that stressor exposure evokes the release of endogenous BLPs at several stress relevant brain regions, including the central nucleus of the amygdala and anterior pituitary gland [2, 210, 297, 301].

Although gastrin-releasing peptide (GRP), a mammalian counterpart of BB, appears to be involved in stress-related responses, the specific physiological role of GRP in mediating anxiety and fear responses remains unclear. Central GRP administration activates the HPA axis as reflected by an increased release of ACTH and CORT [135], and this effect can be completely blocked by pretreatment with a competitive and specific GRP (BB₂) receptor antagonist [212]. Shumyatsky et al., [436] provided evidence demonstrating that the GRP gene is highly expressed in the lateral amygdala, (a region intimately involved in conditioned fear) and that the BB₂ receptors are particularly expressed on gamma-aminobutyric acid (GABA) interneurons [436]. Furthermore, they noted that BB₂ receptor-deficient mice displayed greater and more persistent long-term memory of fear, suggesting that GRP plays a role in the regulation of amygdala-dependent fear-related learning. In line with these findings, we recently demonstrated that intracerebral ventricular (i.c.v.) administration of GRP attenuated fear-potentiated startle, while

its administration to either the prelimbic (PrL) or infralimbic (IL) cortex, as well as the central (CeA) or basolateral (BLA) nucleus of the amygdala, attenuated freezing in a conditioned fear paradigm [30, 321, 322].

In contrast to these findings, Roesler et al., [391] reported that microinjection of a selective BB₂ receptor antagonist directly into the BLA impaired memory retention on an inhibitory avoidance task, suggesting that blockade of BB₂ receptors impairs aversive memory, while we reported that blockade of BB₂ receptors in the IL, CeA or BLA results in sometimes contradictory, dose-dependent results on conditioned freezing. Thus, while there are data supporting a role for BLPs in fear and stress-related responses, current data are sparse and not fully congruent.

The aim of this set of experiments was to further characterize the role of GRP and its receptor in fear-related responses using animal models of conditioned fear. To this end, the effects of i.c.v. administration of GRP, and a BB₂ receptor antagonist, RC-3095, were assessed in rodent paradigms thought to reflect anxiety and/or fear; namely the conditioned emotional response (CER) and the fear-potentiated startle (FPS) paradigms.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec) weighing between 250-275 g at time of surgeries were used. Animals were housed individually in a temperature and humidity controlled environment on a 12-hour light/dark cycle (lights on at

07:00 h) and were permitted 1 week to acclimatize to the vivarium prior to being used.

Throughout the study, all animals had free access to food (Purina Rat Chow) and tap water.

Surgery

Animals were anesthetized with the inhalant halothane at 2.5% and were stereotactically implanted with a stainless steel guide cannula (22 gauge, 5.5 mm length; Plastics One, Roanoke, VA), aimed at the third ventricle (A/P: -4.4 mm; D/V: -4.4 mm; L: 0 mm; obtained from [339]). The cannulae were anchored to the skull with 4 stainless steel screws and dental acrylic. Removable stylets (Plastics One, Roanoke, VA) were inserted into the guide cannulae until the experimental day. Animals were allowed one week recovery before testing. During the recovery period animals were acclimated to handling as well as mock central injection procedures.

Drugs and Injections

All drugs were delivered into the 3rd ventricle in a 3 μ L volume infused over 60-s via an injection cannula (0.5 mm longer than the guide cannula). The injector was connected to a 10 μ L Hamilton Syringe with polyethylene tubing, which delivered the drug at a flow rate of 0.5 μ L/min over a 1-min interval (pump: Harvard Apparatus, MA). Following drug infusion, the injector was left in place for an additional 60 s to ensure diffusion of the drug away from the cannula tip.

GRP (Phoenix Pharmaceuticals, Inc.) and the BB₂ receptor antagonist RC-3095 (D-Tpi6, Leu13 psi[CH₂NH]-Leu14) BB (6-14); Sigma-Aldrich) were each dissolved in Krebs ringer buffered saline solution (KRB) consisting of (in nM; 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻ ascorbate, pH 7.4). The control (vehicle) animals received an equivalent volume of KRB alone. In the GRP study, animals were randomly assigned to 1 of 4 groups (n = 8-10 rats/group): vehicle (KRB); 0.062 nmoles GRP (Low); 0.3 nmoles GRP (Med); 3.0 nmoles GRP (Hi). In the

RC-3095 study, animals were randomly assigned to 1 of 4 drug conditions (n = 8-10/group): vehicle (KRB); 0.3 nmoles RC 3095 (Low); 3.0 nmoles RC 3095 (Med); 9.0 nmoles RC-3095 (Hi).

Conditioned Emotional Response (CER)

Apparatus

The conditioning chamber (Coulbourn Instruments) measured 31 x 25 x 30 cm. The front and back walls were made of clear Plexiglas and two side walls made of stainless steel panels. The floor was composed of 16 stainless steel rods (2 mm diameter, 3 cm apart), which were connected to a Coulbourn Instruments shock generator (model H13-16) that delivered constant current. A Sonalert tone generator (75 kHz, low setting – Coulbourn Instruments) was situated in the top rear panel and provided the conditioning auditory cue.

Procedure

All subjects completed one day of training followed by a day of testing 24-h later. Training for contextual fear occurred one week after surgery, while cued fear training followed 2 weeks from surgery. During the contextual training phase, subjects were placed in the conditioning chamber where they received 6 footshocks (1.0 mA; 1-s in duration) with an average intertrial interval (ITI) of 1-min. Cued fear training included 6 pairings of a 20-s tone with a 1.0 mA (1-s) continuous footshock. The shock was delivered during the final second of the 20-s tone. Again, each trial was delivered at an average ITI of 1-min.

On the test days, rats were infused with the drug 15-min before testing. Contextual fear was assessed (over a 4-min period for the GRP study and over a 15-min period for the RC 3095 study) by placing them in the conditioning chamber where they had previously been shocked.

The difference in timing between the agonist and antagonist condition was based on previous findings that 4 min was sufficient for the agonist to demonstrate effects [322], whereas pilot work using RC-3095 injected i.c.v. indicated that the antagonist would require a longer test time to demonstrate its effects. Freezing behavior (the absence of movement excluding involuntary respiratory movements) was assessed using a time-sampling method. The absence or presence of complete immobility during every 5-s sampling epoch was recorded over the course of the test period. Evaluations of freezing were conducted by trained experimenters blind to the drug condition.

To test for CER in the cued condition, rats were placed in a novel environment similar to the training chamber; however, these chambers were modified in that the walls were covered in black laminate, while the floor was smooth and covered with bedding chips. Animals were allowed a 1-min exploration period and were subsequently presented with the conditioned cue (tone - that had previously been paired with footshock). A total of 15 tones (each 20-s in duration) were presented at 1-min intervals. Freezing was scored as described in the contextual test. Between each training and testing session, cages were cleaned with 70% ethanol.

Fear-Potentiated Startle

Apparatus

The startle apparatus (Coulbourn Instruments) consisted of a sound attenuated chamber containing 2 calibrated platforms (18 x 10 cm) designed to measure the animal's startle response [103]. Animals were placed in a Teflon cage (18.5 x 11 cm), positioned atop the platforms. The cage floor consisted of metal rods (4 mm diameter spaced 1.8 cm apart) that were connected to shock generators. A high frequency speaker mounted (24 cm) above the platforms generated

white noise, while tones were generated by a sonalert model tone generator (75 kHz – Coulbourn Instruments).

Procedure

The fear-potentiated startle training and testing spanned 4 days. On Day 1, rats received 30 random bursts of white noise (95, 110, and 115 db) over 16 min to establish baseline startle amplitudes and acclimatize animals to the startle chamber. On Day 2, animals received their first conditioning session (CS-US pairing); rats received 7 trials (randomized 1 min intertrial interval - ITI) pairing a 4 s tone (75 kHz) with a 0.6 mA, 0.5 s footshock administered during the last 500 ms of the tone. Forty-eight hours later (Day 4), animals were tested for fear potentiation. Briefly, drugs were injected 15 min prior to testing, at which point animals received twenty trials consisting of 110 db white noise bursts (random 1 min ITI), followed by 5 tone-paired noise trials, and finally, 5 noise alone trials. Between tests, cages were cleaned with 70% ethanol.

Histologies

Following completion of the experiment, rats received an overdose of pentobarbital and 1 μ L of India ink (25%) was delivered through the injection cannula. Animals were then sacrificed and their brains were removed and frozen. Locations of the cannulae were verified histologically upon thionin staining of the sections.

Statistics

For CER, data were analyzed separately for the agonist and antagonist conditions, as were the data from the contextual and cued freezing conditions. For the contextual test in the agonist condition, the raw freezing scores were transformed into a percentage of sampling bins during which freezing occurred over the four 1-min intervals. These percentages were then

analyzed using a mixed-measures analysis of variance (ANOVA) with Treatment condition as the between-group measure and Time (minutes 1 to 4) as the within-group measure. For the antagonist contextual condition and the cued tests, percentage of freezing scores were calculated for the 20 1-min bins during the test, and subsequently collapsed over three 5-min time blocks. These values were then analysed using a mixed-measures ANOVA with Treatment condition as the between-group measure and Time (three 5-min blocks) as the within-group measure. Follow-up tests were conducted using Newman-Keuls multiple comparisons ($p < .05$).

Grooming behavior was only scored in the agonist condition as RC-3095 is not known to elicit this behavior (nor was grooming observed during testing), and further, only grooming during the cued portion of the task is presented as animals did not noticeably groom during the shorter-duration contextual task.

For fear-potentiated startle, differences in both baseline amplitude values and fear potentiation were analyzed. To determine fear-potentiation, data from the CS + noise trials was expressed as percent change (mean startle amplitude on CS + noise trials – mean startle amplitude on noise-alone trials/ mean startle amplitude on noise-alone trials x 100) (see [487]). Differences in baseline startle amplitudes as well as % fear-potentiation were assessed using one-way ANOVAs followed by Newman-Keuls multiple comparisons.

Results

Effects of GRP on freezing in the CER

In the contextual CER test, freezing was lower in GRP-treated rats compared to those that received the vehicle treatment ($F_{3,102} = 18.36, p < .0001$; see Figure 1A), and a significant interaction was observed between Drug treatment and Time ($F_{9,102} = 2.47, p = .01$). Follow up analyses revealed that during the first minute, only the high dose of GRP significantly differed

from the vehicle-treated control animals, during the second minute, both high and medium doses significantly differed from controls, while by the third and fourth minutes, all doses elicited significantly lower levels of freezing compared to vehicle-treated controls.

The effects of GRP on freezing elicited by the specific auditory cue demonstrated a similar profile (Figure 1B). Follow up analysis of the Drug treatment and Time interaction ($F_{6,62} = 9.37, p < .0001$) revealed that during the first 5-min, only the high dose of GRP significantly reduced freezing in comparison to vehicle-treated rats, whereas at the second and third 5-min time blocks (10 – 15 mins of testing), both the medium and high doses reduced freezing compared to controls. A similar trend was observed with the low dose, however, this effect did not reach statistical significance.

In terms of grooming, this behavior did not account for a significant proportion of the test duration (see figure 2).

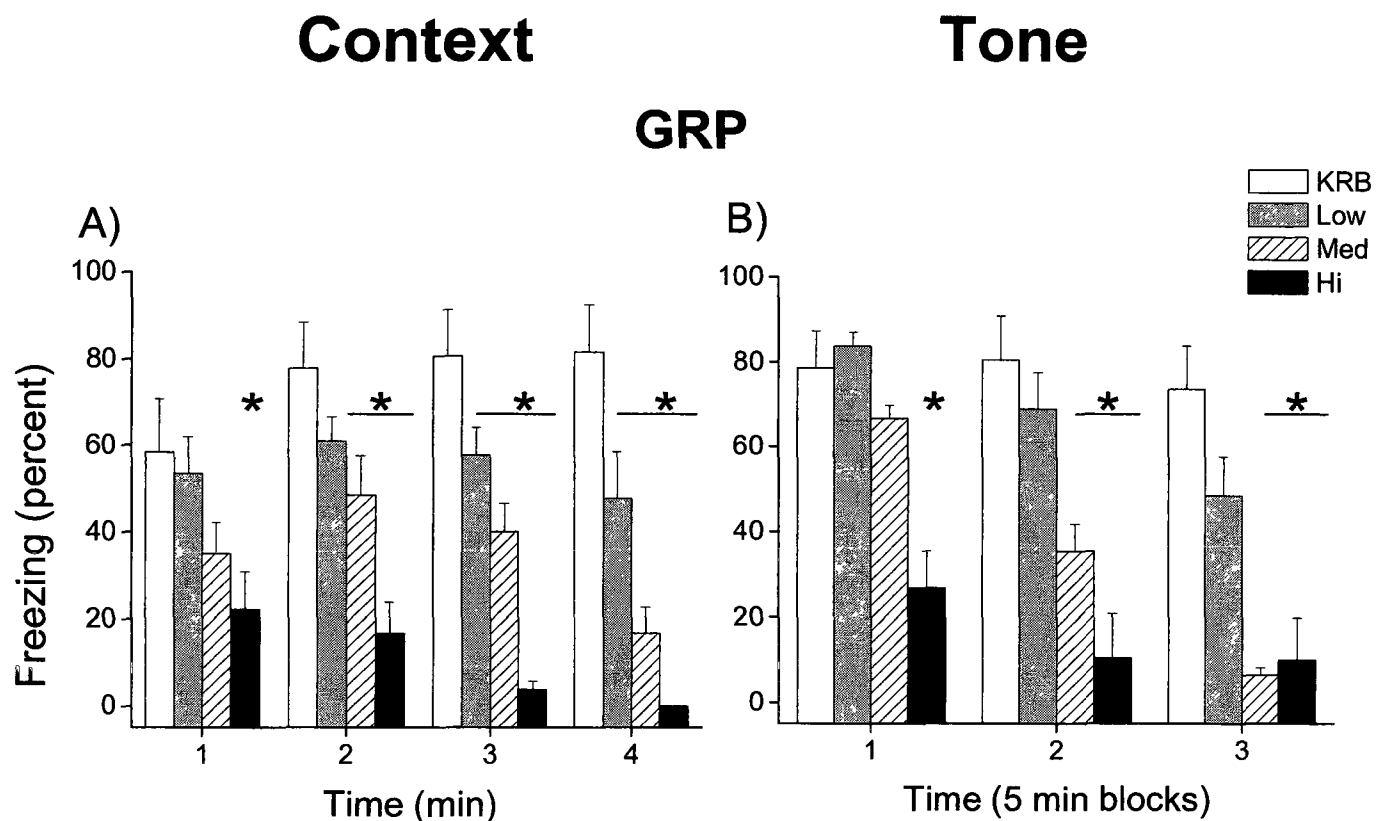


Figure 1. Percentage of time engaged in freezing ($\pm SEM$) following microinjection of GRP (in nmoles [nm]; KRB 0.0 nm; Low - 0.062 nm; Med - 0.3 nm; Hi - 3.0 nm) into the 3rd ventricle in the contextual task of the CER (panel A); and in response to the fear cue applied in a different environment (panel B). The same vehicle controls are depicted across both contextual and cued conditions.

* Significantly different from control (vehicle) group, $p < .05$.

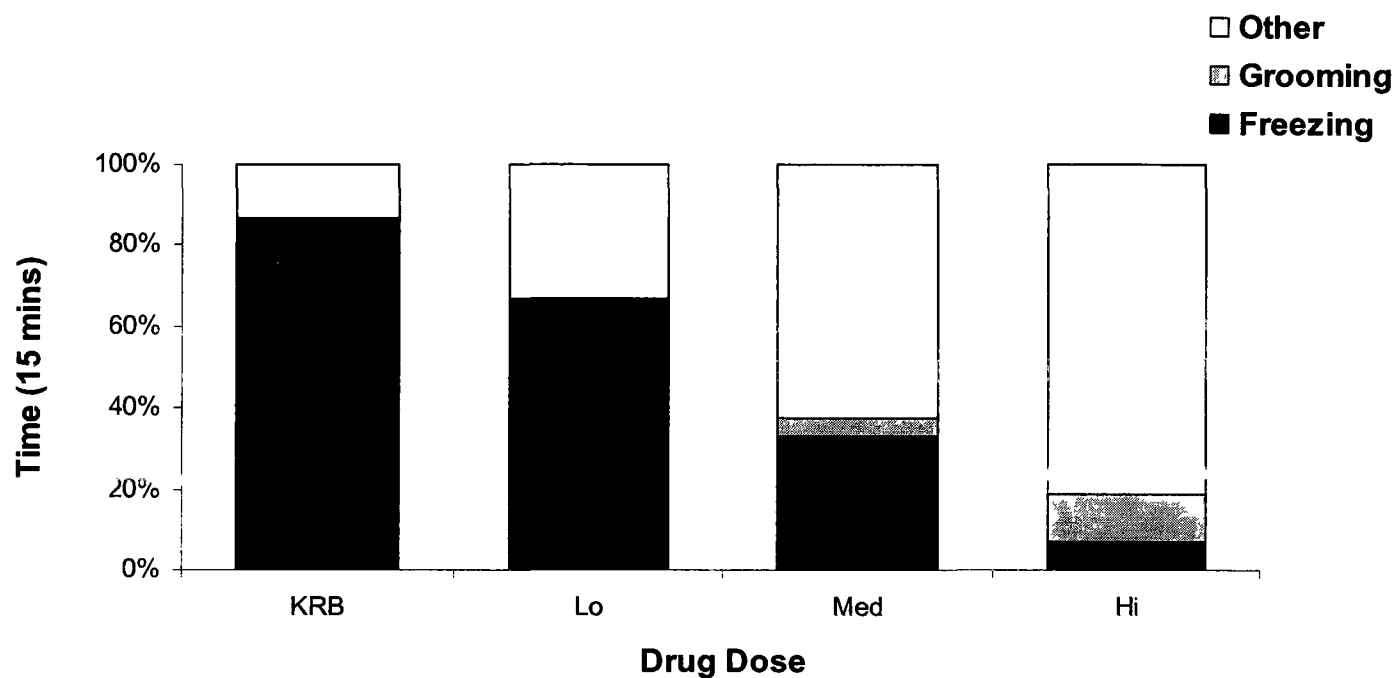


Figure 2. Percentage of time spent freezing, grooming and participating in other behaviors (such as exploring, resting and sleeping) during the 15 min cued task in animals injected with KRB (0.0 nm); Low (0.062 nm); Med (0.3 nm) and Hi (3.0 nm) doses of GRP.

Effects of the BB₂ receptor antagonist RC-3095 on freezing in the CER

Analysis of the effects of RC-3095 in the context condition revealed a significant interaction between the Drug effect and Time ($F_{6,70} = 2.41, p < .05$; see Figure 2A). The follow up analyses revealed that only the high dose of RC-3095 increased freezing relative to the control condition, and this effect was observed during the last 15-min of the test (Time block 3). A similar profile with RC-3095 was observed in the cued condition. Follow up analyses of the significant interaction effect ($F_{6,72} = 2.79, p < .05$; see Figure 2B) revealed that again, only during the last 5-min of the test, the high dose elicited increased freezing relative to control rats ($p < .05$).

Context

Tone

RC-3095

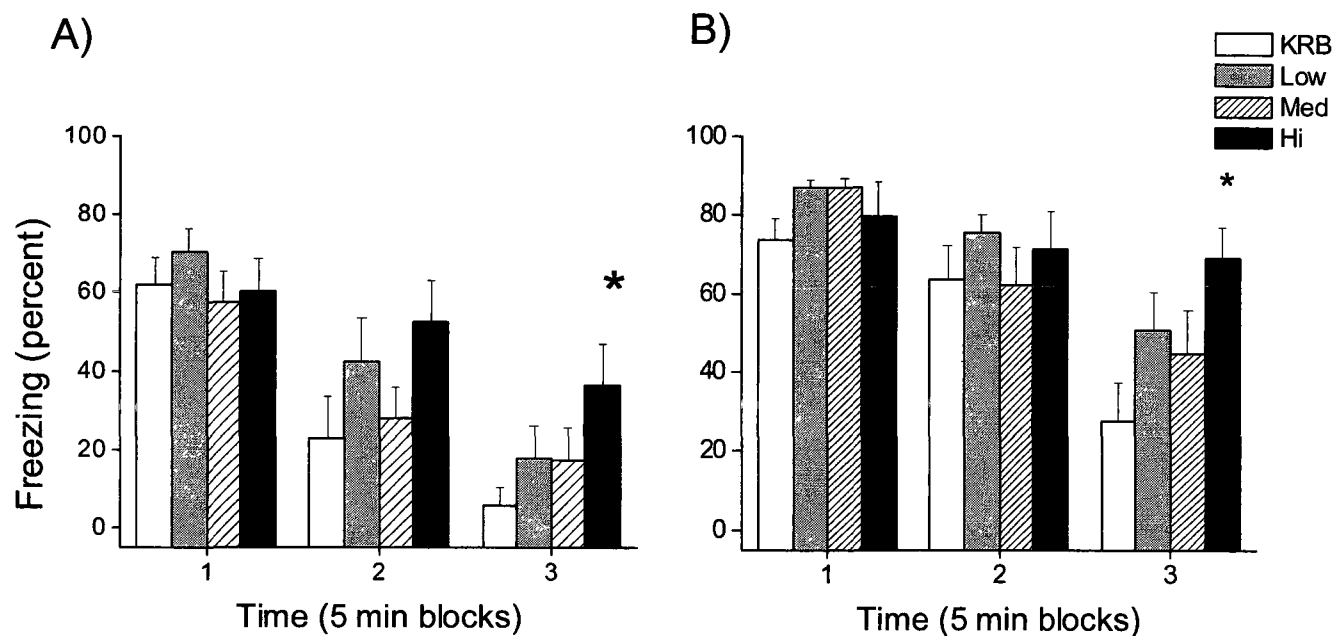


Figure 3. Percentage of time engaged in freezing ($\pm$ SEM) following microinjection of the BB₂ receptor antagonist RC-3095 (in nmoles [nm]; KRB 0.0 nm; Low - 0.3 nm; Med - 3.0 nm; Hi - 9.0 nm) into the 3rd ventricle in the contextual task of the CER (panel A); and in response to the fear cue applied in a different environment (panel B). The same vehicle controls are depicted across both contextual and cued conditions.

* Significantly different from control (vehicle) group, $p < .05$.

Effects of GRP on fear-potentiated startle

As depicted in Figure 3, GRP significantly decreased the expression of fear-potentiated startle ($F_{3, 39} = 2.92, p < .05$), without affecting the baseline startle amplitude (noise alone trials). The follow-up comparisons revealed that rats treated with the medium (0.3 nmoles) or high dose (3.0 nmoles) of GRP displayed significantly reduced startle potentiation relative to the vehicle control group.

Effects of the BB_2 receptor antagonist RC-3095 on fear-potentiated startle

Treatment with RC-3095 had no effect on the expression of fear-potentiated startle. In contrast, however, RC-3095 did have an effect on the baseline startle amplitude ($F_{3, 31} = 3.46, p < .03$). The follow up comparisons revealed that rats treated with the high dose of RC 3095 (9.0 nmoles) displayed a significantly increased startle amplitude during the noise alone trials compared to vehicle-treated rats (see Figure 3).

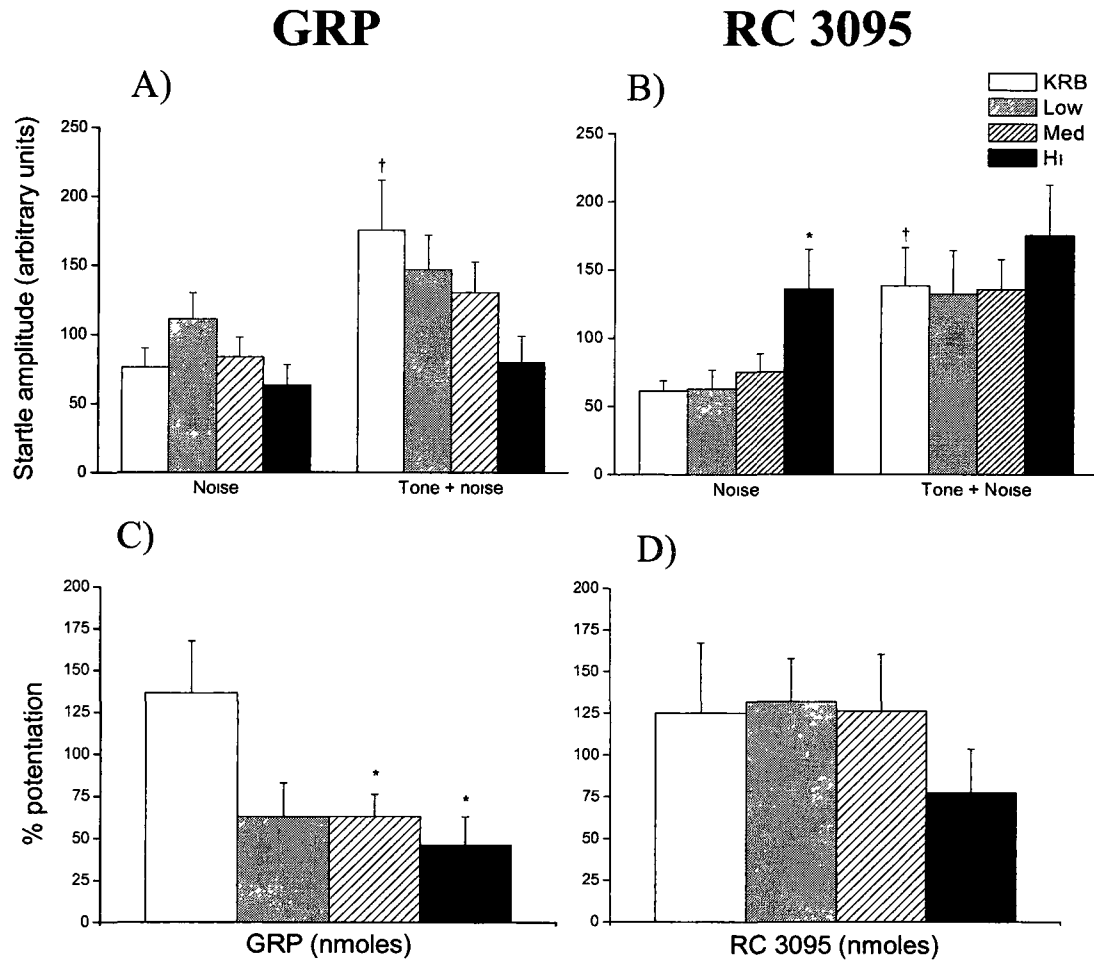


Figure 4. Top panels (A and B): mean startle amplitude (in arbitrary units) $\pm$ SEM for noise alone and tone + noise trials and Bottom panels (C and D): percent fear potentiation in the fear-potentiated startle paradigm in rats treated with either GRP (A and C) injected at 0 nmoles (vehicle: open columns), low dose (0.062 nmoles: grey columns), medium dose (0.3 nmoles: hatched columns) or high dose (3.0 nmoles: solid columns) or RC-3095 (panels B and D), injected at 0 nmole (vehicle: open columns), low (0.3 nmoles: grey columns), medium (3.0 nmoles: hatched columns) or high (9.0 nmoles: solid columns) doses.

* Significantly different from trial-matched vehicle condition $p < 0.05$.

† Significantly different from group-matched noise alone trials at $p < 0.05$.

Discussion

Previous findings concerning the effects on BLPs on stress and fear responses have yielded somewhat inconsistent results [30, 96, 321, 322, 390, 391, 393]. As different paradigms were used across studies, the present study sought to assess the dose-related effects of i.c.v. administration of GRP in several conditioned fear paradigms. As well, we assessed the influence of a BB₂ receptor antagonist, RC-3095, that has not previously been used ventricularly in such paradigms. Consistent with our previous findings [30], i.c.v. administration of GRP reduced fear-potentiated startle (FPS) at both medium (0.3 nm) and high (3.0 nm) doses. In contrast, while the BB₂ antagonist ([Leu¹³-(CH₂NH)Leu¹⁴]-BN) was without effect in our previous study, at the high dose (9 nmole), central administration of RC-3095, a newer and more specific BB₂ antagonist [109], significantly increased basal startle amplitude compared to vehicle-treated controls. This outcome was observed even though the treatment did not influence FPS, suggesting that animals in this group had higher levels of fear from the outset of the test.

In the CER paradigm, GRP significantly and dose-dependently reduced freezing relative to vehicle-treated controls in both the contextual and cue-elicited tests. In contrast, the high dose of RC-3095 blocked the reduction of freezing behavior (a measure of extinction learning) normally observed in this paradigm in both the contextual and cue-elicited tests (relative to that seen in vehicle controls). These findings are in keeping with several lines of evidence suggesting that the administration of GRP reduces fear in fear-related paradigms, or that blockade/deletion of BB₂ receptors leads to either increased fear and/or a blockade in reduction of fear to fear-eliciting cues [96, 436]. For example, Shumyatsky et al., [436] demonstrated that contextual fear was enhanced in BB₂-receptor knockout mice compared to their wild-type counterparts and

Dantas et al., [96] found that at high doses (10 µg), RC-3095 enhanced learning (enhanced fear) in an inhibitory avoidance task.

Studies of BB₂ receptor blockade/deletion have not yielded consistent results. For example, Santo-Yamada et al., [416] showed that peripheral injection of the GRP receptor antagonist [leu¹³-(ψ-CH₂NH)-leu¹⁴]BB impaired inhibitory avoidance learning in mice. Similarly, Roesler et al., [390, 391, 393] found that systemic as well as localized central injection of RC-3905 in rats impaired memory in an inhibitory avoidance paradigm, however, dose-dependent opposing effects have also been observed [96]. We have demonstrated that BB₂ antagonists had dose-dependent effects that depended on the locus of administration [321, 322]. Specifically, administration of low doses (50 ng) of BW3385U89 to the central nucleus of the amygdala resulted in increased contextual freezing, however, at high (300 ng) doses, decreased freezing was observed. Further, at the infralimbic cortex, 50 ng dose decreased freezing to both contextual and cued fear conditioning, whereas at the prelimbic cortex, BW2258U89 was without effect. Finally, using the antagonist RC-3095, decreased freezing was observed in response to contextual cues when administered to the BLA, but had no effect on cue-elicited freezing at this locus.

Together, these findings suggest that non-specific inactivation of BB₂ receptors (via ventricular drug administration) leads to either an increase in fear and/or the diminished ability for learned fear to be extinguished, whereas the effects of site-specific blockade of GRP receptors appears to be dose-dependent and varies with the site of administration. It is possible that at certain stress-relevant brain regions, BB₂ antagonists act in a predictable antagonistic manner, leading to increased fear, but at other loci, these receptors may not be involved in fear-dependent pathways at all. Yet, GRP may recruit other brain systems and act in conjunction with

other neurotransmitters and/or neuropeptides, possibly leading to the differential effects observed in the aforementioned studies.

One interesting finding in the current study was that elevated basal startle amplitudes were observed in the FPS test in rats that received the high dose (9 nmoles) of RC-3095. This suggests that animals were afraid at the outset of the experimental procedure, and therefore, fearful towards contextual cues associated with the testing environment and not necessarily to the pre-conditioned tone. This is interesting in light of the findings suggesting that GRP and its receptors, at least at the level of specific nuclei, appear to be preferentially involved with contextual-based fear. Indeed, when GRP and its receptor antagonists were administered to the IL, PrL, CeA and BLA, cue-elicited drug effects were only observed at the IL, an area critical to cue-elicited extinction learning [374].

It is also important to point out that central injection of GRP (at doses ranging from 0.064 nmol to 6.4 nmol) were shown to elicit grooming- and scratching-type responses [276]. As grooming behavior may interfere with the ability of a rat to freeze, we felt it was necessary to also measure the time spent grooming over the course of the testing period in addition to (and in the same manner as) time spent freezing. As anticipated, animals injected with either the medium (0.3 nmole) or the high (3.0 nmole) dose of GRP did indeed display an increased frequency in grooming compared to those injected with either the low dose of GRP (0.062 nmole) or vehicle. However as clearly depicted in figure 2, the amount of time spent grooming did not noticeably interfere with the amount of time spent freezing. In the instances where rats did groom (with the medium or high dose of GRP), behavior that could be considered ‘other’ made up the vast majority of the total time of the test, where ‘other’ behaviors could include such behaviors as exploring, rearing, resting and sleeping.

In summary, these data lend support to the notion that GRP is involved in conditioned fear responses, and supports the contention by Shumyatsky et al., [436] that GRP and its neural circuitry operate as a negative feedback system that regulates fear. These findings may have important implications for the development of novel therapeutic interventions for clinically significant fear reactions.

Preface to Chapter 2

The first set of experiments provided evidence that administration of BLP analogues and/or antagonists could alter behavior in tests designed to assess either anxiety or fear. We wanted to further explore the role of BLPs (more specifically GRP) in conditioned fear; thus, the aim of this chapter was to determine if exposure to a conditioned-fear paradigm elicits alterations in endogenous levels of GRP in brain regions known to be involved in stress and fear-conditioning processes. In addition to measuring immunoreactive (ir)-GRP, we further assessed changes in immunoreactive (ir)-CRH as evidence exists suggesting a relationship between the release of BB/GRP and CRH in stress-relevant brain areas. Although there are many technological shortcomings with this study, it was designed to be an exploratory pilot project in an attempt to highlight brain areas showing alterations in ir-GRP and/or ir-CRH levels in response to conditioned fear. These brain areas showing differences in peptide concentrations would then be explored further in subsequent studies in order to gain greater insight into the role of GRP (and its subsequent relationship with CRH) in fear.

Chapter 2

Through which central neuroanatomical nodes does gastrin-releasing peptide mediate its effects on fear?

Abstract

Previous research has shown that bombesin-like peptides (BLPs) are involved in the mediation of behavioral responses to stress, anxiety and fear. Their role in fear however, is only beginning to take light. One study by Shumyatsky et al., [436] demonstrated that BB₂-receptor deficient mice showed altered fear behavior to a conditioned freezing paradigm, whilst the previous chapter demonstrated altered fear behavior when BLP analogues and antagonists were administered to the third ventricle. Thus, the current pilot project sought to determine where in the brain these effects might be mediated. We also sought to measure CRH as previous evidence suggests a relationship between BLP and CRH release patterns in stress-relevant brain areas. The results revealed that three regions had significant differences in peptide release; the prelimbic cortex, ventromedial hypothalamus and amygdala. Immunoreactive (ir)-GRP content was significantly lowered at the prelimbic cortex compared to the control condition (tone not associated with shock), while levels of ir-CRH were significantly higher in the right side of animals exposed to the tone/shock (CS). In the ventromedial hypothalamus, the CS lowered ir-GRP content relative to the control condition. However, in the amygdala, left and right differences were seen in both the central and the basolateral nuclei. In both these nuclei, the left side contained significantly higher ir-GRP and CRH concentration, than the right side.

Introduction

Based on our earlier findings implicating bombesin-like peptides (BLPs) in the modulation or mediation of fear-related behaviors and anxiety, the current study sought to determine where in the brain these effects might be mediated. Although BB₁ and BB₂ receptors appear to have distinct distribution patterns [28, 228, 312, 484], both receptor subtypes are present in various stress-relevant brain loci including those affecting HPA axis functioning (hypothalamic structures such as the parvocellular layers of the paraventricular hypothalamic nucleus; the suprachiasmatic; supraoptic; preoptic and mammillary nuclei) [296]. Further, gastrin-releasing peptide (GRP) and its (BB₂) receptors are present in several areas implicated in the stress response; including several limbic structures (e.g. the lateral and central amygdala, hippocampus, and bed nucleus of the stria terminalis) and various hypothalamic nuclei [28, 312, 313, 336, 484, 485, 500]. These peptidergic systems are also functional at several caudal brainstem nuclei including the nucleus of the solitary tract and the parabrachial nucleus [313, 336, 500], which have been implicated in the expression of fear-related behaviors.

As we have previously revealed robust effects of GRP receptor manipulation on fear behavior [30], the aim of this current pilot study was to determine if exposure to a conditioned-fear paradigm elicits alterations in endogenous levels of GRP in brain regions known to be involved in stress and fear-conditioning processes. In addition to GRP, we also measured corticotropin-releasing hormone (CRH), as evidence suggests a relationship between BLP and CRH release patterns in stress-relevant brain areas [301]. Similar to GRP, CRH has also been found in stress-relevant limbic areas [26, 66, 152, 410, 454], and has been shown to modulate fear behavior [110, 159, 180, 251, 258, 427, 457, 494]. Further, BLPs appear to, at least in part, exert their effects through CRH receptors [211, 212]. For example, pretreatment with a-helical

CRH₉₋₄₁ attenuates BB's ability to activate the HPA axis and sympathetic nervous system [135, 210], while i.c.v. pretreatment with GRP potentiates CRH-induced ACTH secretion [331], an effect which is blocked by pretreatment with BB₂ receptor antagonist or with CRH antiserum [23, 135, 331].

Thus, through the measurement of regional levels of immunoreactive (ir)-GRP and CRH in response to recall of conditioned fear, it is our intention to probe the areas that show alterations in peptide content further in future studies, in order to mete out the more specific roles GRP and CRH play in anxiety and fear.

Materials and Methods

Subjects

Male Sprague-Dawley rats weighing 275-300g were individually housed and maintained on a 12 h light/dark cycle (lights on at 07:00 h). Rats were given *ad libitum* access to food (Purina Rat Chow) and tap water.

Apparatus

The conditioning chambers (Coulbourn Instruments) measured 31 x 25 x 30 cm. The front and back walls were made of clear Plexiglas and the two side walls consisted of removable and adjustable metal plates. The grid floor contained 16 metal rods (2 mm diameter, 3 cm apart), which were connected to a Coulbourn Instruments shock generator (model H13-16). The tone was generated by a Coulbourn Instruments Sonalert tone generator (75 kHz) located in the top back panel of the operant chamber.

Procedure

Animals were randomly assigned to one of two conditions (shock or no shock). All subjects completed one day of training followed by a day of testing. During the training phase the subjects were placed in the conditioning chamber and were exposed to 6 pairings of a 20 s tone with a 1.0 mA continuous footshock delivered during the last second of the tone (shock condition only, control condition received the tone alone). Each trial was separated by a random 1-min inter-trial interval.

Testing was carried out the following day and involved first assessing contextual freezing behavior (defined as the complete absence of movement with the exception of respiration for 5 s) in the conditioning chamber in the absence of tone or shock for 3 min. This was followed immediately by a cued freezing session, carried out in distinct clean cages (identical to their home cages). Rats were presented with 5 tone (20 s) presentations in the absence of shock. Researchers blind to the experimental condition scored freezing behavior. Twenty minutes after testing was completed, animals were euthanized by decapitation and trunk blood was collected in EDTA, spun immediately, and plasma samples were aliquoted and stored at -80°C. Brains were removed and frozen with isopropanol on dry ice and stored at -80°C until sectioning.

Tissue Preparation for RIA

Frozen brains were sectioned at 300 μ m using a cryostat and specific coronal planes selected for specific brain regions/sites, according to the Paxinos and Watson atlas [339]. Sections were dissected on an ice-cold platform using blunt dissection. Nine brain regions were isolated including; infralimbic, prelimbic and cingulate cortices, CA1 region of the hippocampus, paraventricular nucleus, ventromedial hypothalamus, basolateral amygdala, central amygdala and periaqueductal grey. Each dissected sample was placed in tubes containing 250 μ L of 0.2 M

acetic acid heated to 80°C for a period of 1 h. The tissue was then homogenized and sonicated (Kontes, Micro ultrasonic cell disrupter, setting 5; Kontes, Vineland, N.J.). Three aliquots were removed, 40 uL was frozen at -20°C and subsequently analyzed for protein content using a BCA Protein Analysis kit (Chromatographic Specialities, Canada) and a PC 800 colorimeter (Brinkmann, Canada). Two 95 uL aliquots were frozen at -80°C, lyophilized and stored at -20°C until the RIA was performed.

Radioimmunoassay (RIA)

Tyr⁴BB was iodinated using a modified version of the technique described by Salacinski et al., [411] and purified using a Sephadex column (Sephadex G-10, 0.6 x 20 cm). The peak fractions were pooled and heated to 80°C for 1 h in the presence of 1 M 1,4-dithiothreitol (DTT) to reverse the oxidation of amino acids. The fraction was then aliquoted and stored at -20°C. The specific activity of the peptide was calculated to be approximately 1200Ci/mmol, using the method of Chiang [80].

On the day of the RIA, the lyophilized aliquots were reconstituted in RIA buffer (0.05 nM phosphate buffered saline, pH 7.4 with 0.25% bovine serum albumin). The detection and quantification of CRH was achieved through a solid-phase high-sensitivity adaptation or modification [253] of the double-antibody liquid phase RIA originally described by Vale and colleagues [471]. GRP was detected using a similar solid-phase RIA [358]. Briefly, protein A/G (Calbiochem, La Jolla, CA)-coated Immulon-4 wells (Dynatech, Chantilly, VA) were incubated with anti-CRH serum (rC70, kindly provided by W. Vale, The Salk Institute, La Jolla, CA) or anti-BB serum (a-BB₂, kindly provided by Dr. T. Moody, NCI, Rockville, MD) for 2 h at 20°C. The specific anti-CRH serum recognizes CRH₁₋₄₁ and cross-reacts poorly with other related peptides including urotensin 1 and urocortin [331]. The GRP antibody recognizes the C-terminal

of bombesin and cross reacts strongly with GRP₁₋₂₇ (110%), bombesin (100%) and GRP₁₈₋₂₇ (82%), but only weakly with substance P or other mammalian BLPs (neuromedin B-10 and neuromedin B-32; <0.1% - [313]). The final dilution volume was 1:100 000. Samples, standards (reconstituted in KRB solution ranging from 0.05 to 250 fmol/well), or blanks were incubated for 24 hr at 4°C. Next, 25ul of assay buffer containing 5000-6000 cpm of [¹²⁵I-Tyr⁰]rCRH (Amersham, Oakville, Ontario, Canada) or [¹²⁵I-Try⁴]BB (iodinated in-house, as per Salacinski et al., [411]) was added to each well and incubated for an additional 24 h period at 4°C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted in a gamma counter (Cobra II Auto-gamma). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 and 2 fmol/well for CRH and BB, respectively.

Plasma ACTH and CORT levels were determined using commercial RIA kits (MP Biomedical, Canada).

Results

The regional peptide concentration of ir-GRP revealed distinct alterations. In the prelimbic cortex, as the ANOVA indicated no hemispheric differences, data from both right and left sides were pooled to determine if differences existed between Shock and no-Shock conditions. One-way ANOVA indicated that rats in the Shock condition had significantly lower levels of ir-GRP ($F_{1,35} = 4.042, p < .05$) than their no-Shock counterparts (Figure 1a). This same trend was observed in the ventromedial hypothalamus whereby the Shock condition again showed significantly lower ir-GRP than controls ($F_{1,35} = 5.722, p < .03$; Figure 1b). In contrast, at the amygdala, there were no differences between the Shock and no-Shock conditions. However, it is of interest to note that there were left and right differences in both the basolateral

($F_{1,22} = 7.542, p < .02$; Figure 1c) and the central amygdala ($F_{1,31} = 7.367, p < .01$; Figure 1d); in both instances, the left side contained significantly higher concentration of ir-BB than the right side. Post-hoc one-way ANOVAs revealed that these lateral differences were detectable only when data were pooled across treatment conditions. Furthermore, in the case of the CeA, these differences were also evident irrespective of whether in the Shock ($F_{1,16} = 7.698, p < .02$) or the no-Shock condition ($F_{1,14} = 10.032, p < .01$). For a complete detailed list of ir-GRP concentrations, refer to Table 1.

GRP

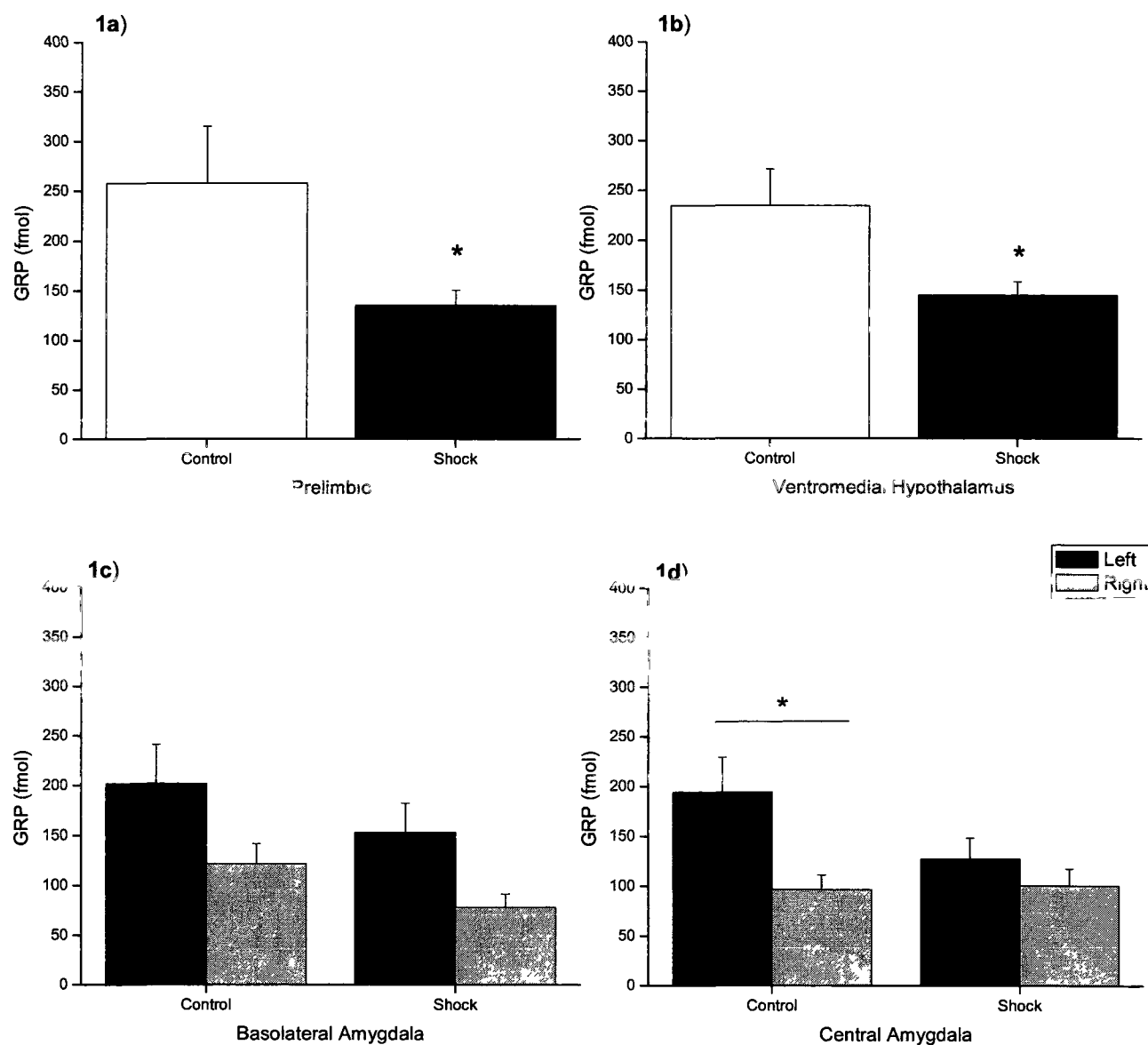


Figure 1. Values represent the mean ($\pm$ SEM) concentrations of ir-GRP measured in each nucleus. a) Depicts a significant decrease in concentration of GRP in the shock compared to no shock controls in the prelimbic cortex. b) Represents the same parameters measured in the ventromedial hypothalamus while c) and d) indicate a significant difference in peptide concentration for the left side and the right side for the basolateral and central amygdala nuclei respectively depicted separately for each condition. Figure d) shows a further significant difference between the left and right ir-GRP levels in the no-shock control condition.

Levels of ir-CRH similarly varied in a region- and treatment-specific manner. In the prelimbic cortex, the difference between the Shock and no-Shock condition approached but did not reach statistical significance ($p = .06$ in overall ANOVA). However, a significant difference was observed between the levels of ir-CRH in the left and right sides ($F_{1,30} = 5.572, p < .02$), with the highest level being detected at the right side (Figure 2a). In the basolateral nuclei, a significant Side x Condition interaction was observed ($F_{1,17} = 4.559, p < .05$). Further analysis showed that in the animals that received Shock, the left BLA showed increased levels of ir-CRH, while the right nucleus showed decreased levels of ir-CRH, when compared to controls (Figure 2b). In the CeA, both a Condition ($F_{1,29} = 7.324, p < .015$) and Side ($F_{1,29} = 10.21, p < .01$) difference was observed. Compared to the no-Shock condition, animals that received the Shock showed markedly higher levels of ir-CRH in the left side, whereas no changes were evident on the right side (Figure 2c). For a complete detailed list of ir-CRH concentrations, refer to Table 1.

Analysis of both CORT and ACTH from animal trunk blood showed no significant differences between animals who had previously received shock and those who had not ($F_{1,29} = 1.164, p < .67$ and $F_{1,29} = 0.183, p > .05$ respectively).

CRH

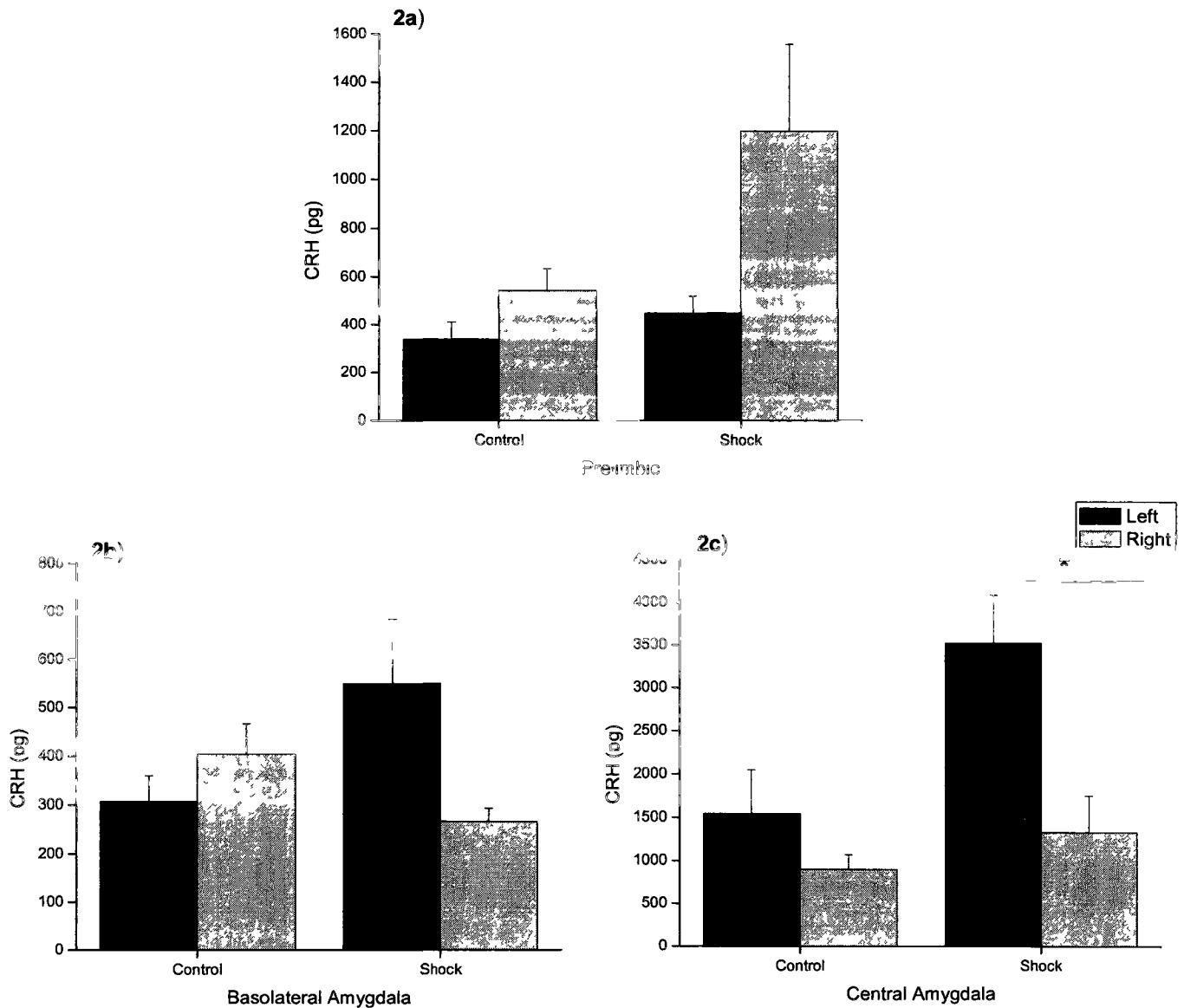


Figure 2. Values represent the mean ($\pm$ SEM) concentrations of ir-CRH measured in each nucleus. a), b) and c) each depict significant differences in peptide concentration for the left side and the right side for the prelimbic cortex, basolateral and central amygdala nuclei respectively, depicted separately for each condition. Figure c) shows a further significant difference between the left and right ir-CRH levels in the shock treatment condition.

Table 1. Summary of RIA results

	Gastrin-Releasing Peptide				Corticotropin-Releasing Hormone			
	<i>Control</i>		<i>Shock</i>		<i>Control</i>		<i>Shock</i>	
	Left	Right	Left	Right	Left	Right	Left	Right
Prelimbic	271.2±93.7	242.8±68.5	131.4±25.9	138.9±17.1*	337.2±71.2	541.5±90.6	447.2±71.4	1196.3±358.5†
Infralimbic	129.7±22.2	138.6±36.1	124.5±21.2	135.5±17.0	599.7±110.4	440.3±108.7	466.6±141.3	525.7±117.4
Cingulate	186.3±51.4	157.7±45.4	112.5±25.4	156.6±46.6	448.9±89.8	629.9±147.8	530.7±121.5	660.6±137.7
Central Amygdala	194.2±34.9	96.6±14.9†	127.4±20.9	100.4±17.0	1541.4±507.6	899.4±170.1†	3519.3±566.6	1324.4±424.8*
Basolateral Amygdala	201.6±39.9	121.1±20.2†	152.7±29.4	78.1±13.2	306.9±52.7	403.3±63.1	549.5±134.5	265.5±27.7
Hippocampus (CA1)	403.7±194.2	140.6±64.5	82.7±32.4	122.8±41.3	200.2±33.0	278.2±100.4	254.2±77.5	286.2±137.1
Ventromedial Hypothalamus	237.2±68.2	231.9±32.2	125.3±13.8	164.0±22.9*	481.0±120.6	976.7±251.1	676.4±176.1	535.0±213.3
Paraventricular Nucleus	231.7±32.6		223.1±35.3		1374.4±353.2		1680.1±559.5	
Periaqueductal Grey	189.4±47.1		155.4±43.8		809.6±203.3		406.1±63.5	

Note. Data represent means ($\pm$ SEM) for concentration of gastrin-releasing peptide and corticotropin-releasing hormone respectively in each nuclei of interest. Asterisks represent significant statistical differences between the shock and no shock (control) conditions ($p \leq .05$), while crosses indicate a significant difference between left side and right side ($\dagger \leq .05$).

Discussion

The present study sought to assess regionally specific fluctuations in the concentration of GRP and CRH in response to a cue that was previously paired to an aversive footshock. Of the various fear-conditioning and/or the stress responsive brain nuclei analyzed, only three regions revealed significant differences; the prelimbic cortex, ventromedial hypothalamus and amygdala.

Results revealed that presentation of a tone previously paired to a shock (CS) significantly lowered the ir-GRP content at the prelimbic cortex (PrL), compared to the control condition (tone not associated with shock). Under the same conditions, levels of ir-CRH were significantly higher in the right side of animals exposed to CS. The PrL has been implicated in limbic-cognitive functions [480], and is thought to play a role in processing cognitive aspects of emotional experiences. This region is also involved in the memory for fearful events (such as a CS previously paired with shock) [82] and is part of a larger system implicated in the extinction of fear-related behaviors, the medial prefrontal cortex (mPFC). In support of the above findings, using a similar micropunch technique, Adamec and colleagues [2] found increased GRP content in the cingulate cortex (the mPFC nucleus which sits dorsal to the PrL) of rats exposed to a cat. However, in the present study, CS-induced differences in the cingulate cortex were not observed, a finding which may be explained by methodological differences between the two studies. In Adamec's study, tissue samples were collected and analyzed in response to an immediate threat, whereas in the current study, samples were collected 24h after stressor exposure in an attempt to measure peptide fluctuations upon *recall* of a stressful event.

In the ventromedial hypothalamus (VMH), exposure to the CS lowered ir-GRP content relative to the control condition. GRP alterations at this locus are not surprising given the

involvement of the VMH in satiety (a process which is mediated by GRP and would be inhibited during fear-eliciting events) [218, 219, 502]. And like GRP, the VMH is also involved in anxiety and fear [64, 90, 112, 459, 460, 522]. Indeed, in the same study as mentioned above, Adamec and colleagues [2] found an increase in GRP at the VMH in response to cat exposure. Again, the discrepancies observed between that study and the current one may be explained by methodological variations.

In the amygdala, left and right differences were seen in both the CeA and the BLA. In both these nuclei, the left side contained significantly higher ir-GRP and ir-CRH concentration than the right side. It is difficult to understand why this might be so, but other studies have found hemispheric differences in amygdala activity pertaining to anxiety/fear [3, 4, 5] and the involvement of the amygdala in fear behaviour is well-established. Further, the aforementioned cat exposure study supports a variation in GRP release at the amygdala in response to fear, as an increase in GRP was observed at the CeA in that study [2]. It is noteworthy that Adamec's study did not find differences in CRH at any of the sites that were of interest in this study.

In summary, the hemispheric differences seen with both CRH and/or GRP in the amygdala and the prelimbic cortex are interesting, as the patterns are consistent across amygdalar nuclei but inversely related across GRP and CRH and between the amygdala and prefrontal cortex. Further, there is ample evidence in the literature implicating the amygdala and medial prefrontal cortex nuclei in fear conditioning processes. Indeed, it has been hypothesized that these two brain areas may form a fear circuit [9, 162, 369, 370, 371]. The findings from the current pilot study, taken together with a plethora of published literature implicating the mPFC and amygdala brain regions in conditioned fear, suggests that these nuclei deserve closer scrutiny in the context of fear conditioning and the role that GRP plays in this circuit.

Preface to Chapter 3

As suggested by the previous pilot study, both the prelimbic cortex and the amygdala demonstrate distinct alterations in ir-GRP and CRH in response to cues associated with an aversive footshock. This is consistent with two lines of research. Firstly, Adamec and his colleagues [2] demonstrated that within 5 min of a traumatic experience (exposure to a cat), the levels of GRP rose at the cingulate cortex (compared to non-exposed controls). The cingulate cortex is part of a system including the prelimbic and infralimbic cortices that make up the medial prefrontal cortex (mPFC), which has been shown to be intimately involved in learned fear [304, 316, 317, 402, 415]. Secondly, Shumyatsky and his colleagues [436] showed that the GRP gene is heavily expressed at the lateral nucleus of the amygdala, a second region intimately involved in conditioned fear [436]. Furthermore, they demonstrated that BB₂ receptor-deficient mice displayed 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.

These results taken together with literature assessing the neurobiology of conditioned fear and post-traumatic stress disorder make the amygdala and mPFC interesting targets for further exploration. For these reasons, the subsequent set of studies were undertaken to assess the behavioral effects of direct microinjections of GRP or its receptor antagonist into the mPFC or the amygdala to further elucidate the role of this peptidergic system in fear.

Chapter 3 – Part I

The Role of Gastrin-Releasing Peptide on Conditioned Fear: Differential Cortical and Amygdaloid Responses in the Rat

Abstract

Rationale: Bombesin (BB), an amphibian peptide, has been shown to affect the expression of the stress response. However, the physiological role of mammalian counterparts of BB in mediating anxiety and fear responses remain to be characterized. *Objective:* This study examined the effects of gastrin-releasing peptide (GRP), a mammalian analogue of BB, and its receptor antagonist, BW2258U89, on conditioned emotional response (CER), using fear conditioning. *Methods:* The effects of these compounds on contextual and cued fear conditioning were assessed following direct bilateral infusions into the prelimbic (PrL) cortex, infralimbic (IL) cortex or central nucleus of the amygdala (CeA). *Results:* GRP (300 ng) microinjected into each of the three target nuclei significantly reduced freezing to contextual cues. Similarly, in the cued portion of CER, GRP administered to the IL cortex significantly reduced freezing. Administration of BW2258U89 resulted in dose-dependent and site-specific effects. At the IL cortex, 50 ng dose decreased freezing to both contextual and cued fear conditioning, at the CeA, the 300 ng dose also decreased freezing, but at the 50 ng dose, it increased contextual freezing. At the PrL cortex BW2258U89 did not affect freezing. *Conclusions:* These results illustrate that i) GRP system(s) can significantly affect the expression of learned fear, ii) some of the relevant brain sites mediating these effects include the PrL, IL and the CeA., and iii) such effects may be dependent upon whether responses were evoked by environmental contextual fear cues or by specific auditory cues that were explicitly paired with an aversive stimulus.

Introduction

Gastrin releasing peptide (GRP) has been implicated in the regulation of emotionally-salient memories, feeding behavior, synaptic plasticity, immune functioning and the pathogenesis of several types of human cancer [71, 126, 279, 296, 390, 404, 436, 481]. GRP is a mammalian analogue of bombesin (BB), an amphibian tetradecapeptide originally isolated from the skin of the frog *Bombina Bombina* [17]. This peptide is heterogeneously distributed throughout the mammalian central nervous system as are the BB₂ receptors that preferentially bind to these peptides.

Based on the effects of GRP and/or BB on anxiety-like behaviors, conditioned fear, and neuroendocrine responses to stressors [94, 296, 436, 505, 507], this peptidergic system has been proposed as a potential therapeutic target for anxiety disorders [389]. Central administration of BB promotes both hypothalamic-pituitary-adrenal (HPA) and sympathetic activation [212]. Furthermore, it modulates expression of corticotropin releasing hormone (CRH) at stress-relevant brain sites, including the central nucleus of the amygdala (CeA) [211]. The administration of GRP to *in vitro* cell cultures of the hypothalamus, the pituitary, or the adrenal cortex provoked the release of CRH, adrenocorticotrophic hormone (ACTH) and corticosterone, respectively [134]. As well, in rodents, pretreatment with GRP receptor antagonists attenuated serotonin release at the hypothalamus under both basal and restraint stressor conditions [133].

In addition to affecting the stress response, there is reason to believe that GRP may influence memory processes, including acquisition of conditioned fear. For instance, systemic administration of BB or GRP improved memory retention in step-down inhibitory avoidance tasks [376, 417], and the blockade of peptide action (using BB₂ receptor antagonists) impaired inhibitory avoidance learning [390]. Interestingly, Rashidy-Pour and Razvani [376] found that

the memory-enhancing effects of systemic BB administration were attenuated by unilateral inactivation of the amygdala using lidocaine immediately following training. Furthermore, a BB₂ receptor antagonist when microinjected directly into either the basolateral nucleus of the amygdala (BLA) or the hippocampus immediately after training, impaired both the short- and long-term retention of the inhibitory avoidance response [391, 393]. Concordant with the contention that GRP may be involved in the mediation of fear, it was shown that the GRP gene is highly expressed in the lateral nucleus of the amygdala (LA) [436]. Moreover, BB₂-receptor knockout mice exhibited enhanced memory storage of fear-motivated tasks as well as enhanced synaptic long-term potentiation [436]. Taken together, these data support the involvement of GRP in emotionally-based learning and points to its potential involvement in long-term aversive memory formation.

In addition to GRP involvement in amygdala-mediated fear responses, levels of this peptide were also elevated at the cingulate cortex in response to distress elicited by predator exposure [2]. The cingulate cortex is part of a system involving the prelimbic (PrL) and infralimbic (IL) cortices that together constitute the medial prefrontal cortex (mPFC). Although relatively scant, evidence suggests that the cingulate cortex may be important in fear-related processes. For instance, lesions to this area produced an increase in fear responses to fear conditioning [316]. Destruction of the IL and PrL cortices however, blocked recall of fear extinction [317, 373], suggesting their role in the storage and regulation of long-term extinction memory. In addition, Milad et al., [305] demonstrated that electrical stimulation of IL neurons reduced freezing to conditioned tones, and that neurons in this area increased responding in response to cues previously extinguished, suggesting a role in extinction recall [304]. Moreover, stimulation of the combined areas resulted in a feedforward inhibition of CeA output neurons,

suggesting that mPFC gates amygdaloid output activity [370]. The current view regarding the functional role of the IL/PrL activity suggests that whereas IL exhibits projection patterns consistent with a role in visceral and autonomic activity, the PrL projection patterns are consistent with a role in limbic or cognitive functions [480].

It has been suggested that the GRP system may serve to draw attention to biologically significant cues or events, such as those posing a threat to survival [301]. Furthermore, it has been posited that this peptidergic system may (directly or indirectly) exert inhibitory control over the processing of conditioned stimuli, thus regulating the excitatory and inhibitory circuits within amygdala nuclei [436]. Preliminary work in our laboratory had shown that central administration of GRP decreased freezing in a conditioned emotional response (CER) task and the startle amplitude in a fear-potentiated startle paradigm. Taken together with data from Shumyatsky [436] revealing that BB₂-receptor deficient mice show increased freezing, we hypothesized that GRP injected into either the cortex (mPFC) or the amygdala (CeA) would decrease freezing in a CER task, while a BB₂ antagonist would exacerbate such responses.

In the present study, we investigated the impact of GRP infusion into the CeA, the PrL or the IL, in the expression of a fear response. As the response to the more general contextual cues and the more specific (auditory) cues explicitly paired with a stressor are thought to involve distinct neural processes [222], we monitored responses to both contextual and specific auditory cues, using the conditioned emotional response (CER) paradigm.

Materials and Methods

Subjects

Sprague-Dawley rats weighing 275-300g were obtained from Charles River Laboratories (St-Constant, Quebec). Animals were doubly housed in plastic cages (45 x 25 x 20 cm) and maintained on a 12-h light/dark cycle (lights on at 07:00 h) in a climate-controlled environment (23°C, relative humidity 60%). Animals had access to food and water *ad libitum* throughout the experiment. All experiments were conducted in accordance with the Canadian Council of Animal Care, and were approved by the animal care committee of the University of Ottawa.

Surgery

Animals were anesthetized with the inhalant, halothane at 2.5%, and were stereotaxically implanted bilaterally with 22 gauge guide cannulae (Plastics One) at the following coordinates from [339]; positioned above the PrL and/or IL cortices: A/P +3.0 mm, L $\pm$ 0.07 mm, D/V -4.2 mm; and CeA: A/P -2.8 mm, L $\pm$ 4.5 mm, D/V -7.2 mm. The length of the injection cannula varied according to D/V position of the target nucleus (see Drugs and Injections section below). Rats received oral acetaminophen (Tylenol; 100-200 mg/kg) for 3 days prior to and 3 days following surgery. In addition, they received rectal Tylenol (50 mg/kg) on the day of the surgery and for 3 days following surgery.

To maintain cannula patency, a removable obturator was inserted into the guide cannula, which protruded 0.5 mm beyond the cannula tip. Animals were given a 7-day recovery period before testing began. During this interval, they were handled daily and obturators were removed and reinserted to familiarize the animals with the handling/injection procedures.

Drugs and Injections

The BB₂ receptor antagonist BW2258U89 (2-Phenylpropanoyl-His-Trp-Ala-Val-D-Ala-His-D-Pro=Phe-NH₂) [314] and GRP (Phoenix Pharmaceuticals, Inc.) were dissolved in Krebs ringer buffered saline solution (KRB) consisting of (in nM; 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻ ascorbate, pH 7.4). The control (vehicle) animals received an equivalent volume of KRB alone.

All drug infusions were delivered via two 28-gauge injectors (Plastics One). Each obturator was replaced with an injection cannula, such that it protruded 1 mm beyond the tip of the guide cannula (PrL and CeA) and 1.8 mm beyond the tip in the IL. The injectors were connected to 10 µL Hamilton Syringes with polyethylene tubing. Drugs were simultaneously delivered to both cannulae at a flow rate of 0.5µL/min over a 1-min interval. Injectors were left in place for a further 1-min period to allow for drug diffusion. In the PrL and IL studies, rats were randomly assigned to 3 groups: vehicle (KRB; n = 9 [PrL]; n = 11 [IL]); GRP (300 ng; n = 8 [PrL]; n = 11 [IL]) and BW2258U89 (50 ng; n = 11 [PrL]; n = 13 [IL]). An additional group that received both BW2258U89 and GRP (n = 13) was included in the IL study. For the CeA, animals were randomly assigned to vehicle (n = 15), GRP (50, 150 or 300 ng; n = 7-11) or the BW2258U89 (antagonist) conditions (50, 150 or 300 ng) (n = 10-14). The number of subjects varied across groups as some animals were removed due to inaccurate cannula placements and outliers (deviating by more than 5 SD from the group and grand means).

Conditioned Emotional Response

Apparatus

The conditioning chamber (Coulbourn Instruments) measured 31 x 25 x 30 cm. The front and back walls were made of clear Plexiglas and two side walls made of stainless steel panels. The floor was composed of 16 stainless steel rods (2 mm diameter, 3 cm apart), which were connected to a Coulbourn Instruments shock generator (model H13-16) that delivered constant current. A Sonalert tone generator (75 kHz, low setting – Coulbourn Instruments) was situated in the top rear panel and provided the conditioning auditory cue.

Procedure

All subjects completed one day of training followed by a day of testing. During the training phase the subjects were placed in the conditioning chamber where they received 6 pairings of a 20-s tone with a 1.0 mA (1-s) continuous footshock. The shock was delivered during the final second of the 20-s tone. Each trial was delivered at an average intertrial interval of 1-min.

On the test day, rats received bilateral infusion of the drug 15-min before testing. In the case of the animals receiving both BW2258U89 and GRP, the antagonist was administered first, 30-min before testing. The agonist was delivered next, 15-min prior to testing. Thereafter, animals were tested for contextual fear over a 4-min period, by placing them in the conditioning chamber (where they had previously been shocked), however, this was now done in the absence of the tone or shock. Freezing behavior (the absence of movement excluding involuntary respiratory movements) was assessed over a 4-min session, using a time-sampling method. The absence or presence of complete immobility during every 5-s sampling epoch was recorded.

Evaluations of freezing responses were conducted by trained experimenters blind to the drug condition. Immediately following contextual testing, the animals were assessed for the cued response. To that end, rats were transferred to a clean Plexiglas cage (similar to their home cage) and presented with the conditioning cue (tone - that had previously been paired with footshock). A total of 20 tones (each 20-s in duration) were presented at 1-min intervals. A 1-min acclimatization period was allowed, prior to the onset of tone presentation. Freezing was scored as described in the contextual test. Between each training and testing session, cages were cleaned with 70% ethanol.

Histologies

Following behavioral testing, animals were given an overdose of pentobarbital and were perfused with a 10% formalin solution. A 25% India ink solution (0.5 μ l) was injected into each cannula in the manner the drugs were infused. Brains were then removed and stored in a 10% formalin solution at room temperature (minimum 4-h) and were subsequently transferred into a 10% sucrose solution for at least 18-h prior to sectioning. Brains were frozen with carbon dioxide prior to sectioning, and were stained using Cresyl Violet. Cannula placements for each animal were verified histologically and animals for which at least one cannula had reached the nucleus of interest were included in the data analysis (see Figures 1A and B).

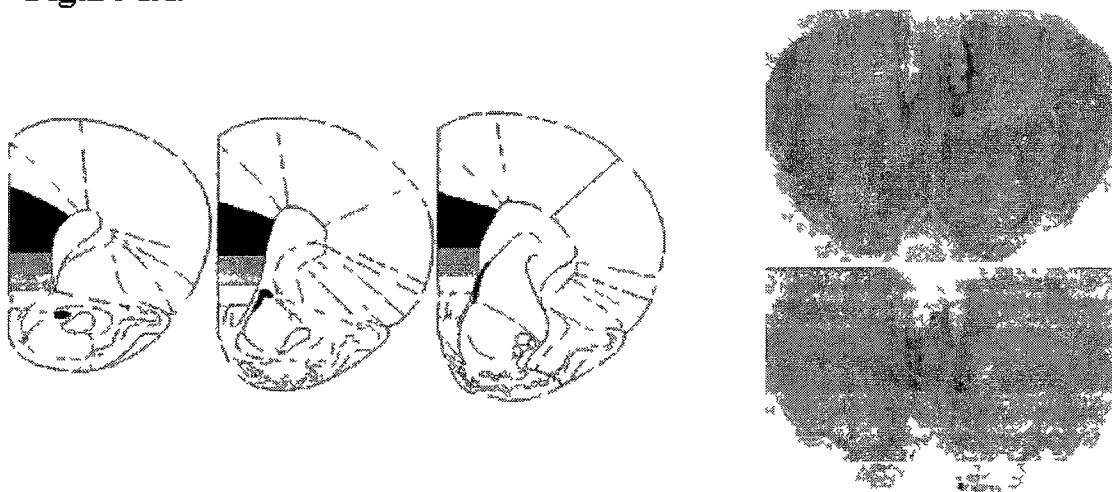
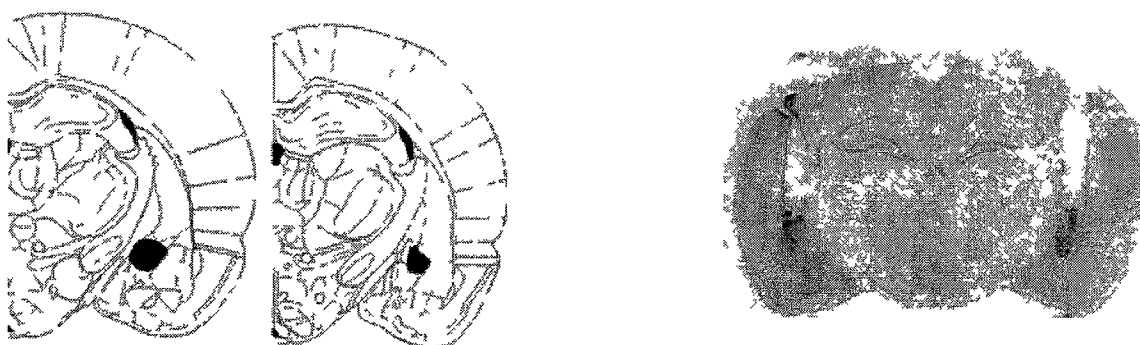
Figure 1A.**Figure 1B.**

Figure 1. Diagram of the acceptable planes (taken from [339]) for placement showing the PrL cortex, shaded black, and the IL cortex, shaded gray (1A). Diagram of the acceptable planes (taken from [339]) for placement showing the CeA, darkened, (1B).

Statistical Analysis

Data were analyzed separately for the agonist and antagonist conditions, as were the data from the contextual and cued freezing conditions. For the contextual tests, the raw freezing scores were transformed into a percentage of sampling bins during which freezing occurred over the four 1-min intervals. These percentages were then analyzed using a mixed-measures analysis of variance (ANOVA) with Treatment condition as the between-group measure and Time (minutes 1 to 4) as the within-group measure. Follow-up tests were conducted using Newman-Keuls multiple comparisons ($p < .05$). For the cued test, percentage of freezing scores were calculated for the 20 1-min bins during the test, collapsed over four 5-min time blocks. These values were then analyzed using mixed-measures ANOVA with Treatment condition as the between-group measure and Time (four 5-min blocks) as the within-group measure. Newman-Keuls multiple comparisons were again used for follow-up analyses.

Results

Effects of GRP or GRP antagonist infusion into the PrL

The effects of GRP infused into the PrL were examined in both the contextual and cued conditions of the CER test. The mixed-measures ANOVA revealed that in the contextual portion of the test, freezing was lower in GRP-treated rats than in those that received the vehicle treatment ($F_{1,15} = 5.872, p = .028$; see Figure 2A). In contrast, freezing elicited by the specific auditory cue did not differ significantly between groups (Figure 2B). Although there was a trend suggestive of suppressed cue-elicited freezing in the GRP (compared to the control) condition,

this effect did not reach statistical significance; most likely due to relatively low levels of freezing seen in the group implanted at this site.

In contrast, BW2258U89 failed to significantly affect the expression of fear in either the contextual or cued conditions (Figures 2C and D).

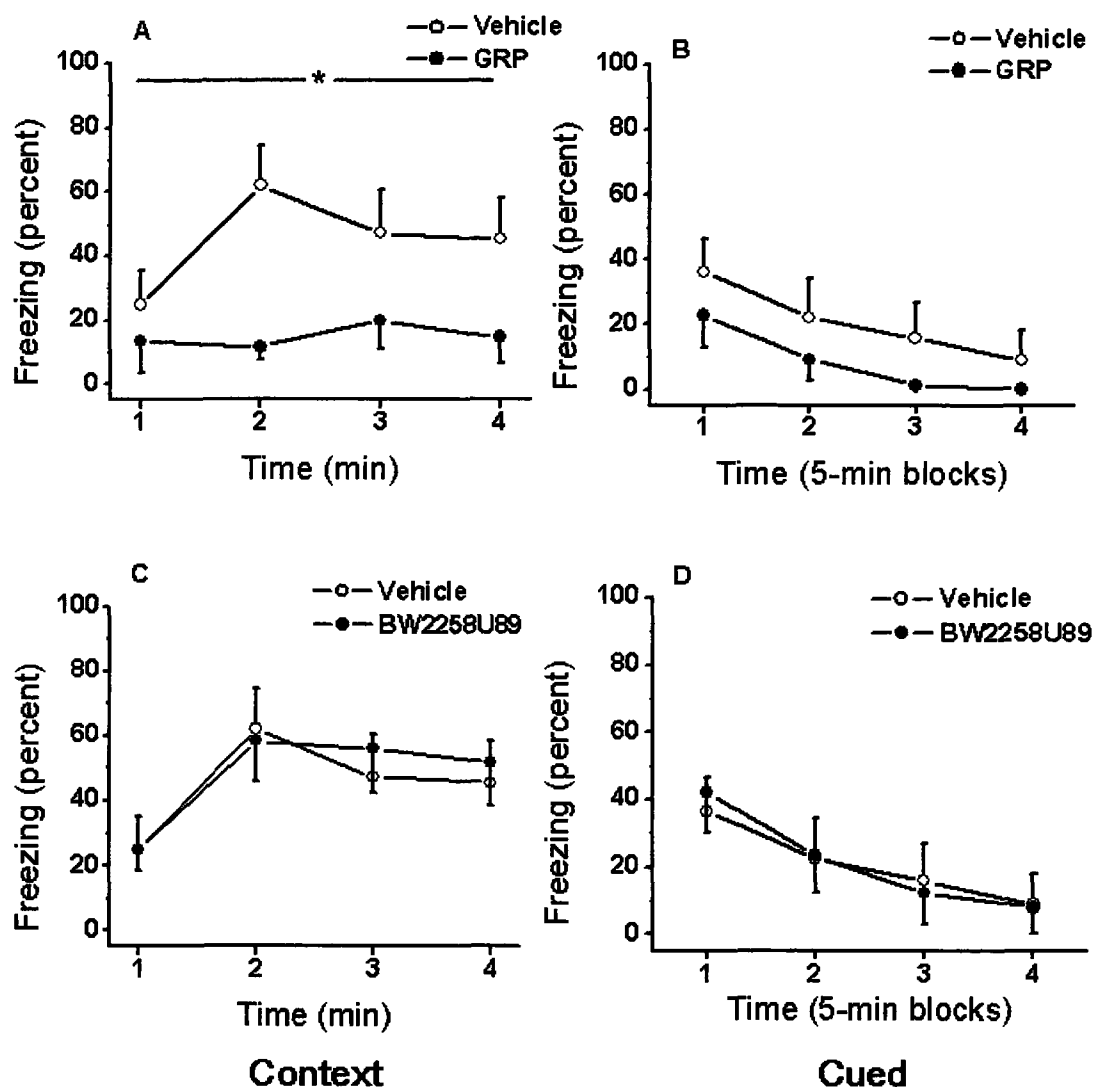
Figure 2.

Figure 2. Percentage of time engaged in freezing ($\pm$ SEM) following microinjection of GRP or vehicle into the PrL in the contextual task (2A), and in response to the fear cue applied in a different environment (2B). Panels 2C and 2D depict the percentage time engaged in freezing in the contextual and cued tests respectively, following microinjection of BW2258U89 or vehicle into the PrL. The same vehicle controls are depicted across both GRP and BW2258U89 conditions.

* Significantly different from control (vehicle) group, $p < .05$.

Effects of GRP or GRP antagonist infusion into the IL

As seen with GRP administration in the PrL, microinjection of this peptide into the IL revealed a significant Treatment effect in the contextual portion of the CER test ($F_{1,20} = 12.405$, $p = .002$). While the interaction with Time was not significant, post-hoc Newman-Keuls multiple comparisons indicated that this effect was attributable mainly to a between group difference during the final 3 min of the test (see Figure 3A).

The effect of GRP on freezing in the cued portion of the CER test was also significant, ($F_{1,20} = 5.318$, $p = .03$). One-way ANOVA were conducted to test for simple effects within the four 5-min time blocks. These analyses revealed that the tone significantly increased freezing in vehicle-treated animals, and that the freezing response in GRP-treated rats was significantly reduced (relative to vehicle-treated controls) during the first three 5-min blocks of time. During the fourth time block no treatment differences were apparent (Figure 3B).

Interestingly, as in the case of GRP, infusion of the BB₂ receptor antagonist into the IL significantly decreased contextual freezing, ($F_{1,22} = 12.291$, $p = .002$); however, it failed to reduce the expression of fear in the cued condition (Figures 3C and D).

When the agonist and the antagonist were co-administered, no significant effects were observed in either the contextual or cued conditions (Figures 3E and F).

Figure 3.

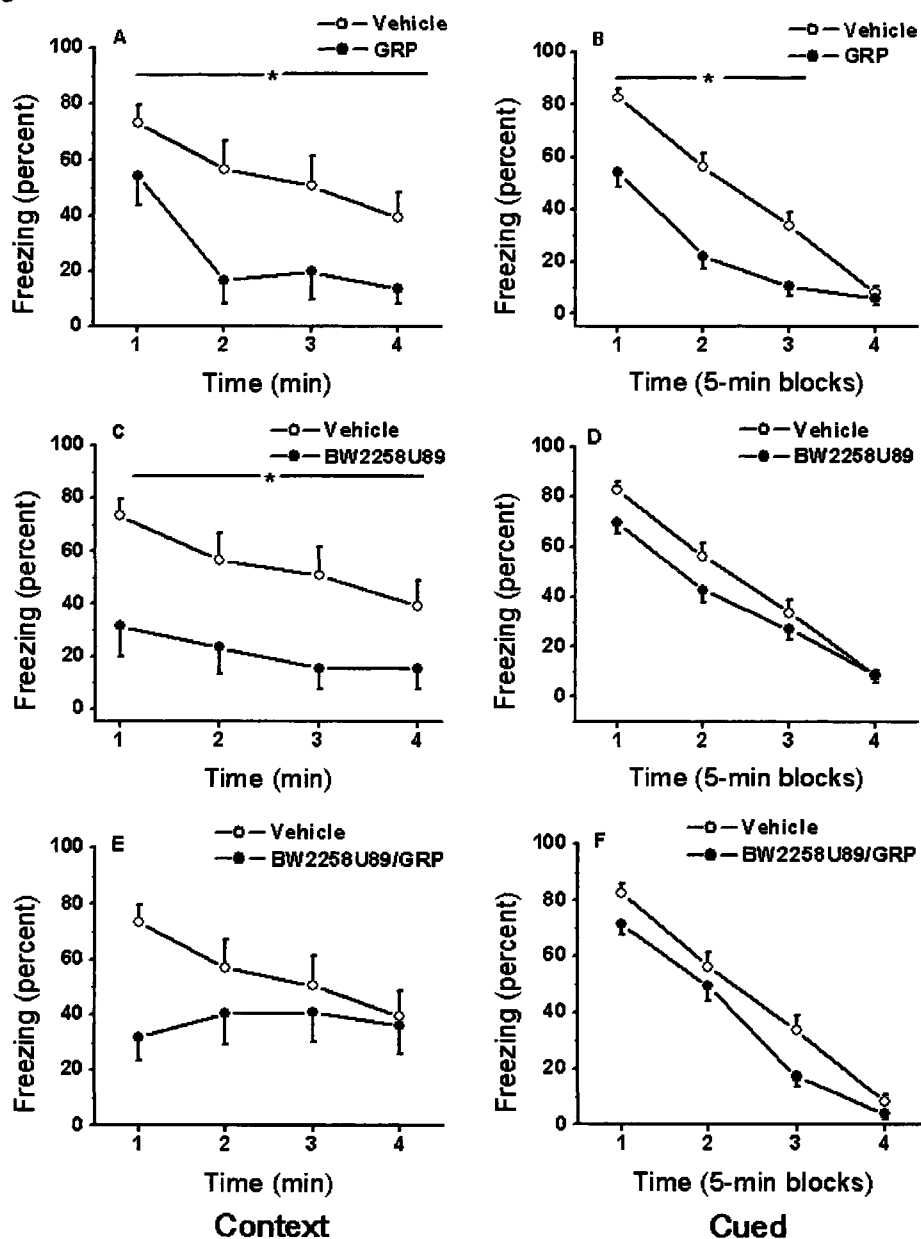


Figure 3. Percentage freezing (±SEM) following microinjection of GRP or vehicle into the IL cortex in the contextual task (3A); and the cued task (3B); and microinjection of BW2258U89 or vehicle into the IL in the CER contextual task (3C) and in the cued CER task (3D). Figures 3e and f represent the percentage of time engaged in freezing in the contextual and cued tasks respectively, following microinjection of both the antagonist and agonist. The same vehicle controls are depicted across both GRP and BW2258U89 conditions.

* Significantly different from control (vehicle) group, $p < .05$.

Effects of GRP or GRP antagonist infusion into the CeA

The effects of GRP at the CeA were examined in both the contextual and cued conditions of the CER test. Mixed-measures ANOVA revealed that there was no significant Treatment x Time interaction in contextual freezing ($F_{9,111} = 1.699, p > .05$). Again, given the *a priori* hypothesis, further analyses using ANOVA revealed that administration of the 300 ng dose of GRP significantly reduced freezing ($p < .05$) in the second minute of the test (in comparison to vehicle-treated controls), and freezing in this group of rats remained consistently low throughout the 4-min contextual condition (see Table 1).

Table 1. Effects of BB₂ receptor agonists (GRP) and antagonists (BW2258U89) on the percentage of time engaged in freezing in the contextual condition.

Treatment	Context			
	1	2	3	4
Vehicle	30.0 ± 5.1	66.7 ± 8.5	53.9 ± 10.8	52.8 ± 11.0
GRP				
50 ng	35.4 ± 12.0	51.0 ± 11.1	46.9 ± 12.8	58.3 ± 13.7
150 ng	32.1 ± 5.9	72.6 ± 9.7	55.9 ± 12.0	38.1 ± 17.4
300 ng	25.8 ± 8.3	28.8 ± 11.6*	30.3 ± 10.2	28.0 ± 11.2
BW2258U89				
50 ng	34.5 ± 6.5	72.0 ± 7.7	75.6 ± 4.3	88.7 ± 4.7*
150 ng	25.8 ± 9.4	36.7 ± 11.2	31.7 ± 13.0	29.2 ± 12.3
300 ng	17.5 ± 6.1	20.8 ± 10.6*	45.0 ± 12.5	34.5 ± 6.5

Note. Data represent mean percentage freezing ± SEM across 4-mins (1-min represented in each column). * Significantly different from respective vehicle condition at $p < .05$.

Effects of the GRP in the cued portion of the CER paradigm were difficult to assess or interpret, as the freezing response appeared to be abolished by cannulation at this site (see discussion - data not shown).

The effects of the GRP antagonist on contextual freezing varied as a function of the Treatment x Time interaction ($F_{9,135} = 3.195, p = .0015$). Follow-up comparisons revealed that during the second minute of the context test, rats treated with the higher dose (300 ng) of the BB₂ receptor antagonist exhibited significantly lower levels of freezing (in comparison to the control condition). In contrast, by the fourth minute of the contextual test, rats that received the lower dose (50 ng) of the antagonist exhibited significantly higher levels of freezing relative to vehicle-treated controls (Table 1). Responses in the cued condition were not assessed due to low levels of freezing (see discussion).

Discussion

Although GRP has been implicated in the formation or expression of emotionally-salient memories [390, 391, 416, 417, 436], few studies have examined the pharmacological effects of GRP at specific brain nuclei thought to be involved in fear and/or anxiety. The current study investigated the modulation of conditioned emotional response (freezing behaviors) in response to GRP or its receptor (BB₂) antagonist microinjected directly into specific cortical and amygdaloid sites (PrL, IL and CeA). Consistent with a role for GRP in fear/anxiety [436], rats microinjected with GRP (300 ng) into the PrL or IL cortices exhibited decreased contextual freezing. A similar effect was evident when GRP was administered into the CeA, but this effect

appeared to be less pronounced. The effects of the BB₂ receptor antagonist appeared to be more site specific in that although BW2258U89 (50 ng) administration into the PrL failed to affect the freezing response, it reduced freezing when infused into the IL. Furthermore, at the CeA, depending on the doses used, it produced either a mild enhancement or a mild reduction in this response.

In general, these findings appear consistent with those of other researchers investigating the role of GRP on learning and memory in paradigms involving aversive motivation. The reduction of contextual freezing following administration of the BB₂ antagonist (in the IL and CeA) is in accord with the findings of Santo-Yamada et al., [416], who showed that peripheral injection of the GRP receptor antagonist [leu¹³-(ψ-CH₂NH)-leu¹⁴]BB impaired inhibitory avoidance learning in mice. Similarly, Roesler et al., [390, 391, 393] found that systemic as well as localized central injection of RC-3905 in rats, impaired memory in this paradigm. The current findings at first may appear inconsistent with those of Shumyatsky et al., [436] who demonstrated that contextual fear was enhanced in BB₂-receptor knockout mice compared to their wild-type counterparts, and with those of Martins et al., [273] who showed that rats treated with the BB₂-antagonist, RC-3095, spent significantly more time in the closed arms of an elevated plus maze (indicative of higher levels of anxiety). However, it is noteworthy that at the CeA, the BB₂-receptor antagonist (50 ng dose) elicited a comparable effect, but only towards the end of the contextual test, suggesting that the fear-related memory trace was protracted in these animals, or that the antagonist diminished extinction of the contextual fear response. This outcome is also consistent with Shumyatsky's observations that BB₂-knockout mice expressed enhanced long-term potentiation (LTP) in the amygdala, as well as a long-lasting enhancement of conditioned fear. Furthermore, the suppression of freezing induced by GRP administered

across various brain regions, supports the contention by Shumyatsky et al., [436] that GRP and its neural circuitry operate as a negative feedback system that regulates fear.

It is difficult to reconcile the fact that some of the effects of the GRP antagonist were, under certain conditions, similar to those of GRP itself. In this context, it should be noted that some GRP antagonists have intrinsic agonistic activity [73, 109, 220, 405, 489], which may become evident at certain doses, in a site specific manner. Furthermore, as with other peptides, BB and GRP have been shown to have an inverted-U dose-response curve for inhibitory avoidance task [126], and in a more recent report, Roesler's group [96] likewise demonstrated that whereas at a lower dose (1 μ g), RC-3095 impaired memory retention in an inhibitory avoidance task, at a higher dose (10 μ g at the dorsal hippocampus CA1 region) it enhanced memory retention. Such differential effects may translate into modulation of distinct neural circuits and behavioral outcomes.

The finding that BW2258U89 was ineffective in moderating conditioned fear when administered into the PrL, but affected behavior when administered into the IL and CeA, again suggests site-specific actions of the antagonist. This outcome seems plausible as extinction-related processes appear to be more strongly related to IL as compared to the PrL neurons [374]. Furthermore, it is possible that at this site of lower receptor density [205, 227, 311, 313, 336, 484], the dose of the antagonist used may not have been optimal for receptor blockade. Furthermore, given that the antagonist deployed in our study (BW2258U89) may not be a pure antagonist [220], it may in effect, have contributed to the expression of its intrinsic activity by masking its antagonistic action.

Although one of the objectives of the present investigation was to assess the effects of GRP and GRP antagonist at the CeA, implantation of cannula at this site affected the CER such

that freezing levels in response to the conditioning cue were markedly attenuated. Thus it was difficult to discern differential drug effects. This potentially confounding effect was not a spurious observation, as subsequent studies yielded similar outcomes (unpublished data). This was a site-specific confound, as level of freezing was not affected when cannulae were implanted at either the PrL or IL, suggesting that critical fiber tracts explicitly involved in response to CS-US associations may have been disrupted by CeA cannula insertion. Indeed, as in the present study, others have also documented that moderate fiber-sparing lesions to the CeA disrupt freezing to a tone, but not to contextual stimuli [222]. Thus, it is possible that cued effects of GRP at the amygdala may only be measured from projection fibers arriving at the CeA. It should also be noted that the effects observed in these studies could possibly be due to disruption in locomotor abilities; however this seems unlikely as both Martins et al., [273] and our own research (unpublished data) found no difference in locomotor and exploratory behaviors in an open field arena.

While the present findings suggest that GRP plays a role in the expression of learned fear, the underlying mechanisms through which this peptide acts remain largely speculative. Shumyatsky et al., [436] reported that GABAergic (gamma-aminobutyric acid) inputs provide both feedback and feedforward information, which in turn, may determine how both excitatory and inhibitory information is processed within neuronal pathways of the amygdala. Further, they found increased GABA excitation upon application of GRP in the LA, and showed that this GABA-mediated inhibition played an important role in the induction of LTP. Thus, it is likely that activation of amygdala GABAergic interneurons provoked by GRP release may reduce the firing from the CeA and decrease the stimulation of brain regions that control behavioral and endocrine aspects of the stress-response. It is of interest that GRP and CRH are co-released at

the CeA during both appetitive and stressful manipulations [301], and that CRH and GABA may have reciprocal effects on neuronal functioning [170]. Thus, it might be considered that interactions involving GRP, CRH and GABA may have important clinical implications, particularly as decreased levels of GABA (or disorganized regulation of GABA_A subunits) have been reported in several stress-related disorders, including depression, panic and generalized anxiety disorders [41, 224, 306], as well as in cortical regions of suicide victims [294].

In terms of neurocircuitry mediating the conditioned emotional response, it is important to note that the amygdala and mPFC share reciprocal connections, and it has been suggested that the mPFC gates or inhibits responses from the CeA via projections through either the lateral amygdala or through intercalated cells located between the LA and CeA [304, 305]. It has been suggested that GRP may be co-released with glutamate from pyramidal neurons, which subsequently synapse on both principle cells and on a subpopulation of inhibitory GABAergic interneurons located within the LA [436]. Thus, it is possible that the administration of GRP in the mPFC cortex may activate glutamatergic fibers synapsing in the LA, creating a signaling circuit between the mPFC and amygdala mediating fear-mediated processes. This work suggests that GRP and related peptides may play a physiological role in the mediation or modulation of conditioned fear and that GRP (or BB₂) receptor ligands may represent therapeutic targets in the treatment of anxiety related disorders.

Chapter 3 – Part II

Effects of Gastrin-Releasing Peptide Agonist and Antagonist Administered to the Basolateral Nucleus of the Amygdala on Conditioned Fear in the Rat

Abstract

Rationale: Bombesin (BB)-like peptides have been shown to affect neuroendocrine and neural functions related to the stress response and the modulation of conditioned fear. In line with this view, central administration of gastrin-releasing peptide (GRP; a mammalian analogue of BB) or its receptor antagonist (D-Tpi6, Leu13 psi[CH₂NH]-Leu14) BB⁽⁶⁻¹⁴⁾ (RC-3095) modulate conditioned fear. *Objective:* The present study examined the effects of bilateral infusions of GRP or its receptor antagonist (RC-3095) into the basolateral nucleus of the amygdala (BLA) on the conditioned emotional response. *Methods:* The effects of GRP (150, 300 and 600 ng/0.5µl) and/or RC-3095 (50, 500 and 1000 ng/0.5µl) on contextual and cued fear conditioning were assessed following direct bilateral infusion of these compounds into the BLA. *Results:* Both GRP (300 ng/0.5µl) and RC-3095 reduced freezing during the contextual testing period, but did not influence responding in the cued test. Although both compounds reduced freezing in the contextual paradigms, at a moderate dose, pretreatment with RC-3095 attenuated the GRP-elicited decrease in contextual freezing. *Conclusions:* It appears that manipulation of GRP at the BLA may influence the expression of learned fear, and that these effects preferentially influence contextual versus cue-dependent emotional responses.

Introduction

The amygdala has long been implicated in subserving emotional responses and disorders [230], although different aspects of the amygdala may serve different functions related to fear or anxiety [15, 99, 101, 230]. Classical conditioning models, whereby a neutral conditioned stimulus (CS), such as a tone is paired with an aversive or unconditioned stimulus (US) such as a shock, have been used to study fear learning processes (for a history see [230]). Based largely on this research, a working hypothesis has evolved suggesting that the lateral/basolateral amygdaloid complex (BLA) represents a key site where CS-US associations are formed. Information from the BLA is then relayed to the central nucleus of the amygdala (CeA) for the expression of conditioned fear responses [138, 178, 199, 206, 353, 472].

The involvement of corticotropin releasing hormone (CRH), especially at the amygdala, has been the focus of considerable research in fear and anxiety [8, 129, 248, 425, 438, 466]. It has been suggested that bombesin (BB), an amphibian tetradecapeptide originally isolated from the skin of the frog *Bombina bombina* [17], may either directly or through its actions on CRH, influence fear and/or anxiety [296]. Indeed, the mammalian analogue of BB, namely gastrin-releasing peptide (GRP), has been shown to influence fear-related responses [30, 293, 436], while GRP within the BLA appears to be particularly salient in this regard [436]. In this vein, Shumyatsky and colleagues [436] showed that the GRP gene is highly expressed in the lateral nucleus of the amygdala (LA), and mice lacking the GRP receptor (BB₂-receptor) displayed enhanced memory for fear-motivated tasks in conjunction with enhanced synaptic long-term potentiation [436].

Further evidence for of the involvement of GRP in fear-related memory comes from studies using inhibitory avoidance tasks. For example, post-training systemic administration of

GRP attenuated the loss in memory elicited by scopolamine and hypoxia, and blockade of the peptide's action (using BB₂ receptor antagonists) impaired inhibitory avoidance learning [390, 416]. Furthermore, bilateral microinjections of a BB₂ receptor antagonist (RC-3095) into either the hippocampus or BLA immediately after training, impaired both the short- and long-term retention of the inhibitory avoidance response [391, 393].

Recent evidence from our laboratory has demonstrated that GRP administered either intraventricularly (i.c.v.), or directly into the central nucleus of the amygdala (CeA) or ventral medial prefrontal cortex, attenuated expression of fear (namely freezing) in a conditioned emotional response (CER) paradigm that involved either contextual cues (relating to the testing environment) or cues (tones) explicitly paired with a painful stimulus (shock) [30, 293, 322]. In contrast, intraventricular administration of a GRP receptor antagonist (RC-3095) increased freezing in a CER paradigm. However, when microinjected directly into the infralimbic cortex, RC-3095 reduced freezing [293]. Dose-related effects were seen at the CeA, whereby high doses reduced freezing and low doses increased freezing.

Given the potential involvement of the GRP system in the modulation of the fear response, and the importance of the BLA in the acquisition of a fear response, it was of interest to establish whether alterations of GRP within the BLA would influence the expression of fear-related behaviors. It was hypothesized that GRP would reduce freezing behavior elicited by both contextual and conditioned cues, whereas the BB₂ receptor antagonist RC-3095 would increase the expression of these fear-related behaviors.

Materials and Methods

Subjects

Sprague-Dawley rats were purchased from Charles River Laboratories (St-Constant, Quebec). Weighing 275-300g upon arrival, animals were housed in pairs in standard plastic cages (45 x 25 x 20 cm) until surgery. Lighting was maintained on a 12-h light/dark cycle (lights on at 07:00 h) in a climate-controlled environment (23°C, relative humidity 60%). Animals had access to food and water *ad libitum* throughout the experiment. All experiments were conducted in accordance with the Canadian Council of Animal Care guidelines, and were approved by the animal care committee of the University of Ottawa.

Surgery

Animals were stereotaxically implanted bilaterally with 22 gauge guide cannulae (Plastics One) at the following coordinates taken from Paxinos and Watson [339]; A/P -3.1 mm, L + 5.3 mm, D/V -7.7 mm. All surgeries were performed using the inhalant halothane (2.5%) as anesthesia. For pain control, rats received oral Tylenol (100-200 mg/kg) for 3 days prior to and 3 days following surgery. In addition, they received rectal Tylenol (50 mg/kg) on the day of the surgery and for 3 days following surgery. To maintain cannula patency, removable obturators were inserted into the guide cannulae. Animals were given a 7-day post-surgical recovery period before testing began.

Drugs and Injections

GRP (Phoenix Pharmaceuticals, Inc.) and the BB₂ receptor antagonist RC-3095 (D-Tpi6, Leu13 psi[CH₂NH]-Leu14) BB (6–14); Sigma-Aldrich) were dissolved in Krebs ringer buffered

saline solution (KRB) consisting of (in mM; 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻ ascorbate, pH 7.4). The control (vehicle) animals received an equivalent volume of KRB alone.

Drugs were simultaneously delivered to both cannulae via two 28-gauge injectors (Plastics One), which protruded 1.5 mm beyond the tip of the guide cannulae. The injectors were connected to 10 μ L Hamilton Syringes with polyethylene tubing, which delivered the drugs at a flow rate of 0.5 μ L/min over a 1-min interval. Injectors were left in place for a further 1-min period to allow for drug diffusion. Animals were randomly assigned to 4 drug/dose conditions in the antagonist (RC-3095) study: 0 (KRB vehicle; n = 12-16); 50 ng (n = 13-14); 500 ng (n = 14-15); or 1000 ng (n = 9-15). In the GRP study, animals were randomly assigned to 5 dose groups: 0 (KRB vehicle n = 15); 150 ng (n = 12); 300 ng (n = 9-11); or 600 ng (n = 8). The fifth group received both RC-3095 (500 ng) and GRP (300) as described below (n = 12). The number of subjects varied across groups as some animals were removed due to inaccurate cannulae placements, loss of headcaps and outliers (deviating by more than 5 SD from the group and grand means).

Conditioned Emotional Response (CER)

Apparatus

The conditioning chamber (Coulbourn Instruments; Allentown, PA) measured 31 x 25 x 30 cm. The front and back walls were made of clear Plexiglas and two side walls made of stainless steel panels. The floor comprised of 16 stainless steel rods (2 mm diameter, 3 cm apart), which were connected to a constant current shock generator (Coulbourn Instruments

model H13-16). A sonalert tone generator (Coulbourn Instruments; 75 kHz, low setting) was situated in the top rear panel and provided the conditioning auditory cue.

Procedure

All subjects completed one day of training followed by a day of testing 24-h later. During the contextual training phase, subjects were placed in the conditioning chamber where they received 6 footshocks (1.0 mA; 1-s in duration) on a random schedule with an average intertrial interval (ITI) of 1-min. Cued fear training comprised the delivery of 6 pairings of a 20-s tone with a 1.0 mA (1-s) continuous footshock. The shock was delivered during the final second of the 20-s tone. Again, each trial was delivered at an average ITI of 1-min.

On the test days, rats received bilateral infusion of the drug 15 min before testing. In the case of the animals receiving both RC-3095 and GRP, the antagonist was administered first, 30 min before testing, followed by the agonist 15 min prior to the test onset. Contextual fear was assessed over a 15-min period by placing them in the conditioning chamber where they had previously been shocked. Freezing behavior (the absence of movement excluding involuntary respiratory movements) was timed using the software program ODlog (Macropod Software). Trained experimenters blind to the drug condition conducted evaluations of freezing responses. To assess the CER (in the cued condition), rats were transferred to a novel environment of similar dimensions, but visually and texturally distinct from the training chamber. Specifically, black laminate covered the walls, and the floor was smooth (instead of rod-grid floor) and covered with bedding chips. Animals were allowed a 1-min exploration period and were then presented with the conditioned cue (the tone that had previously been paired with footshock). A total of 15 tones (each 20-s in duration) were presented at 1-min intervals (20 s tone + 40 s ITI).

Freezing was scored as described in the contextual test. Chambers were cleaned with 70% ethanol between each training and testing session.

Histology

Following the completion of testing, animals were euthanized with an overdose of euthansol (65mg/ml), and were perfused using a 10% formalin solution. After perfusion, a 25% India ink solution (0.5µl) was injected through each cannula in the manner the drugs were infused. Brains were then removed and stored at room temperature in a 10% formalin solution (minimum 4-h) and were subsequently transferred into a 10% sucrose solution for at least 18-h prior to sectioning. Brains were flash frozen with carbon dioxide prior to sectioning, and were stained using Cresyl Violet. Cannula placements were verified histologically and those animals with at least one cannula (of the bilateral pair) correctly positioned were included in the data analysis (see Figures 1A and B).

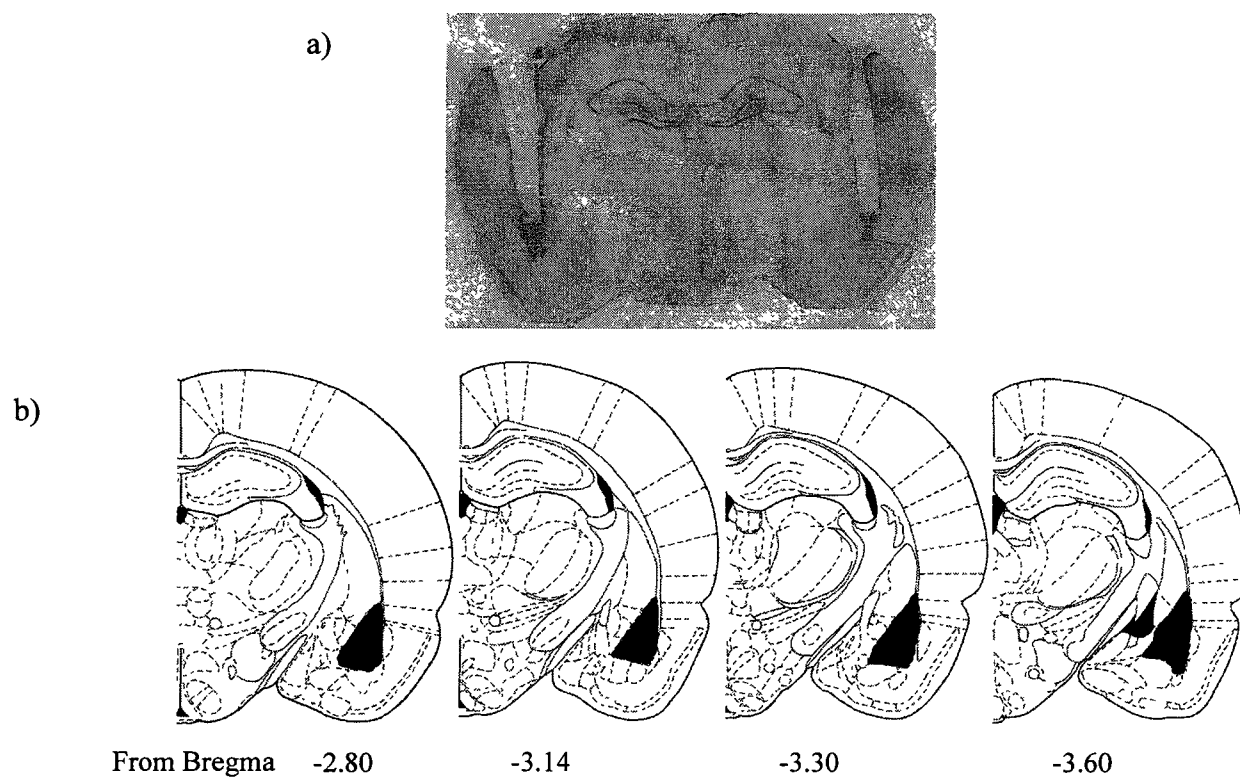


Figure 1. Diagram of the acceptable planes (taken from [339]) for placement showing the BLA cortex shaded black.

Statistical Analysis

Data were analyzed separately for the agonist and antagonist studies, as were the data from the contextual and cued freezing conditions. For each condition, Student's t-tests were run between animals with off-target placements and vehicle-treated animals as a positive control measure. Raw freezing scores were transformed into a percentage of time spent freezing within each 1-min bin. The 15 1-min bins were then collapsed over three 5-min time blocks. These values were then analyzed using a mixed-measures ANOVA in which drug treatment served as the between subjects variable, and time blocks the within-subjects variable. Follow-up tests were conducted using Bonferroni pairwise comparisons to maintain the α at .05.

Results

The effects of GRP infused into the BLA were examined in both the contextual and cued conditions of the CER test. The mixed-measures ANOVA revealed that in both the contextual and cued conditions, overall, freezing decreased over time, ($F_{2,88} = 63.55, p < .0001$ and $F_{2,102} = 69.97, p < .0001$, respectively), indicating that over time the anxiety/fear response was subject to extinction. Although GRP did not elicit a significant Treatment x Time interaction in the contextual task (Figure 2a), given our *a priori* hypothesis, one-way ANOVAs were conducted to test for simple effects within the three 5-min time blocks. These analyses indicated that GRP reduced freezing during the second and third 5-min periods ($F_{4,44} = 4.11, p = .006$ and $F_{4,44} = 2.82, p = .03$, respectively). Follow-up analyses confirmed that in the second and third trial blocks these effects were significant at each dose of the GRP treatment in comparison to vehicle (see Figure 2a).

The RC-3095 treatment significantly reduced contextual freezing over time ($F_{2,88} = 32.66$, $p < .0001$), and this change was qualified by group membership ($F_{6,88} = 2.32$, $p = .04$). In the context condition, rats that received RC-3095 exhibited lower levels of freezing relative to vehicle-treated animals during the first 5-min time block ($F_{3,44} = 5.65$, $p = .002$) and the third 5-min time period ($F_{2,102} = 4.76$, $p < .006$), and a similar trend approaching significance was observed during the middle 5-min time period. As illustrated in Figure 2c, and confirmed by post-hoc comparisons, during Block 1 and 3, animals injected with each of the doses of RC-3095 demonstrated significantly less freezing than did rats injected with vehicle ($ps < .05$). During the second trial block, animals that received the Low (50 ng/0.5 μ l) and Medium (500 ng/0.5 μ l) doses of RC-3095 demonstrated significantly less freezing than those animals treated with vehicle.

It is particularly significant that although RC-3095 attenuated the diminution of freezing over time, as did GRP itself, the antagonist attenuated the decline of freezing ordinarily promoted by GRP. As seen in Figure 2a, the actions of the antagonist were modest during the first trial block, and became significant on the second block, and by the third block, where freezing was virtually absent, the RC-3095 treatment no longer influenced the action of GRP.

In response to the conditioned cue, decreased freezing was apparent over time regardless of the GRP (Figure 2b) and RC-3095 treatments (Figure 2d) ($F_{2,102} = 69.97$, $p < .0001$ and $F_{2,112} = 99.15$, $p < .0001$ respectively). Neither of these agents influenced the freezing in response to the cue, nor did the drug treatments interact with time following treatment.

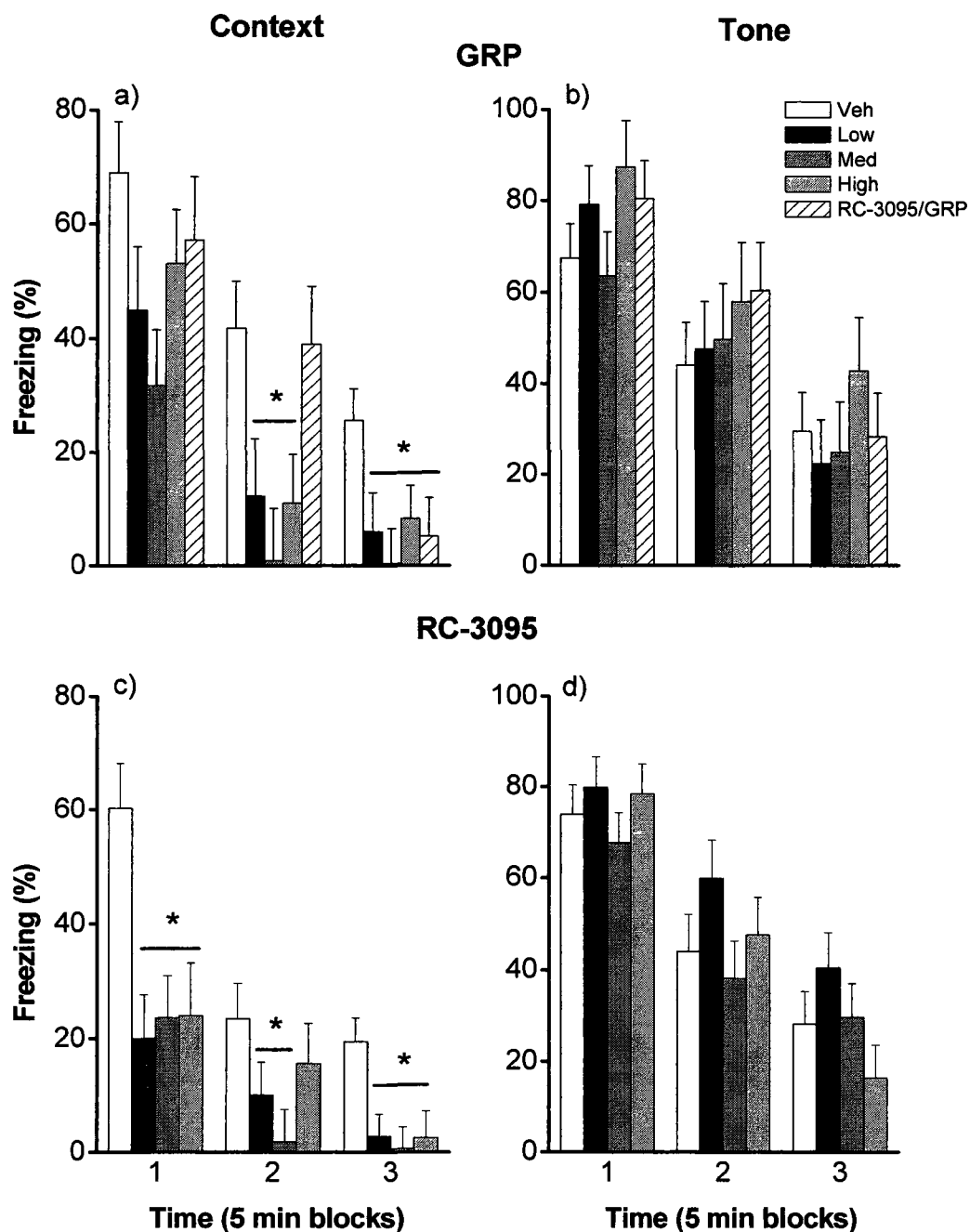


Figure 2. Percentage of time engaged in freezing ($\pm$ SEM) following microinjection of GRP (150, 300 and 600 ng/0.5 μ l), RC-3095 (500 ng/0.5 μ l) in combination with GRP (300 ng/0.5 μ l), or vehicle into the BLA in the contextual task (2a), and in response to the fear cue applied in a different environment (2b). Panels 2c and 2d depict the percentage time engaged in freezing in the contextual and cued tests respectively, following microinjection of RC-3095 (50, 500 and 1000 ng/0.5 μ l) or vehicle into the BLA. The same vehicle controls are depicted across both contextual and cued conditions within each experiment.

* significantly different from control (vehicle) group, $p < .05$.

Students t-tests to determine differences in target placements showed no significant differences between animals with off-target placements and vehicle-treated rats in the agonist condition ($n = 6$), across any time block. It is noteworthy that the majority of off-target placements were outside of the amygdala, often situated dorsal or lateral to the BLA, and the off-target animals were evenly dispersed between the 5 treatment conditions. In the antagonist study, there were 3 animals with off-target placements, all of which had received the high dose of RC-3095. These placements were either too central or too posterior, such that the injection was administered to the lateral ventricle. Student's t-test revealed significant differences from the vehicle-treated animals during the first time block, ($t_{(73)} = -2.093, p < .05$), and third time block, ($t_{(73)} = -1.938, p = .05$), but differences were not evident during the second time block ($p > .05$). These findings follow closely with that observed in animals with on-target placements.

Discussion

Our earlier studies had demonstrated that central administration of GRP (intraventricularly or localized at specific amygdaloid or cortical sites), attenuated the expression of fear in both cued and contextual conditions [30, 322]. However, responses to a GRP receptor antagonist were more complicated. Specifically, i.c.v. administration of RC-3095 increased freezing in the CER paradigm (unpublished data, but see [293]), whereas localized microinjection of the BB₂ antagonist, BW2258U89, into the infralimbic cortex attenuated the freezing response [322]. The effects of BW2258U89 at the CeA were biphasic in that high doses reduced freezing and low doses increased freezing [322].

In the present study, bilateral microinfusion of either GRP or the GRP antagonist RC-3095 into the BLA (in contrast to the CeA) significantly reduced the freezing response elicited

by contextual cues, but had no effect on explicit cues (conditioned tone). Interestingly, despite the similar behavioral effects of the agonist and antagonist, when RC-3095 was administered prior to infusion of GRP, the decreased freezing due to GRP was attenuated.

The attenuation in freezing observed following GRP injection is consistent with our previous research involving both the conditioned emotional response and the fear-potentiated startle response, implicating a role for GRP in fear/anxiety [30, 293, 322]. Indeed, GRP consistently reduced fear responses in both paradigms whether injected into the ventricles or site specifically into the CeA, infralimbic or prelimbic cortices.

In contrast to these effects, Shumyatsky et al., [436] reported that among GRP receptor knock-out mice, conditioned freezing was increased. Moreover, it was recently demonstrated that i.c.v. administration of the GRP antagonist, RC-3095, also lead to enhanced freezing in this paradigm [293]. Thus, comparable effects are observed by the modulation of the GRP receptor through receptor knockout strategies and ventricular administration of GRP antagonists. However, taken together with our previous findings involving the prelimbic and infralimbic cortices, it seems that the effects of the antagonist vary across different brain regions.

It is uncertain whether the results reported by Shumyatsky et al., [436] reflect the potential widespread (across multiple brain regions) decline of GRP receptors, or whether the results of the present investigation were unique to the particular dose used. Indeed, it has been reported that high vs. low doses of GRP antagonists may elicit opposite effects with respect to inhibitory avoidance. Specifically, bilateral administration of a 1 μ g dose of RC-3095 to the dorsal hippocampus impaired inhibitory avoidance training, whereas a 10 μ g dose enhanced inhibitory avoidance memory [96]. Similarly, several studies have reported agonistic effects of a peptide antagonist for the CRH receptor [40, 290, 375, 439, 498, 520]. Moreover, we observed a

similar trend using neuromedin B (a related peptide from the bombesin family), such that both neuromedin B and its receptor antagonist elicited similar effects in a fear conditioning paradigm [30]. Thus, partial-agonistic effects are not uncommon in response to peptide antagonists, and indeed, several GRP antagonists demonstrated intrinsic agonistic properties [73, 109, 220, 405, 489]. These dose-dependent and/or site-specific effects may help reconcile the fact that in the present investigation the GRP antagonist effects were similar to those of GRP itself. Given that BB_2 receptors are highly concentrated in the BLA [436], it is plausible that much lower doses of the RC-3095 antagonist may elicit effects opposite to that of GRP itself.

Unlike the effects observed in the contextual fear paradigm, in the present study neither GRP nor its antagonist influenced freezing in response to cues that had been explicitly paired with shock. Inasmuch as GRP manipulations at the CeA in our previous study [322] elicited behavioral effects distinct from those in the present investigation, where GRP and the GRP antagonist were injected into the BLA, the possibility exists that the varied GRP actions may involve distinct pathways within the amygdala, and these may be relevant to the differential effects observed with respect to contextual and cue-related freezing. Indeed, it has been reported that cue-related and contextual conditioning are mediated by both overlapping and distinct amygdaloid circuitry [148], and that additional (or alternative) neural circuits may be recruited to consolidate extinction learning in an environment different from the CS-US training environment [181]. Further, it is possible that the effect of the intra-BLA infusion on expression of contextual conditioning was related to indirect actions in some brain area other than the BLA. For example, Roozendaal and McGaugh [399] demonstrated that functioning within the dorsal hippocampus, an area critical for contextual but not cued fear expression [348], interacts with the BLA [399] in affecting performance in a fear-related task. Specifically, it was observed that hippocampal

infusion of a glucocorticoid agonist enhanced memory for inhibitory avoidance and that lesioning the BLA could block this effect. Likewise, administration of a glucocorticoid antagonist prior to training disrupted memory in a Morris water maze test, and again lesions of the BLA blocked this attenuation. As reciprocal connections exist between the BLA and hippocampus, it is possible that GRP and its receptor antagonist could be exerting their effects on contextual freezing via hippocampal pathways. The present findings do not speak to this directly, but are in line with this view given the differential effects observed between contextual versus cued conditioning.

The findings of the present investigation ought to be distinguished from those of other studies that have assessed GRP involvement in fear-related processes. In the present study, RC-3095 was administered prior to testing (in what was essentially an extinction paradigm in which the diminution of freezing over time was assessed). From this perspective, it is possible that BB₂ receptor blockade reduces contextually-related fear/anxiety or enhances extinction learning involving a contextually-dependent anxiety response. Other investigators [249] demonstrated that microinjection of RC-3095 in the hippocampus *after* extinction training, impaired later extinction learning, suggesting the GRP manipulations impair the memory of extinction learning. Consistent with the view that GRP antagonism may disrupt fear-related memory, it was reported that localized central injection of RC-3095 (into the BLA or the hippocampus) immediately after inhibitory avoidance training, impaired aversive memory [96, 391, 393]. Conversely, systemic and i.c.v. administration of GRP agonists improved memory retention in step-down inhibitory avoidance tasks [126, 376, 417, 496]. As Roesler et al., [96, 390, 391, 393] typically administered drugs immediately after training, they conclude that RC-3095 disrupts memory consolidation.

There is the possibility that drug infusions into the amygdala might disrupt the expression of freezing behavior itself rather than effects on fear memory [475, 476]. However, this seems unlikely given that rats demonstrated normal freezing behavior in the cued conditioning task. Further, it seems unlikely that the drug disrupted freezing by increasing locomotion or by state-dependent generalization decrements given that only context fear was affected by the drug infusion.

Although the mechanisms underlying the observed peptidergic action(s) remain largely speculative, it is possible that freezing in the present study was inhibited through actions on GABAergic (gamma-aminobutyric acid) neurons. Indeed, Shumyatsky [436] demonstrated that GRP receptors are located on GABAergic interneurons within the BLA, and that excitation of these interneurons increases inhibition of principle neurons. Similarly, GRP was found to depolarize hippocampal interneurons *in vitro*, resulting in an increase in frequency and amplitude of spontaneous inhibitory post-synaptic currents [237], while Andrews et al., [19] showed that GRP increases extracellular levels of GABA in the hippocampus through stimulation of BB₂ receptors. Moreover, Dantas et al., [96] found that at high doses (10 µg/side), the post-training administration of RC-3095 to the dorsal hippocampus enhanced 24 h memory for inhibitory avoidance training and this enhancement was prevented by a pretraining infusion of an otherwise inert dose of the GABA_A receptor agonist muscimol.

A second possible mechanism for the observed results involves interactions with another related peptidergic system, namely CRH. The BLA contains ample CRH receptors [76, 79, 474], and projects to the central nucleus of the amygdala, an area critical for the expression of fear-related behaviors [99, 234]. Central administration of GRP elicits and/or potentiates the activation of HPA axis hormone secretion [135, 160, 221, 331], including corticosterone [135].

This effect appears to be mediated by CRH and is blocked by pretreatment with the CRH receptor antagonist α -helical CRH [135]. Moreover, central bombesin administration elicits the release of CRH from the median eminence [211]. Together these findings support the view that the action of GRP on stress-related (or in this instance fear-related) processes may be mediated in part by increasing CRH activity.

The findings of the present investigation, and those suggesting GRP involvement in memory processes are not necessarily at odds with one another. It is certainly possible that BB-related peptides have multiple effects involving different neural circuits that could potentially subserve fear and anxiety states, particularly in the memory formation/consolidation of fear-conditioning and extinction-related processes. In addition, we previously suggested that activation of the GRP system may serve to draw attention to biologically significant cues or events, such as those posing a threat to survival [301]. Future studies might seek to more extensively delineate the site-specific drug or peptide effects by looking at other nuclei related to fear and/or anxiety states, and the interaction of this peptidergic system with other neurotransmitter systems.

Preface to Chapter 4

The previous three chapters have established that GRP is indeed involved in conditioned fear, however, little is known about the brain's response to this type of aversive assault. It has been suggested that GRP may serve to draw attention to biologically significant cues or events, such as those posing a threat to survival [212]. Furthermore, the view was expressed that GRP may act through other systems to provide feedback and feedforward inhibitory control of the processing of conditioned stimuli, thus providing regulatory control between excitatory and inhibitory circuits within amygdala nuclei [436]. Given these considerations, the present study sought to explore the endogenous release profile of GRP in response to fear conditioning in hopes to subsequently elucidate the neuroanatomical and functional mechanisms underlying this GRP/fear relationship. To this end, Chapter IV sought to determine if GRP is released at the mPFC and amygdala in response to fear-provoking stimuli and further, what effects the recall of fear conditioning is imparting on the release of CRH.

Chapter 4

***In Vivo* Levels of Corticotropin-Releasing Hormone and Gastrin-Releasing Peptide at the Basolateral Amygdala and Medial Prefrontal Cortex in Response to Conditioned Fear in the Rat**

Abstract

Given the modulatory effect of exogenously administered corticotropin-releasing hormone (CRH) and gastrin-releasing peptide (GRP) on conditioned fear, the present study sought to measure the fear-induced endogenous release of CRH and GRP at the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA) using *in vivo* microdialysis. Rats were divided into 2 training conditions; tone only (cue), or tone paired with shock. The day after conditioning, animals were tested for fear by scoring freezing behavior in response to the tone alone in cages different from the cages they were previously conditioned in. Freezing was scored for 10 min. Dialysates were collected in 20 min intervals for 2h prior to testing to establish baseline values and were continued uninterrupted for 3h post-testing. Analyses of dialysates revealed that both CRH and GRP release at the BLA increased over time and that peptide release was significantly higher in animals that had previously received shock relative to rats that had not previously been stressed. Further, the release of CRH and GRP was significantly correlated with freezing behavior such that levels of freezing (an indication of fear in the rat), appeared to be related to the observed release in peptides, depending on whether animals were shocked or not. These effects were not observed in the mPFC. It appears that at the BLA, the release of CRH and GRP are related to fear. These peptides may be a marker for fear and/or they may be involved in determining the emotional salience of an aversive event.

Introduction

The amygdala and the prefrontal cortex have both been implicated in the development and maintenance of conditioned fear. Using classical conditioning models, where a neutral conditioned stimulus (CS), such as a tone is paired with an aversive or unconditioned stimulus (US), such as a shock, the specific contributions of different cortical and amygdala nuclei to the induction of fear and anxiety has become clearer. In this regard, it has been suggested that the basolateral amygdala (BLA), which comprises the lateral, basolateral and basomedial nuclei, is a key site where the interface of CS-US associations are formed [68, 93, 232, 265]. Information from the BLA is relayed to the central nucleus of the amygdala (CeA) where it regulates the expression of conditioned fear responses [138, 178, 199, 206, 353, 472]. The medial prefrontal cortex (mPFC), which encompasses the cingulate, prelimbic and infralimbic cortices, shares reciprocal connections with both the BLA and CeA and is recruited during the extinction of conditioned fear [304, 305, 316, 317, 370]. Indeed, it is believed that the mPFC gates or inhibits amygdalar activity through the lateral amygdala and/or through intercalated cells located between the BLA and CeA [304, 305].

Increasing evidence has implicated amygdalar corticotropin-releasing factor (CRH) as subserving, in part, behavioral responses indicative of stress and/or anxiety [114, 204, 455] and/or fear [191, 238]. For example, microinfusion of either NBI27914 or α -helical CRH₉₋₄₁, both selective CRH₁ antagonists, into the CeA blocked footshock-induced freezing [25, 455]. Similar results were observed in response to intra-BLA infusions of the CRH₁ antagonist DMP696 [191], but a corresponding role for CRH receptors in the CeA was not observed in response to this specific antagonist. Using an inhibitory avoidance task, Liang and Lee [244] found that post-training administration of CRH to the amygdala led to enhanced task retention.

In contrast, microinjections of α -helical CRH₉₋₄₁, into either the BLA or CeA immediately after training in the inhibitory avoidance task, produced deficits in retention in the BLA treated animals, but not in rats that had the antagonist injected into the CeA [398]. Further, microinjections of the CRH₁ antagonist, antalarmin, into the BLA of mice immediately preceding social defeat resulted in the reduction of defensive behaviors to a non-aggressive intruder upon testing the next day [387].

Like CRH, gastrin-releasing peptide (GRP), the mammalian equivalent of the tetradecapeptide, bombesin (BB), has also been associated with stress and/or fear-related pathology. Amygdaloid and cortical sites appear particularly relevant in this respect. The GRP gene is highly expressed in the lateral nucleus of the amygdala (LA) and enhanced memory for fear-motivated behaviors was evident in mice lacking the GRP receptor (BB₂-receptor) [436]. Moreover, acute stressor exposure elicited the release of BB-like peptides from the CeA and induced a significant elevation of GRP levels at the cingulate cortex/mPFC [2, 297, 301]. As predicted, administration of GRP (intraventricularly [i.c.v.] or localized to specific amygdaloid or cortical sites), attenuated the expression of fear (as seen by reduced levels of freezing) in response to contextual cues (i.e., in a context in which animals had previously been exposed to shock), and to a tone that had previously been paired with a shock [30, 321, 322]. Conversely, we observed that i.c.v. administration of RC-3095, a BB₂ receptor antagonist, increased freezing in this paradigm [293]. Localized microinjection of the BB₂ antagonist, however, elicited a more complicated pattern of behavioral responses. Specifically, administration of the BB₂ receptor antagonist BW2258U89 into the infralimbic cortex attenuated the freezing response [322], whereas the effects of BW2258U89 at the CeA were biphasic in that high doses reduced freezing and low doses increased freezing [322]. Administration of RC-3095 to the BLA significantly

reduced the freezing response, but this outcome was observed only to contextual cues and not to the conditioned tone.

Despite the complex relations between GRP alterations and fear responses, both the CRH and BB/GRP systems have been proposed as novel therapeutic targets for anxiety disorders [185]. In an attempt to clarify the role of CRH and GRP peptidergic systems in fear responses, we examined the effect of conditioned fear on *in vivo* CRH and GRP release at the medial prefrontal cortex (mPFC) and BLA while simultaneously assessing freezing behavior associated with conditioned fear cues. .

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec) were maintained on a 12h light/dark cycle (lights on at 07:00 h) in a climate-controlled environment (23°C, relative humidity 60%). Weighing approximately 275-300g upon arrival, animals had free access to food (Purina Lab Chow) and water throughout the experiment and were doubly housed in standard plastic cages (45 x 25 x 20 cm) until surgery. All experiments were conducted in accordance with the Canadian Council of Animal Care, and were approved by the animal care committee of the University of Ottawa.

Surgery

Animals were anesthetized with Halothane at 2.5% and were stereotaxically implanted with intracerebral guide cannula (MD-2250; Bioanalytical Systems Inc.) at the following coordinates; mPFC: A/P +2.8 mm, L + 0.6 mm, D/V -3.7 mm (Figure 1a); BLA: A/P -3.1 mm, L + 5.3 mm, D/V -7.2 mm (Figure 1b), based on [339]. For pain control, rats received oral

acetaminophen (Tylenol: 100-200 mg/kg) for 3 days prior to and 3 days following surgery. In addition, they received rectal Tylenol (50 mg/kg) twice daily beginning on the day of surgery and for 5 days thereafter. To maintain cannula patency, removable stylets were inserted into the guide cannulae. Animals were given a 7-day recovery period before testing began.

Experimental Design and Procedures

Behavioral Training Procedure

After surgical recovery (a minimum of 7 d), animals completed one day of training followed by a day of testing 24 h later. Rats were divided into 2 conditions; one group received 6 trials of a 20s (75 kHz) tone paired with a 1s, 1mA footshock (given during the final second of the tone), whereas the second group received the tone but were not shocked. The conditioning chamber (Coulbourn Instruments) measured 31 x 25 x 30 cm. The front and back walls were made of clear Plexiglas and two side walls made of stainless steel panels. The floor was composed of 16 stainless steel rods (2 mm diameter, 3 cm apart), which were connected to a Coulbourn Instruments shock generator (model H13-16) that delivered constant current. A sonalert tone generator (75 kHz, low setting – Coulbourn Instruments) was situated in the top rear panel and provided the conditioning auditory cue.

In vivo Microdialysis

After the training session (which lasted approximately 10 min), animals were transferred to individual microdialysis chambers (25 x 35 x 34 cm) that served as home cages, and allowed to acclimate overnight before testing. The chambers had a hinged front door and a smooth floor covered with bedding. The cage top was modified to allow the attachment of a liquid swivel assembly, which was subsequently tethered to the animal's headcap, permitting animals to move

freely. The tether assembly was removable and allowed investigators to transfer the animal to the testing cages with minimal disruption and a continual sample collection before, during and after the transfer of the rat. Animals were allowed *ad lib* access to food (Purina Lab Chow) and water during the course of the experiment.

The experiment was conducted 24 h after training, during the light phase of the diurnal cycle (0800 – 1700 hr). Rats were briefly (approximately 3 min) anesthetized with isoflurane and stylets were replaced with microdialysis probes (MD-2200 - Bioanalytical Systems Inc). The probe had a 2 mm membrane with a 30 KD cutoff and protruded 2 mm from the bottom of the guide cannula. Dialysates were collected in a medium of Bovine Serum Albumin (0.2%) and Krebs ringer buffered saline solution (KRB) consisting of (in mM); 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻ ascorbate, pH 7.4 [310]. After a 1 h stabilization period, *in vivo* dialysates were collected from animals (every 20 min) in their home cages (from either the right side mPFC or right side BLA) at a flow rate of 2 μ L/min (Model 22, Harvard Apparatus pump). The right side was targeted as there is considerable evidence linking the right hemisphere (as opposed to the left) more directly with stress-regulatory systems [6, 453]. Samples were immediately transferred to dry ice and subsequently stored at -80°C until analysis.

Behavioral Testing Procedure

After a baseline collection period of 2 h, animals were transferred to a novel environment similar to the training chamber, however, these chambers were modified in that the walls were covered in black laminate, while the floor was smooth and covered with bedding chips. Animals were allowed a 1 min exploration period and were subsequently presented with the conditioned tone. A total of 10 tones (each 20 s in duration) were presented at 1 min intervals. Freezing behavior was timed by trained experimenters using the software program ODlog (Macropod

Software). Immediately following this testing period, animals were returned to their home cages. The collection of dialysates was not interrupted during this testing period and continued for a further 3 h.

Histologies

Following the completion of behavioral testing and dialysate collection, animals were euthanized via an overdose of euthansol (65mg/ml). Brains were removed and frozen using dry ice and a brain block prior to sectioning, and were stained using Thionin. Cannula placements for each animal were verified histologically and only animals in which probes were correctly positioned were included in the data analysis (see Figures 1A and B).

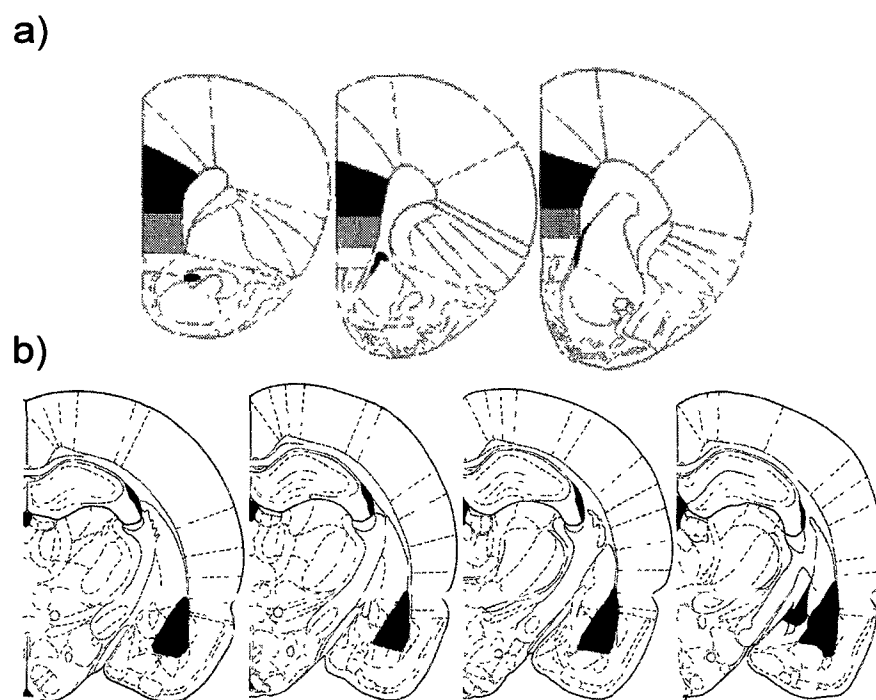


Figure 1. Diagram of the acceptable planes (taken from [339]) for placements, showing a) the prelimbic cortex shaded in black and the infralimbic cortex shaded in grey of the mPFC (A/P +2.8 mm, L + 0.6 mm, D/V -3.7 mm) and b) the BLA shaded black (A/P -3.1 mm, L + 5.3 mm, D/V -7.2 mm).

Radioimmunoassay

The determination of CRH and GRP was performed by radioimmunoassay as reported previously [301]. Briefly, protein A/G (Calbiochem, La Jolla, CA)-coated Immulon-4 wells (Dynatech, Chantilly, VA) were incubated with CRH (rC70, kindly provided by W. Vale, Salk Institute, LaJolla, CA) or GRP antibody (α -bombesin kindly provided by T. Moody, NCI, Rockville, MD) for 2 h at room temperature. The specific anti-CRH serum recognizes CRH₁₋₄₁ and cross-reacts poorly with other related peptides including urotensin 1 and urocortin [331]. The GRP antibody recognizes the C-terminal of bombesin and cross reacts strongly with GRP₁₋₂₇ (110%), bombesin (100%) and GRP₁₈₋₂₇ (82%), but only weakly with substance P or other mammalian bombesin-like peptides (neuromedin B-10 and neuromedin B-32; <0.1% - [313]). The final dilution volume was 1:100 000.

After incubation, the wells were washed three times with wash buffer. Samples, standards (reconstituted in KRB solution ranging from 0.05 to 250 fmol/well for CRH and 0.25 to 512 fmol for GRP), or blanks (to determine non-specific binding) were incubated for 24 h at 4°C. Next, 25ul of assay buffer containing 5000-6000 cpm of [¹²⁵I-Tyr⁰]rCRH (GE Healthcare, Canada) or [¹²⁵I-Tyr⁴]BB (iodinated in-house, as per [411]) was added to each well and incubated for an additional 24 h period at 4°C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted using a gamma counter (Canberra Packard, Cobra II Auto-gamma, model D5002). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 and 2 fmol/well for CRH and BB (GRP), respectively.

Statistical Analysis

Data (peptide and behavior) were analyzed separately for the mPFC and BLA. Basal differences in CRH and GRP as a function of previous shock exposure were determined through a mixed-measures analysis of variance (ANOVA) wherein Condition (tone paired with shock vs. tone alone) was considered the between-group variable and Time as the repeated variable. The levels of the assayed substances were expressed as the raw concentrations found in the microdialysates (mean $\pm$ SEM).

To analyze the relationship between behavioral (freezing) data and peptide concentrations, raw freezing scores were transformed into a total percentage of time spent freezing for the entire 10 min period for each animal. The mean freezing values were then correlated with mean peptide levels for each animal using Pearson's r . In this instance, animals were not distinguished according to their group membership as it is presumed that the level of freezing is indicative of the animal's level of fear. Further, it is not uncommon for animals in the control condition to exhibit fearful behavior, or for animals in the test condition (receiving shock) to not exhibit fear in this type of paradigm. Thus, the sole purpose of this analysis was to determine if a correlation exists between levels of fear (as determined by freezing behavior) and interstitial peptide levels. In cases where a significant correlation was found, this relationship was followed up using analysis of covariance (ANCOVA) to determine whether levels of fear (as determined by freezing) were related to the different peptide effects observed between animals that received shock treatment and those that did not.

From animals included in the statistical analyses, there were occasional missing values attributable to accidental sample loss, assay error, or flow problems; in these instances the mean of the sample prior to, and after, the missing value was inserted.

Results

Effects of previous shock exposure on interstitial levels of CRH at the BLA and mPFC

Immunoreactive CRH (ir-CRH) levels increased over time in both the BLA ($F_{12,108} = 15.97, p < .0001$; Figure 2a) and in the mPFC ($F_{12,132} = 11.78, p < .0001$; Figure 3a). However, a significant difference in ir-CRH levels between animals who received shock ($n = 5$) and those who did not ($n = 6$), was observed only in the BLA ($F_{1,9} = 5.41, p < .05$; Figure 2a).

Relationship between freezing behavior and CRH peptide levels

To determine if a relationship existed between interstitial CRH levels and fear behavior, a Pearson correlation was conducted on mean peptide levels and mean freezing scores. A significant correlation was found between ir-CRH and freezing scores within the BLA ($r = .62, p < .01$; Figure 2b) but not within the mPFC ($r = .09$; Figure 3b), indicating that as fear levels rise (as indicated by freezing), so too do amygdalar levels of CRH. When ANCOVA was run using mean basal ir-CRH peptide levels as the dependent variable, shock treatment as the independent variable and freezing as the covariate, all significant differences previously observed in ir-CRH levels between animals that received shock and those that did not, were lost ($F_{1,8} = .52, p > .05$). This suggests that the level of freezing animals exhibited is related to the observed peptide release in shocked vs. non-shocked animals.

BLA

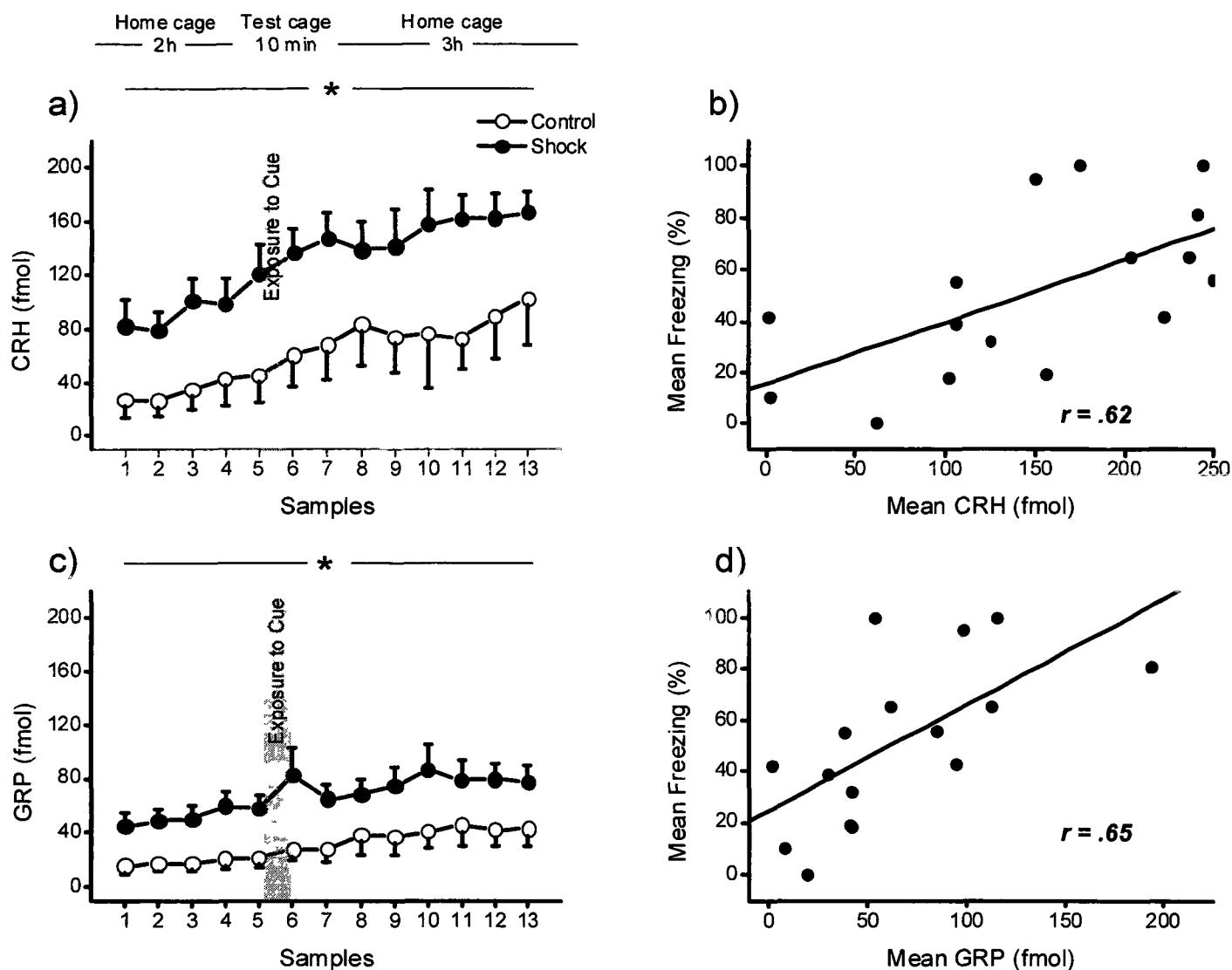


Figure 2. Effect of conditioned fear on peptide release at the BLA and freezing behaviour 24h post-training. Data are means ($\pm$ SEMs) for all images. Panel a) represents the mean CRH release at the BLA in response to previous fear conditioning. Panel b) shows the correlation between mean CRH levels released at the BLA and mean freezing for all animals, a significant correlation was found ($r = .62$; $p < .05$), such that as peptide levels increased, so too did freezing. Panel c) represents the mean GRP release at the BLA in response to previous fear conditioning. Panel d) shows the correlation between mean GRP levels and mean freezing for all animals, again, a significant correlation was found ($r = .65$; $p < .05$).

* Significantly different from control (no shock) group, $p < .05$.

mPFC

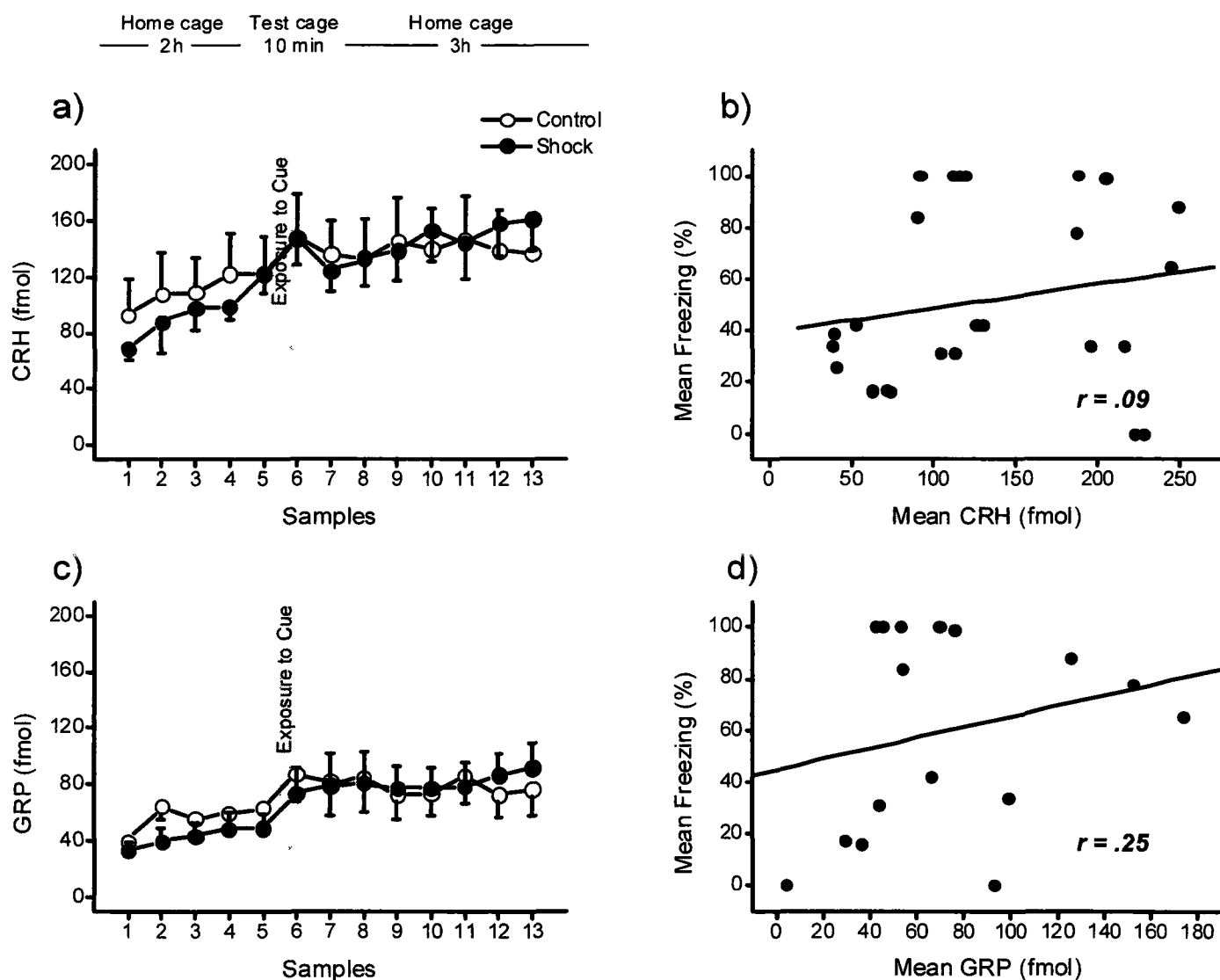


Figure 3. Effect of conditioned fear on release of CRH and GRP at the mPFC. Data are means ($\pm$ SEMs) for all images. Panel a) represents the mean CRH release at the mPFC in response to previous fear conditioning. No significant differences were observed between groups. Panel b) shows the correlation between mean CRH levels released at the mPFC and mean freezing levels for all animals, no significant correlation was found. Panel c) represents the mean GRP release at the mPFC in response to previous fear conditioning. No significant differences were observed between groups. Panel d) shows the correlation between mean GRP levels and mean freezing for all animals, no significant correlation was found.

Effects of previous shock exposure on interstitial levels of GRP at the BLA and mPFC

Similar to that observed with CRH, immunoreactive GRP (ir-GRP) raw peptide levels increased over time both in the BLA ($F_{12,156} = 6.37, p < .0001$; Figure 2c) and in the mPFC ($F_{12,168} = 5.82, p < .0001$; Figure 3c). As in the case of CRH, differences between animals who received shock ($n = 8$) and those who did not ($n = 8$) reached significance in the BLA, but were again absent in the mPFC (Figure 3c). Notably, animals that received shock had significantly higher mean peptide concentrations in the BLA ($F_{1,13} = 6.38, p < .05$; Figure 2c), than control animals, albeit a significant interaction was absent.

Relationship between freezing behavior and GRP peptide levels

Interestingly, as in the case of CRH, in the BLA (Figure 2d) but not the mPFC ($r = .25$; Figure 3d), the overall increase in ir-GRP was significantly correlated with freezing behavior ($r = .65, p < .01$). When ANCOVA was run using mean basal ir-GRP peptide levels as the dependent variable, shock treatment as the independent variable and freezing as the covariate, again, all significant difference were lost ($F_{1,12} = .05, p > .05$). This, again, suggests that freezing is related to the observed peptide release in shocked vs. non-shocked animals.

Discussion

It has been reported, based on studies using microdialysis, push-pull perfusion and post-mortem tissue, that CRH and GRP release at the CeA were increased in response to both a restraint stressor and to a “naturalistic” stressor comprising predator exposure [2, 210, 297, 300, 301, 350]. Nevertheless, it has been suggested that the involvement of amygdala CRH in anxiety responses may vary as a function of the stressor to which animals had been exposed [298]. Specifically, it was suggested that the primary aversive stimulus and the cues associated with this

stressor may engage different neural circuits [298]. Thus, rather than evaluating responses to primary stressors, the objective of the current study was to assess the effects of an explicit cue that had previously been paired with footshock on the endogenous release of CRH and GRP at both the BLA and mPFC. Analyses of dialysates collected *in vivo* from the BLA revealed that interstitial levels of both CRH and GRP were significantly elevated in response to a conditioned fear stimulus relative to non-shocked (exposure to tone alone) controls. Moreover, the increase of CRH and GRP were significantly correlated with freezing behavior and it appeared that the levels of freezing observed in the animals could be related to the variability in release of these peptides, depending on whether the animals were shocked or not.

These data are commensurate with previous reports demonstrating elevated CRH and GRP in response to stressor exposure [2, 295, 297, 300, 301, 350], and while other studies have shown release of CRH and/or GRP immediately following stressor exposure, the current study showed a persistent increase of these peptides (as measured 24 hours after acute stressor exposure). It is possible that the protracted elevated peptide levels observed reflect a sustained response to the previous stressor experience. Indeed, the stressor applied earlier may have resulted in the sensitization of neurochemical processes such that later insults (including those associated with probe insertion or with being taken to the test area), was sufficient to result in greater neurochemical changes. Such effects have been reported with respect to forebrain monoaminergic changes as well as CRH variations at the CeA [20, 254]. In addition, we have previously shown a protracted increase of GRP at the anterior pituitary 5 days after exposure to chronic repeated restraint, but persistent elevations of CRH were not observed [295].

It is noteworthy that in the present study, an immediate increase in CRH and/or GRP release following presentation of the cue was not observed in rats that had previously received

the cue-shock pairings. Although this finding was somewhat surprising in light of previous reports showing a stressor-elicited increase in peptidergic release [91, 297, 300, 301, 302, 350], there are several possible explanations which may help to reconcile this discrepant finding. For example, in the current study, the stressor (footshock) was administered 24 h previously, whereas in other studies [91, 297, 300, 301, 302, 350] the stressor was administered during the sampling period. It is possible that the recall of the conditioned stressor as a consequence of cue exposure, does not elicit an immediate rise in CRH and/or GRP. It is equally possible that the recall of a stressful or fear-eliciting event is not powerful enough to elicit a spike in peptide release in animals already exhibiting sustained peptide elevations. Supporting this contention, we previously showed that the peptidergic release elicited by an appetitive event (palatable food intake) was considerably greater in control animals (with lower basal peptide levels) as compared to rats that had previously been exposed to repeated restraint (with elevated basal peptide levels) [295]. A third explanation is that these peptides (at the level of the BLA) do not respond to cue-elicited conditioned fear, but more robustly respond to contextual components of conditioned fear. It is noteworthy that we previously showed that exogenous administration of GRP to the BLA led to a reduction in freezing to contextual-based conditioning, but had no effect on cue-elicited freezing [321]. Finally, it also ought to be considered that the relatively long sampling periods (20 min) did not provide the resolution necessary to detect modest cue-elicited peptidergic changes. Indeed, a 10 min sampling period may have been more effective [297, 302].

Interestingly, a strong correlation was revealed between levels of fear (freezing), and mean CRH and GRP levels in the BLA. These peptides are not likely involved with freezing behavior directly, as peptide levels were elevated and remained elevated for the duration of the

experiment, even while animals appeared calm, resting and sleeping. Importantly however, some animals that had not previously received shock exhibited strong freezing responses after cue presentation, whereas other animals that had been shocked did not freeze in the subsequent test. Thus, the correlation between CRH/GRP levels and freezing might be an indication of the animals' level of fear/distress (irrespective of the previous days' experiences) and underscores innate individual differences in fear/distress between animals. Thus, higher levels of CRH and GRP in the BLA may be a marker for elevated fear/distress irrespective of the animals' prior experiment-related experiences. Consistent with this view, Lehner et al., [240] recently showed significantly increased levels of CRH-related immunostaining at the BLA in rats that exhibited high levels of freezing (high responders) during a contextual fear test compared to low responding rats. It is unclear however, whether peptide levels increase as an adaptive response to fear/distress, or whether the increase of peptide levels results in the phenomenological experience of fear/distress.

Analyses of dialysates collected *in vivo* from the mPFC revealed that release of both CRH and GRP also increased across time, just as they did in the BLA. However, this increase was not significantly higher in animals that had previously received shock, nor was the increase in GRP significantly correlated with freezing behavior. This stands in contrast to a previous report showing that animals exposed to a cat showed elevated levels of GRP in micropunched tissue from the cingulate cortex [2]. Aside from the fact that the present study sampled primarily prelimbic and infralimbic cortices rather than cingulate tissue, *in vivo* microdialysis allows for determination of dynamic peptide changes as opposed to that captured at a single time point when postmortem tissue is assayed. The choice of examining the infralimbic and prelimbic cortices in the present study (as opposed to the cingulate cortex) was based on the abundant

literature linking the infralimbic (and to a smaller extent prelimbic) cortex to extinction processes [304, 305].

It is difficult to determine why both CRH and GRP demonstrated protracted elevations 24h after stressor exposure. Given that the release of CRH increases in response to exogenously administered BB/GRP [134, 211], as do plasma levels of ACTH, corticosterone, norepinephrine and epinephrine [211], it is possible that elevated levels of GRP and/or CRH serve to modulate the HPA axis in an effort to protect the organism. This could explain why endogenously, levels of GRP and CRH are high when the animal is in a higher state of emotional arousal (as witnessed by the positive correlation with freezing). In general, CRH receptor antagonists and receptor knockdown strategies attenuate fear and anxiety responses (for a review see [457]), whereas administration of CRH exacerbates these emotions [54, 115]. In contrast, GRP and its receptor antagonists elicit the opposite effects; exogenous administration of GRP consistently reduced conditioned freezing, while administration of GRP receptor antagonists result in sometimes increased freezing, no effect or decreased freezing, depending on the locus of administration [30, 293, 321, 322]. In effect, it may be that CRH and GRP act in a serial or collaborative manner.

It is also possible elevated levels of GRP serve to consolidate the memory of the stressor experience. For example, administration of GRP and/or its amphibian counterpart BB, enhances memory formation in an inhibitory avoidance task [126, 376, 417], whereas RC-3095, a GRP receptor antagonist, blocks 24h memory retention [96, 390, 391, 393]. This hypothesis, however, seems unlikely as the memory impairing effects of RC-3095 are observed only if drugs are administered immediately after training. If drugs are given 2h after training, the memory impairing effect is not observed, suggesting that GRP elicits its memory consolidation effects within a 2h time window of training [393]. In the present study, protracted elevations of peptide

were observed 24h after the training session, beyond the time window when memory consolidation would have occurred.

The present study relied exclusively on peptide release, and it remains to be determined whether the stressor procedures influence mRNA expression of CRH and GRP, as well as that of their receptors. This perspective is reinforced by the finding that the initial stressor experience had sustained effects (either as a carry-over of the initial treatment or a reflection of a sensitized response) on peptide release. In this regard, the sustained peptide release also precluded the determination of an unadulterated index of CRH and GRP release in response to cues associated with the stressor. Nevertheless, the present findings are consistent with the view that GRP at the BLA, like CRH, may contribute to fear/anxiety responses associated with aversive events.

Preface to Chapter 5

The final experiment completing this dissertation was designed to determine whether a functional GRP peptidergic pathway exists between the amygdala and mPFC. To this point, we have demonstrated behavioral modifications to conditioned fear as a consequence of exogenous administration of BLPs to the aforementioned brain areas, as well as reported fluctuations in endogenous levels of both GRP and CRH at the BLA in response to the recall of fear conditioning. There is ample evidence suggesting that the mPFC and amygdala communicate with each other during fear conditioning processes, and in fact, it is thought that the mPFC perhaps ‘gates’ amygdalar output in this process. It is yet unknown, however, whether the nuclei in the mPFC communicate with the amygdala using the GRP peptidergic system as a communication link. For this reason, the IL cortex and BLA were chosen as the loci to investigate further in this study, and GRP along with its receptor antagonist (RC-3095 in this instance), were microinjected into the IL cortex while dialysates were collected downstream at the BLA for measurement of GRP and CRH. Thus, this study was designed as an attempt to demonstrate a GRPergic and/or GRP/CRH functional link between the IL and BLA, which might in turn, be involved in the behavioral and neurochemical alterations observed to date in response to fear-related behaviors.

Chapter 5

Effects of Prefrontal Administration of Gastrin-Releasing Peptide (GRP) or its Receptor Analogue RC-3095 on Amygdalar GRP Release

Abstract

The basolateral amygdala (BLA) is a key structure involved in the formation of associations developed during the acquisition of the conditioned fear response, whereas the infralimbic (IL) cortex appears to be important for extinction-related learning. Gastrin-releasing peptide (GRP) or its receptor antagonists can modulate the conditioned fear response when exogenously administered at these sites, and increased release of this peptide at the BLA occurs in response to conditioned fear recall. The present study sought to determine whether a functional pathway utilizing GRP exists between the IL cortex and BLA, and if so, would this pathway also influence amygdalar CRH, owing to reports suggesting an interrelationship between these peptides. It was observed that administration of either GRP (300ng/.05µl) or RC-3095 (a GRP receptor antagonist; 500ng/.05µl dose) into the IL cortex, increased interstitial levels of GRP relative to animals that received vehicle alone (controls) or animals that received both GRP and RC-3095 concomitantly in the same vehicle (300ng/.05µl and 500ng/.05µl respectively). Administration of GRP or its receptor antagonist to the IL cortex did not affect CRH release. It is suggested that these peptides may act in a serial or collaborative manner but at the IL-BLA pathway reported here, they do not appear to functionally interact at the doses used in this study.

Introduction

Whereas the amygdala appears to be involved in the development of a conditioned fear response [100, 272], there is evidence that the infralimbic cortex (IL) is involved in the extinction of conditioned fear [304, 305]. These brain regions seem to share functional roles and reciprocal neural connections [406], wherein the IL cortex inhibits amygdaloid processing [304, 305, 338]. It has been suggested that input from glutamatergic neurons to the lateral/basolateral amygdala (BLA), synapse on gamma-aminobutyric acid (GABA) interneurons located both within the BLA and in intercalated cells located between the BLA and central nucleus (CeA) of the amygdala [338, 370].

The discovery of a preponderance of gastrin-releasing peptide (GRP) as well as GRP receptors at the lateral nucleus of the amygdala (LA), specifically in the vicinity of GABAergic interneurons [436], suggests a molecular mechanism for the role of this peptidergic system in conditioned fear [436]. In addition, receptors for GRP are also found in regions that constitute the afferent pathways of auditory conditioned fear which project to the LA [436]. We observed that GRP injected into either the IL cortex, the CeA or the BLA, reduced freezing in a fear conditioned paradigm, whereas GRP receptor antagonist(s) had mixed results; reducing freezing via injections into both the IL and BLA, and eliciting dose-dependent effects from injections into the CeA [321, 322]. Moreover, in animals that had been conditioned to fear a tone previously paired with a shock, a protracted and sustained elevation of GRP release from the BLA was evident upon testing 24 h after the conditioning session [320].

Interestingly, GRP appears to demonstrate a similar release pattern to corticotrophin-releasing hormone (CRH), a peptide involved in the behavioral and neuroendocrine response to stress [114]. Indeed, in response to a tone previously paired with a shock, protracted and

sustained elevations of both CRH and GRP were observed in dialysates collected from the BLA [320]. Moreover, events such as restraint stress, consumption of a palatable snack or even anticipation of snack presentation, elicit similar release patterns for GRP and CRH [300, 301]. As well, anatomical overlap exists with respect to these peptides and a functional linkage appears between these two peptidergic systems. In this regard, several of the endocrine and behavioral effects of exogenous bombesin (BB – the amphibian counterpart to GRP) administration (e.g., hypothalamic-pituitary hormone activation and anorectic actions) are blocked by pretreatment with CRH [212, 358], whereas central administration of BB significantly reduced endogenous CRH levels at several brain sites including the CeA, while increasing the release of CRH from the median eminence and anterior pituitary [211].

The present study was conducted to elaborate upon the relationship between these two peptidergic systems, as well as to assess whether a functional pathway utilizing GRP exists between the IL cortex and BLA. To this end we assessed whether administration of either GRP or its receptor antagonist, RC-3095 (D-Tpi6, Leu13 psi[CH₂NH]-Leu14 BB⁽⁶⁻¹⁴⁾) into the IL cortex would affect the release of GRP and/or CRH at the BLA.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec) weighing approximately 275-300g upon arrival, were maintained on a 12h light/dark cycle (lights on at 07:00 h) in a climate-controlled environment (23°C, relative humidity 60%). Animals were doubly housed in standard plastic cages (45 x 25 x 20 cm) until surgery and had free access to food (Purina Lab Chow) and water. The experiments were conducted in accordance with the

Canadian Council of Animal Care, and were approved by the animal care committee of the University of Ottawa.

Surgery

Anesthesia was induced with isoflurane in 100% oxygen. Animals were prepared for surgery by shaving the surgical area and applying topical anesthetic. For pain control, rats received oral acetaminophen (Tylenol; 100-200 mg/kg) for 3 days prior to surgery and received rectal Tylenol (50 mg/kg) at the onset of surgery. Saline (5 c.c.) was administered subcutaneously to maintain optimal fluid levels. Animals were subsequently implanted stereotaxically with intracerebral microdialysis probes (MD-2200; Bioanalytical Systems Inc.) in the right-side BLA at the following coordinates: A/P -2.9 mm, L + 5.4 mm, D/V -9.0 mm [339] and with 22 gauge guide cannulae (Plastics One) at the IL: A/P +3.0 mm, L $\pm$ 0.7 mm, D/V -4.7 mm. The right side was targeted as there is evidence linking the right hemisphere (as opposed to the left) in stress-regulatory systems [6, 453]. Guide cannula aimed at the IL were secured with two stainless steel screws and dental acrylic to allow for removal of the stereotaxic arm and subsequent insertion of injectors, while the microdialysis probes remained held in place by the stereotaxic arm during the course of the experimental procedure. Rats were placed on a heated pad to maintain core body temperature at 37.5°C throughout the duration of the experiment. During surgery isoflurane was kept at about 3.0% and during microdialysis sampling isoflurane was maintained at 2.0%.

Drugs and Injections

The GRP receptor antagonist RC-3095 (Sigma-Aldrich) and GRP (Phoenix Pharmaceuticals, Inc.) were dissolved in Krebs ringer buffered saline solution (KRB) consisting

of (in mM; 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻ ascorbate, pH 7.4). The control (vehicle) animals received an equivalent volume of KRB alone. All drug infusions were delivered via two 28-gauge injectors (Plastics One), which protruded 1.5 mm beyond the tip of the guide cannula into the IL. The injectors were connected to 10 μ L Hamilton Syringes with polyethylene tubing. Drugs were simultaneously delivered to both cannulae at a flow rate of 0.5 μ L/min over a 1-min interval. Injectors were left in place for a further 1-min period to allow for drug diffusion. All drug doses were based on efficacy observed in previous studies [293, 321, 322].

Microdialysis

Microdialysis probes (MD-2200) were purchased from Bioanalytical Systems Inc. (BAS Inc.) and contained a 2 mm membrane with a 30 KD cutoff. Dialysates were collected in a medium of Bovine Serum Albumine (0.2%), Bacitracin (0.1%) and KRB (as outlined above). All dialysates were collected using a Harvard Apparatus pump at a flow rate of 2 μ L/min every 20 min. One hour after probe insertion (previously shown to be a sufficient length of time for stabilization [301]), samples were collected in 20 min intervals and were immediately transferred to dry ice and subsequently stored at -80 degree Celsius until analysis.

Two hours after sample collection commenced, animals were injected with either KRB alone (to serve as a control group), GRP (300 ng/0.5 μ L), RC-3095 (500 ng/0.5 μ L) or both GRP and RC-3095 administered simultaneously within the same injection medium (300 ng/0.5 μ L and 500 ng/0.5 μ L respectively). Samples were collected for an additional 3 hours after drug injections.

Histologies

At the completion of dialysate collection, animals were euthanized via an overdose of isoflurane followed by decapitation. Brains were collected and frozen on dry ice prior to sectioning, and were then stained using Thionin to verify injection sites and probe placements. Only animals for which all placements were accurate were included in the data analysis (see Figure 1).

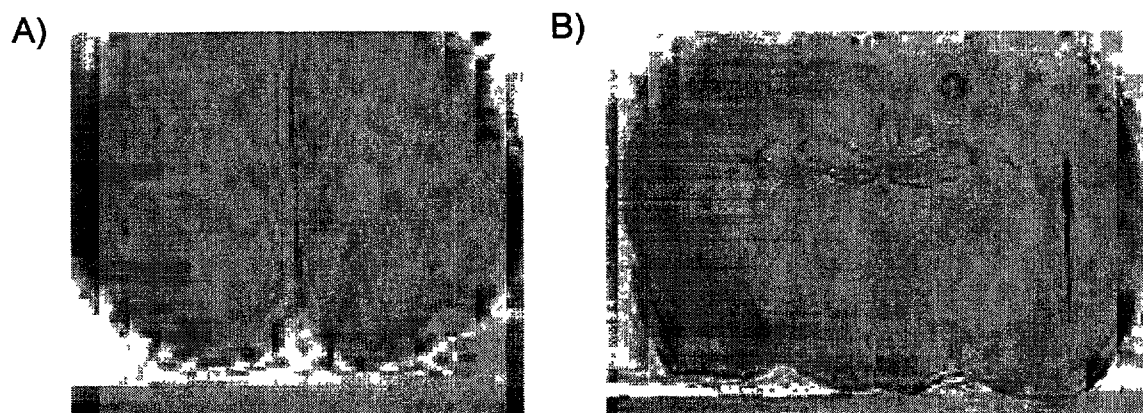


Figure 1. Anatomical localization of A) drug injection sites aimed at the IL cortex and B) microdialysis probes aimed at the right BLA. Placements considered off-site were excluded from the analysis.

Radioimmunoassay

The detection and quantification of immunoreactive CRH (ir-CRH) and immunoreactive GRP (ir-GRP) was performed by high-sensitivity solid-phase radioimmunoassay as reported previously [301]. Briefly, protein A/G (Calbiochem, La Jolla, CA)-coated Immulon-4 wells (Dynatech, Chantilly, VA) were incubated with CRH (rC70, kindly provided by W. Vale, Salk Institute, LaJolla, CA) or GRP antibody (α -bombesin kindly provided by T. Moody, NCI, Rockville, MD) for 2 h at room temperature.

After incubation, the wells were washed three times with wash buffer. Samples, standards (reconstituted in KRB solution ranging from 0.05 to 250 fmol/well for CRH and 0.25 to 512 fmol for GRP), or blanks (to determine non-specific binding) were incubated for 24 h at 4°C. Next, 25 μ l of assay buffer containing 5000-6000 cpm of [125 I-Tyr⁰]rCRH (GE Healthcare, Canada) or [125 I-Tyr⁴]BB (iodinated in-house, as per [411]) was added to each well and incubated for an additional 24 h period at 4°C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted using a gamma counter (Canberra Packard, Cobra II Auto-gamma, model D5002). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 and 2 fmol/well for CRH and BB (GRP), respectively.

The specific anti-CRH serum recognizes CRH₁₋₄₁ and cross-reacts poorly with other related peptides including urotensin 1 and urocortin [331]. The GRP antibody recognizes the C-terminal of bombesin and cross reacts strongly with GRP₁₋₂₇ (110%), bombesin (100%) and GRP₁₈₋₂₇ (82%), but only weakly with substance P or other mammalian bombesin-like peptides (neuromedin B-10 and neuromedin B-32; <0.1% - [313]). The final dilution volume was 1:100 000.

Statistical Analysis

Peptide levels in the five dialysate samples collected prior to drug injection were averaged for each animal and served as a baseline measure (denoted as 100%) of pre-injection interstitial peptide levels. As there is appreciable within group variability in dialysate constitution inherent in microdialysis designs, this process normalizes and reduces within-group variability. All sample values were then expressed as a percentage change from this baseline value and data were assessed using a mixed-measures ANOVA with Treatment group as the between group measure and Sample (across time) as the within group measure. Follow-up comparisons of the main effects or means comprising the simple effects of significant interactions were conducted using Bonferroni t tests to maintain the α at .05.

Results

Figure 2A depicts interstitial levels of ir-GRP (expressed as a percentage of baseline values) at the BLA under basal conditions and following microinjection of either GRP ($n = 8$), the GRP antagonist RC-3095 ($n = 8$), both GRP and RC-3095 combined ($n = 8$), or vehicle ($n = 6$) bilaterally into the IL. Analysis of ir-GRP levels within the right BLA revealed a significant main effect of Time ($F_{9,234} = 13.87, p < 0.0001$) and Treatment group ($F_{3,26} = 10.56, p < 0.0001$), as well as a significant Treatment group x Time interaction ($F_{27,234} = 3.03, p < 0.0001$). The follow up tests of the simple effects of this interaction indicated that at each 20 min sampling period after the drug injection, a significant increase of GRP relative to baseline was observed in both the GRP and RC-3095 treated groups. From the third sample onward, the GRP release in rats treated with GRP exceeded that of control animals. Rats that received the antagonist also exhibited elevated ir-GRP levels, although this outcome was only statistically significant at the fifth sample onward. Interestingly, the elevated ir-GRP elicited by GRP was entirely eliminated

in rats that received administration of GRP + RC-3095. Thus, while either GRP or its antagonist increased GRP, their combination precluded the GRP rise.

In terms of the simple effects of Treatment group, an analysis of both the GRP and RC-3095 conditions revealed that from sample 1 onwards after the injection, the samples were significantly increased from their mean pre-injection baseline value.

In contrast to the GRP changes, as depicted in panel 2B, the interstitial levels of ir-CRH at the BLA under basal conditions and following microinjection did not differ as a function of the treatments rats received. (p 's > .05).

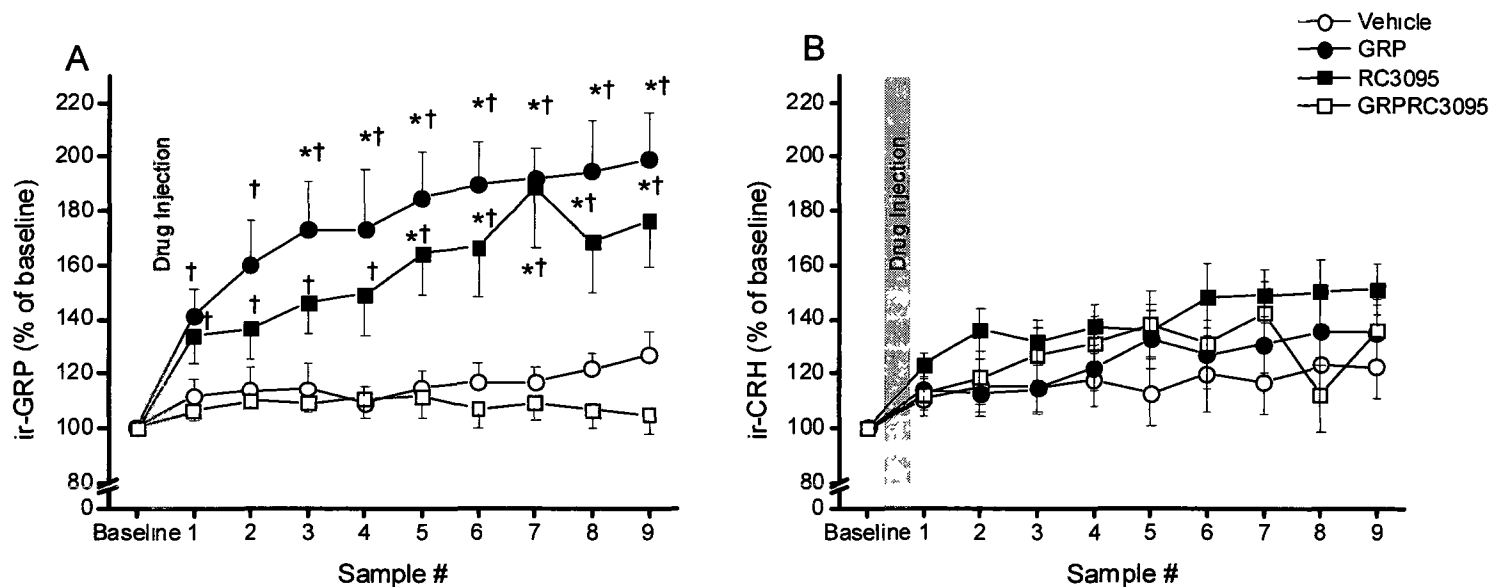


Figure 2. Interstitial levels of A) immunoreactive (ir)-GRP or B) ir-CRH at the BLA following administration of either GRP (300ng/μl), RC-3095 (500ng/μl), both GRP and RC-3095 combined (300ng/μl and 500ng/μl respectively) or vehicle (KRB alone) into the IL cortex. The five baseline values from each of the subjects immediately preceding injections were averaged and defined as 100%. All values were then expressed as a percent of that baseline.

† significantly different from respective baseline sample at $p < 0.001$.

* significantly different from (sample-matched) vehicle control condition at $p < 0.05$.

Discussion

It was previously shown that microinjection of either GRP or its receptor antagonist into either the IL cortex or BLA reduces freezing in a conditioned fear paradigm [321, 322]. Moreover, presentation of fear cues (following training in a conditioned fear paradigm) elicits increased release of interstitial levels of ir-GRP in the BLA relative to animals who were not previously trained using tone-shock pairings [320]. Based on these findings, the present study sought to determine whether a functional pathway utilizing GRP exists between the IL cortex and BLA. Furthermore, as manipulations of GRP can affect CRH levels ordinarily elicited by a stressor [212, 358], the current study also sought to examine the effects of intra-IL administration of GRP and its receptor antagonist on the downstream amygdalar release profile of CRH.

Administration of both GRP and RC-3095 into the IL cortex resulted in a rise in GRP release at the BLA. However, these effects were blocked by the simultaneous administration of GRP and RC-3095. This paradoxical finding is consistent with our previous reports indicating that at both the IL and BLA, the effects of the GRP receptor antagonists were similar to those of GRP itself [321, 322]. Indeed, at both sites, injection of GRP or its antagonist resulted in a significant decrease in freezing elicited by contextual cues in the conditioned emotional response paradigm. Moreover, when RC-3095 and GRP were administered together (RC-3095 injected into the BLA 15 min after GRP, which was administered 30 min prior to testing), the effects were similar to that observed in the vehicle-treated control rats. We previously suggested that these seemingly contradictory results might reflect dose-dependent effects and/or that the antagonist might, in fact, have acted as a partial agonist. These explanations are in keeping with the current results and lend support to the notion that RC-3095 when administered in isolation,

exhibits partial agonist effects, however when given in combination with GRP, it appears to act as a true receptor antagonist.

A similar phenomenon has been reported with several other drugs including buspirone, aripiprazole, buprenorphine and norclozapine. Drugs such as these act as partial agonists, in that they exhibit both agonistic and antagonistic effects. When partial agonists are present along with full agonists, the partial agonist acts as a competitive antagonist, competing for receptor occupancy. This results in a net decrease in receptor activation from what is observed by the full agonist alone [523]. Thus, it is possible that when RC-3095 is given in conjunction with GRP, antagonist effects are observed, however when given alone, agonistic effects are observed.

Our results also suggest that there is indeed a functional pathway between the IL and BLA that utilizes GRP, insofar as manipulation of the upstream target, the IL, with exogenously administered GRP results in downstream, amygdalar, GRP peptidergic effects. This is not surprising given that the IL cortex and amygdala seem to share functional roles and reciprocal neural connections [406]. What is not known however, is whether this pathway from the IL to the BLA is direct (i.e., unisynaptic), or whether this pathway utilizes other systems (such as GABAergic and/or serotonergic systems) en route to the BLA.

Surprisingly, administration of GRP or RC-3095 to the IL had no effect on the downstream release of CRH at the BLA. As previously mentioned, we had shown that i.c.v. administration of BB/GRP elicited the release of CRH at the median eminence/arcuate nucleus and decreased levels of ir-CRH at several regions including the CeA [211]. It is noteworthy however, that at the CeA, the decreased levels of CRH were only observed following administration of a low (0.25 μ g) but not a high dose of BB/GRP (0.5 μ g) indicating that the ability of BB/GRP to influence CRH release at this site may be dose related. This same dose

profile may also hold true for other amygdalar regions, including the BLA, although this contention remains to be verified. Alternatively, of course, it is possible that GRP injection into the IL simply does not influence release of CRH at the BLA, suggesting that the GRP-induced release of CRH may be site-specific.

In summary, although administration of GRP or RC-3095 appears to have no effect on CRH release at the BLA, endogenous GRP release at this site was enhanced by either the administration of GRP or RC-3095, an effect that was blocked when the agonist and antagonist were co-administered. The fact that the release profile was similar following administration of either GRP or its antagonist parallels our earlier behavioral findings [321, 322] and lends support to the view that the GRP receptor antagonists that we utilized (at least at particular doses) has partial agonist effects. As well, the data support the notion that a functional pathway utilizing GRP exists between the IL and BLA, although this does not imply that this pathway utilizes only GRP. Nevertheless, the present findings provide additional evidence for the involvement of GRP in stress and anxiety-related disorders by virtue of its role in brain areas known to be involved in these disorders.

General Discussion

Summary of findings

Mental health is a fundamental dimension of overall health. It can affect physical well-being, influence how we feel, think, perceive, communicate and understand as well as impact our interaction with society in general. Poor mental health or mental illness is associated with significant distress and impaired functioning. It creates economic burdens in the way of lost

productivity and days of work, medical costs, and the need for added social support and is often associated with attached negative stigma [463].

Anxiety disorders account for a considerable portion of mental health burden and a prominent feature consistent across anxiety disorders is uncontrollable fear. Thus, studying the fear system might be necessary to understanding anxiety disorders, particularly post-traumatic stress disorder, which involves a component of learned fear. The central tenet of this dissertation was to more fully understand the neurobiological basis of learned fear by focusing on neuropeptides which may be linked to the underlying processes involved in learned (or conditioned) fear, namely BLP's, and to a lesser extent, CRH.

The first series of experiments sought to address whether BLP's played a significant role in fear and/or anxiety. Central administration of NMB antagonists (BIM 23127 and the mixed BB₁/BB₂ receptor antagonist, PD176252) into the third ventricle appeared to reduce anxiety in an elevated plus maze, whereas NMB itself reduced fear-potentiated startle without affecting basal startle amplitudes, suggesting a role for NMB in both anxiety and fear. GRP in contrast, appeared to have specific effects only in fear-related responses. Central administration of GRP significantly reduced fear-potentiated startle, an effect that was consistent in both Chapter I studies, while RC-3095, the BB₂ antagonist did not increase fear potentiated startle, but at high doses, increased basal startle amplitudes significantly relative to vehicle-treated controls. This suggests a heightened overall fear to the testing environment and procedure, not just to the conditioned cue (in this case a tone). It is noteworthy that this effect was not observed with administration of the BB₂ receptor antagonist [Leu¹³-(CH₂NH)Leu¹⁴]-BN, but the dosage utilized may have precluded this outcome.

In the conditioned emotional response paradigm, freezing was reduced by i.c.v. administration of GRP (relative to vehicle-treated rats) in both the contextual-based test and in the cue-elicited fear test. Conversely, the reduction in freezing usually observed in this paradigm (as observed in the control rats) was blocked by administration of the BB₂ antagonist, RC-3095. These results are in accordance with those seen with BB₂ receptor knockout mice [436], where enhanced freezing was observed in both contextual and cue-elicited CER tasks.

The fact that no effects of GRP administration (or its antagonist) were observed in the EPM, which tends to measure general anxiety more than fear, is supported by other research demonstrating no involvement of the GRP pathway in either the EPM or Morris water maze (a non-emotional memory task) [436, 505] (but see also [96]), suggesting that GRP and its receptors are selectively involved in aversive and learned fear-related memory, and not as much in non-aversive, non-emotionally based memory processes.

Given the findings of Chapter I, that BLP's play a role in anxiety and fear-based responses, the next chapter sought to explore learned fear responses in greater detail and was designed as a pilot project in order to define which areas of the brain might be responsible for the effects observed in Chapter I. These studies, using micro-dissection techniques in conjunction with conditioned fear, highlighted several areas of 'interest', namely the ventromedial hypothalamus, amygdala and prefrontal cortices, which might be involved in the genesis of fear-responses. Given the abundant literature tying the amygdala and prefrontal cortex not only to anxiety disorders in general and to fear in particular (for reviews see [367, 368, 371, 478, 479]), but also to each other in an hypothesized fear circuit [9, 162, 369, 370, 371], the remainder of this dissertation focused primarily on these two cortical areas.

In this regard, the experiments in Chapter III addressed the effects of BLP microinjection into the various nuclei of the amygdala and mPFC. As NMB appeared to be less involved in fear-based responses and current highly competitive antagonists were not readily available for the BB₁ receptor, this chapter focused only on the effects of administration of GRP and BB₂ receptor antagonists. At the central nucleus of the amygdala, several doses of GRP and the BB₂ receptor antagonist BW2258U89 were microinjected, however only the most effective doses were carried over into the PrL and IL experiments; whereas at the BLA, dose response curves were again conducted, this time using GRP and the BB₂ receptor antagonist RC-3095. In keeping with the findings observed with i.c.v. administration, GRP reduced freezing compared to vehicle-treated controls regardless of the site of administration in the contextual fear-based tests. Interestingly, co-administration of both the antagonist and the agonist blocked the effects observed with administration of the agonist alone. This was true for contextual-based tests at both the IL and BLA cortical sites (note that agonist/antagonist co-administration was not done at the PrL or CeA). Effects of GRP administration on freezing in the cued portion of the test were only observed when the drug was microinjected into the IL. The same was not true of the PrL, CeA or BLA cortices.

Effects observed on freezing behavior after microinjection of BB₂ receptor antagonists were mixed. BW2258U89 was completely without effect at the PrL, reduced freezing to contextual cues when administered to the IL and had dose-dependent effects on contextual freezing at the CeA (low dose increased freezing; high dose decreased freezing). While at the BLA, RC-3095 reduced freezing to the context, but was without effect in response to the cue, as observed in the PrL, IL and CeA studies.

To summarize the experiments in Chapter III, it appears that manipulation of the GRPergic pathway may influence the expression of learned fear, however these effects appear to preferentially influence contextual versus cue-dependent emotional responses at the BLA, CeA and PrL. Cued fear-based learning appears to involve the GRP pathway at the IL cortex alone.

It is difficult to determine why site-specific injections of GRP or BB₂ receptor antagonists (with minimal exception) appear to preferentially disrupt contextual-based freezing. It is also not immediately evident why cued-based learned fear is only affected by intra-IL administration of GRP or its receptor antagonist, whereas intra-BLA drug administration had no effect on this type of learned fear. This is particularly perplexing given the abundance of BB₂ receptors found in the LA (where cued fear associations are said to be developed) and scarcity of receptors located within the IL [28, 205, 312, 313, 436, 484, 485]. There are, however, several possible explanations for this phenomenon. Notably, of course is that it is possible that BB₂ receptors located within the BLA are not involved in modulating the behavioral response (freezing) to cue-based conditioned fear.

The strongest evidence suggesting a role for GRP in conditioned fear comes from Shumyatsky et al., [436], who reported an abundance of GRP-encoding genes located within the LA, AB and in regions that convey fearful auditory information to the LA. They also found GRP receptors located on GABAergic interneurons within the LA (which inhibit the firing of principal neurons in the LA), enhanced LTP of these neurons, and a greater and more persistent long-term memory of both contextual and cue-based conditioned fear by using BB₂ receptor knockout mice. These authors presented convincing evidence that a GRPergic signaling network exists within the LA which specifically inhibits memory for learned fear. However, the link between the involvement of BB₂ receptor-expressing GABAergic interneurons within the LA and

enhanced freezing behavior in BB₂ knock-out mice, is based on *in situ* hybridization, immunohistochemical and electrophysiological evidence pointing towards LA receptor involvement. As using receptor knock-out strategies involve the complete elimination of receptors, it is difficult to conclude with certainty where the observed behavioral effects might be originating from. Thus, it is possible that effects on cue-elicited freezing behavior are not, in fact, reliant on GRP receptors in the BLA, and thus, drugs administered to this complex might be ineffective for this type of behavior.

Alternatively, it is possible that the timing of drug administration in our studies precluded an effect on the GRP signaling pathway suggested by Shumyatsky's group. For example, in our studies drug administration was delivered prior to the recall of fearful events to determine if they have anxiolytic and/or anxiogenic properties. Perhaps to induce drug effects on freezing behavior associated with CS-US associations in this nucleus however, the optimal time of drug delivery may have been immediately after training in order to disrupt consolidation of the CS-US associated memory trace. To date, GRP has been shown to affect both fear behaviors (via drug administration prior to behavioral testing) and memory consolidation and reconsolidation (via drug delivery immediately following training and/or following reactivation of the memory trace). In fact, Roesler et al., [391] demonstrated disruption of memory consolidation to an inhibitory avoidance task when RC-3095 was administered to the BLA. Thus, the lack of drug effects on cue-elicited freezing behavior when drug was administered to the BLA in the current study may have been due, in part, to the timing of drug administration.

A third possibility to explain the lack of cue-elicited behavioral effects in the BLA is a methodological explanation. Due to the nature of microinjections, there is almost invariably some damage to the brain tissue at the site of injection and there is always variability in the

location of the injection site. We chose to inject into the ‘BLA complex’ as it offered a larger target for surgical implantation of cannulae and allowed us to include rats with injection sites in all nuclei associated with the BLA complex. The problem with this approach, however, is that BB₂ receptors were reported to be localized in the LA and AB [436], (although BLA receptors were found by others [205]). It is possible that insufficient drug reached these sites which precluded seeing an effect. Further, in animals with more ventral cannula placements, extensive damage might have occurred to the LA, precluding any drug effect on cue-elicited freezing behavior. This argument seems unlikely however, as tone-shock associations remained intact (i.e., animals learned the fearful association between the tone and shock and exhibited robust freezing), suggesting that neurons in this area remained functional.

A fourth explanation involves the nature of context versus cue-elicited testing for recall of the fearful training event. We previously reported that GRP might be involved in determining the salience of an aversive event [301]. This could include the assembly of contextual clues surrounding an aversive event in order to develop and cultivate the emotion-based memory trace for the event. In support of this line of reasoning, several studies on the role of GRP in fear utilized contextual-based tests of fear and/or tests that had a contextual component. For example, in the FPS paradigm utilized in Chapter I, animals that received an i.c.v. injection of GRP did not show significant differences in startle amplitude between their baseline values and when the previously conditioned tone was presented, thus, neither the tone nor the environment elicited significant fear. Conversely, rats that received the antagonist in this study had significantly higher *baseline* values, but not higher tone-potentiated values. Thus, animals receiving the antagonist were already fearful to the contextual cues to the environment, but not more so to the conditioned tone. Further, studies utilizing inhibitory avoidance also contain a

contextual component to the test; animals learn to avoid the area of the testing environment in which they had previously received a shock. Several studies have shown that BB₂ receptor antagonists modulate this form of emotional fear-based learning, suggesting that BB₂ receptors are involved in the formation of the fear-based memory trace [96, 249, 250, 289, 390, 391, 393, 394, 477]. Finally, animals trained in the CER task who received a shock (paired with a tone) demonstrated significantly elevated basal levels of interstitial GRP at the BLA measured 24 h after training (compared with tone exposed non-shocked controls – see Chapter IV). This suggests that the animals were sensitized to further danger, possibly on alert for contextual clues suggestive of further danger. Taken together, these results suggest that when an animal is experiencing a fearful event, GRP is recruited to examine and synthesize the elements of the emotion-based event to build a memory trace. Thus, it is possible that GRP is involved in building the contextual components of the memory trace, and less involved in remembering the association of a tone with the onset of a shock (CS-US association).

This view, however, leads to the question of why an effect was observed to the cue in the IL cortex, an area not known to be involved in forming associations between a tone and a shock. The IL cortex is involved in extinction [371, 373, 374], which essentially comprises learning that a tone no longer predicts a shock. Building on the argument above, it is possible that GRP is recruited for building the *new* memory trace associated with the *extinction* of the tone. Thus, as the tone is being presented to test for the recall of earlier training, it is being presented in the absence of shock. Thus, extinction learning is occurring. If drug administration occurs prior to extinction learning, then it is possible that the drug may be *enhancing* this process, and as such augmenting the process of memory trace formation for extinction. If this were true, then IL effects would be expected to enhance extinction learning, as observed in our studies, but effects

at the BLA would not necessarily be observed as this nucleus might be involved more in the formation of US-CS associations, rather than the *unlearning* of these associations (or learning of a new extinction-based experience), which is the task of the IL. To support this line of reasoning, BLA injections would have to be administered during or immediately after the training session to test for enhanced (and/or blocked) CS-US association formation.

It is equally possible that effects were observed on cued conditioning with intra-IL drug administration via the GRPergic pathway established in Chapter 5. If this perspective regarding LA damage through intra-BLA microinjections is correct, then it is plausible that effects were observed with intra-IL administration as this injection elicited the downstream release of GRP from the BLA that may have been necessary for drug effects to occur. To test this hypothesis, intra-IL injections of GRP/BB₂ receptor antagonists would have to be administered with the simultaneous pharmacological blockade or inhibition of BLA activity. A study of this design might help clarify the contextual versus cued effects observed with GRP site-specific administration and the interaction between the IL and BLA involving this peptide system.

The final explanation involves the possibility that GRP actions may involve distinct pathways within the amygdala (and/or the recruitment of additional or alternative nuclei). Contextual and cue-related conditioning are mediated by both overlapping and distinct amygdaloid circuitry [148]. With respect to contextual-based conditioning, the hippocampus and BLA appear to share reciprocal functional neurocircuitry [348, 399]. For example, lesions to the BLA block hippocampal drug-induced enhancements for memory of inhibitory avoidance [399]. Likewise, BLA lesions disrupt drug-induced impairments of memory in the Morris water maze task (a hippocampal-based task). Thus, it is possible that additional (or alternative) neural circuits may be recruited to consolidate extinction learning in an environment different from the

CS-US training environment, as in the case of cue-elicited conditioning [181]. In summary, it is possible that the effect of the intra-BLA drug infusion on expression of contextual fear was elicited indirectly through the recruitment of hippocampal pathways, but, additional pathways to aid in modulation of cue-elicited fear were not recruited. The present findings do not speak to this directly, but are in line with this view given the differential effects observed between contextual versus cued conditioning.

In summary, the studies presented in Chapter III shed light on the role GRP and its receptors play on conditioned fear when these receptors are manipulated at the various nuclei within the mPFC and the amygdala. Several consistencies were observed between nuclei and with those observed with intraventricular drug administration; however, several inconsistencies were brought to light as well. The most obvious of these inconsistencies is why contextual effects were observed across all nuclei, but cue-elicited effects were observed only in a nucleus not traditionally known to be involved with CS-US associations; and further, the nucleus widely accepted to be involved with CS-US associations and reported to have a fear-based GRP signaling circuit, showed no drug-induced effects on freezing. These discrepancies have been addressed, but there still remains the question of why the antagonist elicited anxiogenic effects (increased freezing) when administered to the third ventricle, but elicited anxiolytic effects (decreased freezing), much like the agonist itself, when administered to the targeted nuclei in Chapter III. These discrepancies will be discussed further along with the issues that arose from the experiment in Chapter V.

To this point, we had determined that the exogenous administration of BLP's impacted fear and anxiety, and we chose to explore GRP and BB₂ receptors exclusively at areas known to be involved in learned fear. In the fourth chapter, we sought to explore the *endogenous*

consequences of fear conditioning on GRP. To this end, the study in Chapter IV was designed to measure GRP released at the mPFC and amygdala in response to previously conditioned fear-provoking stimuli. As our laboratory has designed a method of measuring GRP dialysates in conjunction with CRH in a double assay, and as these two peptides appear to have a relationship in the stress/fear/anxiety response (as outlined in the introduction), the current study also measured the effects of the recall of fear conditioning on the release of CRH at the mPFC and amygdala.

Both CRH and GRP peptide release rose steadily throughout the day of the experiment, but what was interesting was that animals that had been conditioned 24 h earlier with a foot shock in conjunction with a tone, had higher basal levels of peptide from the outset of the day. Moreover, mean peptide release for both GRP and CRH was positively correlated with mean levels of freezing, consistent with the view that level of fear played a significant role in the level of peptide release at the BLA (of course, as these findings were correlational, causal conclusions can not be made based on these data alone). Finally, CRH and GRP increases were observed in the peptide samples from both the mPFC and BLA in response to tone exposure in the samples collected immediately after testing. Thus, it is likely that this increase was in response to exposure to the stressful stimulus (i.e., exposure to the testing environment, tone etc.).

A recent study by Lehner et al., [240], indicated that rats that had the lowest freezing response in a conditioned fear paradigm, also demonstrated higher levels of GABA in the BLA, compared to animals that exhibited high levels of freezing. It is noteworthy that GRP receptors are located on GABAergic interneurons at the lateral/basolateral amygdala [205, 436] and it has been postulated that through binding to GRP receptors on these interneurons, GRP leads to GABA release, thus creating a negative feedback circuit [436].

This scenario thus raises the question of why the highest GRP and CRH peptide levels were observed in animals demonstrating the highest level of fear (freezing). One would think that, in the case of GRP in particular, higher levels of peptide would lead to an increased activation of GABAergic signaling pathways and thus an *enhanced* negative feedback signal eliciting *lower* levels of freezing. This contradiction, however, may be explained by the findings of prolonged (up to 3 h) reduction of extracellular GABA levels in mice that had undergone conditioned fear, suggesting that GABA release is either reduced, or uptake from extracellular spaces is increased during retrieval or expression of conditioned fear [449]. Indeed, it is difficult to interpret these findings given that increased interstitial peptide and/or GABA levels may reflect a) an increased synthesis of the peptide/neurotransmitter and/or b) decreased release of the peptide/neurotransmitter, or c) increased peptide/neurotransmitter release accompanied by even greater rate of its synthesis. Similarly, decreased peptide/neurotransmitter levels may indicate a) decreased synthesis and/or b) increased release or c) increased synthesis accompanied by an even greater rate of release. Moreover, the lack of fluctuations in tissue peptide/transmitter levels may be ambiguous as this could reflect either no change in the rate of synthesis and/or release or rate of release being matched by increased rate of peptide synthesis. Taken together, it is certainly possible that high levels of GRP were observed in high freezing responders in an attempt to activate GABAergic neurons to return to homeostasis. Measurement of concurrent GABA release in the same animals would have shed light on this difficulty, however these data are lacking.

It is noteworthy that Lehner et al., [240] demonstrated a positive correlation between levels of freezing and serotonin (5-HT) immunostaining in the BLA, such that rats that had higher levels of freezing exhibited greater 5-HT immunostaining. Interestingly, these authors

likewise observed higher levels of CRH and c-fos immunocytochemical substrates in the BLA of rats with greater freezing. Given that both CRH and GRP receptors have been found to interact with 5-HT [133, 167], it seems likely that CRH and GRP may elicit their effects on conditioned fear through the recruitment of serotonergic pathways in addition to the GABAergic systems. This possibility is outlined in further detail below.

Given that the GRP-elicited changes of the fear response are likely mediated, in part, by nuclei within the mPFC and the amygdala, the final chapter was designed to determine whether a functional pathway utilizing GRP exists between the IL cortex and the BLA. When GRP or the BB₂ antagonist RC-3095 was administered to the IL cortex, significant elevations in interstitial levels of GRP were observed in the BLA. Further, when the agonist and antagonist were administered concomitantly, no effects of drug administration were observed relative to vehicle controls. Thus, the agonist and antagonist evoked similar peptide release responses from the BLA, but when administered together, the effects of one drug appear to block the effects of the other and a vehicle-like effect was observed. This is somewhat surprising as one would intuitively expect that an agonist and antagonist would have opposing neurochemical drug effects and/or the effects of antagonist administration would block any observed agonist effects (such as those observed when the drugs were administered to the third ventricle in Chapter I).

It is unclear why an antagonist and agonist would elicit similar behavioral and neurochemical effects, but the most parsimonious explanation as outlined in the discussion in Chapter 5, would be that the antagonist was demonstrating partial agonistic effects. This is not surprising given previous studies reporting agonistic effects from various GRP receptor antagonists [73, 96, 109, 220, 405, 489]. Partial agonists can display both agonistic and antagonistic effects by exhibiting partial efficacy at the receptor relative to a full agonist, or by

acting as a competitive antagonist, competing with the full agonist for receptor occupancy, thus producing a net decrease in the receptor activation observed with the full agonist alone [523]. Alternatively, the antagonist and agonist may form an active bifunctional compound molecule which exhibits a similar dose-response curve to that of a partial agonist [523]. It is unclear what is happening in this instance with BB₂ receptor antagonists, but similar results have been observed behaviorally by Roesler's group [96].

Surprisingly, administration of GRP or RC-3095 to the IL had no effect on the downstream release of CRH at the BLA. Our previous research had shown that i.c.v. administration of BB/GRP elicited the release of CRH at the median eminence/arcuate nucleus and dose-dependently decreased levels of ir-CRH at the CeA such that low (0.25 µg) but not high (0.5 µg) doses of BB/GRP attenuated this release [211]. This suggests that the ability of GRP to influence CRH release at the BLA may also be dose related, although this contention has yet to be verified. It is also possible that GRP injection into the IL simply does not influence release of CRH at the BLA, suggesting that the GRP-induced release of CRH may be site-specific.

Possible Mechanisms of BLP Actions

GABA

While the underlying mechanisms mediating BLP effects on learned fear are currently not well understood, evidence is emerging linking the actions of GRP through that of GABA inhibition. High densities of BB₂ receptors were found on GABA interneurons in the LA, leading to an increase in inhibition of principle neurons at this location [436]. Further, bombesin induces depolarization of GABA inhibitory interneurons in hippocampal slices [237]. This finding is supported by immunohistological evidence reporting BB₂ receptors not only located in

the LA, but in the CeA and BLA as well [205]. These authors further reported BB₂ receptors on GABAergic neurons in these regions, lending support to the earlier study by Shumyatsky and colleagues [436]. It should be noted, however, that at the LA, only a subpopulation of cells expressing BB₂ receptors are GABAergic neurons, raising the possibility that other BB₂ receptor-expressing neurons may be located in this area as well [205].

A limited number of behavioral reports have also emerged linking the effects of GRP drug administration with GABAergic neurotransmission. For example, BLA administration of muscimol (a GABA agonist), prior to training in an inhibitory avoidance task, blocked the memory impairing effects of post-training systemic RC-3095 administration [391]. This suggests that RC-3095 may be exerting its effects through BLA GABAergic neurons. Similar results were observed at the hippocampus. In this study [96], an otherwise ineffective (pre-training) dosage of muscimol blocked the post-training inhibitory avoidance memory effects of hippocampal administered RC-3095. Alternatively, Andrews et al., [19] reported increased release of GABA at the ventral hippocampus of freely moving rats in response to reverse hippocampal perfusion of GRP. In fact, these authors observed almost a 40% increase in GABA in the recovered dialysates and a concomitant reduction in seizure activity (in seizure-prone mice) with increased GRP-induced GABAergic inhibition. In general, evidence is emerging supporting the activation of GABAergic inhibitory neurotransmission as a possible mechanism by which GRP exerts its effect on fear.

Serotonin

Aside from GABA, BLPs are also thought to mediate their effects through other neurotransmitter systems. Most notably, there is substantial evidence suggesting that BLPs

influence stress, anxiety and fear responses through interactions with 5-HT, a neurotransmitter known to play an important role in the response to stressor exposure [77, 493]. For example, *in vitro* studies have demonstrated that NMB and GRP receptor activation leads to depolarization of a subpopulation of dorsal raphe 5-HT neurons by acting on postsynaptically located receptor linked K^+ currents [351, 501]. A similar effect was observed in hypothalamic slices. BB (but not NMB) was found to decrease K^+ evoked release of 5-HT from hypothalamic rat brain slices by as much as 25% [420].

Using receptor knock-out strategies, immunohistochemical analysis of brain sections revealed that 5-HT expression levels in the DRN were elevated in BB_1 receptor deficient mice as compared to their wild-type counterparts [508]. And whereas wild-type mice showed an enhanced expression of 5-HT in response to restraint stress, the same was not observed in the BB_1 knockouts. Further, BB_1 receptor knockout mice were used to study anxiety in a marble burying task, a test which is thought to be modulated by 5-HT. BB_1 receptor deficient mice demonstrated decreased marble burying (suggestive of lowered anxiety) [507]. Additionally, immunohistochemical analysis of brain sections from these animals revealed an elevation in 5-HT expression in the DRN relative to wild-type controls, and a significant downregulation of the expression of 5-HT_{1A} gene expression was observed in tissue at the whole brain level in these mutant mice [507]. Thus, there is evidence consistent with the view that impairment of the BB_1 receptor system could lead to a dysregulated 5-HT response to stress.

Pharmacological evidence also points to a functional interplay between BLPs and 5-HT. For example, we showed that microinjection of NMB into the dorsal raphe nucleus is anxiogenic (as assessed in the social interaction test), whereas injection of PD 176252 into this same region is anxiolytic [292]. In this study, intra-DRN microinfusion of the PD 176252 suppressed,

whereas its agonist (NMB-30) promoted, the *in vivo* release of 5-HT in the ventral hippocampus. In parallel, the suppressed social interaction elicited by intra-DRN administration of NMB was attenuated by a systemically administered 5-HT_{2C} (but not 5-HT_{1A}) receptor antagonist [292]. Further, blockade of BB₂ receptors led to an attenuation of the stressor-induced activation of the serotonergic system and the HPA axis [133]. This study also revealed increased 5-HT release following GRP administration, an effect that was blocked by a BB₂ antagonist. It is noteworthy that α -helical CRH₉₋₄₁, a CRH antagonist, had no effect on this GRP-induced enhancement of 5-HT release. It is also 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 [75, 95, 203].

Studies of conditioned fear using *in vivo* microdialysis demonstrated the increased release of 5-HT (but not 5-HIAA) in response to conditioned fear both at the mPFC and at the BLA [516, 521], and Inoue et al., [193] found increased 5-HIAA, but not 5-HT, at these regions in response to foot-shock stress. In addition, it appears that an increased availability of 5-HT at the mPFC reduces fear behavior [165], whereas when selective serotonin reuptake inhibitors (which are anxiolytic in conditioned fear stress) were applied, they appeared to exert their effects at the level of the amygdala, and not at the mPFC [196]. These studies support the involvement of 5-HT in conditioned fear at both the mPFC and the amygdala, however evidence linking the two brain regions in a serotonergic conditioned fear pathway is sparse. One study utilizing high sensitive (HS) and low sensitive (LS) rats (as predetermined by a 'flinch-jump' response to painful stimuli) showed that by pharmacologically blocking prefrontal serotonergic innervation (at the dorsomedial part of the prefrontal cortex) in HS rats, 5-HTergic lesion significantly decreased freezing behavior, enhanced c-FOS expression in the dorsomedial PFC and increased

the concentration of GABA in the BLA. In contrast, for LS rats an increase in the duration of freezing was observed, in addition to increased c-FOS expression in both the CeA and BLA and a decrease in GABA concentration in the BLA. Moreover, in naïve rats (not exposed to shock or test animals), c-FOS expression was not altered in LS and HS rats. Thus, it appears that the 5-HT system modulates conditioned fear at both the PFC and amygdala, and although it is not yet clear how, it is possible that a pathway exists for this neurotransmitter system linking these two brain areas.

In summary, it seems that endogenous BLPs at the DRN evoke the release of 5-HT at the ventral hippocampus, leading to anxiogenesis in behavioral models such as social interaction; a finding that could indicate that antagonism of these compounds could represent a novel therapeutic target for anxiolytic drug agents. Further, although ample evidence exists linking BLPs with the actions of 5-HT, and suggesting that both are involved in conditioned fear at the level of the mPFC and amygdala; there is not yet enough evidence suggesting that these two peptidergic/neurotransmitter systems interact with each other within this limbic/fear system.

Corticotropin-releasing hormone

Ample evidence points to BLP involvement in the HPA response, probably through interactions with CRH (extensively reviewed in the introduction); although this interaction has not yet been clearly delineated. BLPs appear to activate the HPA axis and sympathetic nervous system, an effect which is attenuated in animals pretreated with α -helical CRH [210]. Similarly, α -helical CRH pretreatment blocks the GRP-induced increases in plasma ACTH and corticosterone [135]. Further, i.c.v. GRP pretreatment potentiates CRH-induced ACTH secretion [331], an effect that was blocked by pretreatment with a BB₂ receptor antagonist or with CRH

antiserum [23, 135, 331]. Thus, it appears that these peptides are somehow functionally related, however, they do act exclusively from one another. For example, a BB/GRP-induced increase in plasma catecholamines was apparent [56, 72, 212, 330] when BB/GRP were administered to the ventricles and/or NTS. When administered to the CeA, however, CRH elicited this increase of circulating catecholamines whereas BB administration was ineffective. Thus, it appears, at least at the level of the CeA, that CRH and BB/GRP may work in diverging pathways and likely do not have identical roles.

Evidence garnered from the experiments in this dissertation has supported the contention that there is a functional relationship between BLPs and CRH in fear/anxiety-induced situations. These studies have provided preliminary evidence suggesting that this relationship is not necessarily an interdependent one, at least in terms of conditioned fear. In the pilot project in Chapter 2 significant peptide differences were observed for both CRH and GRP in both the PrL and the amygdala nuclei, suggesting that each of these areas recruit CRH and GRP during the recall of fear conditioning, however diverging patterns were observed at each nuclei. The study in Chapter 4 showed that CRH and GRP have similar release profiles from the BLA during the recall of fear conditioning, and further, that they both appear to correlate positively with animal's level of fear and both appear to be protracted 24h after CS-US conditioning. This suggests that CRH and GRP work in parallel to one another, possibly in a redundant manner or in consequence to one another. For example, it is possible that when one system is activated, the other is recruited as well. It is impossible to determine from the current data however, which peptide might lead this cascade of events.

The study in Chapter 5 showed that a pathway between the mPFC and BLA, two areas in which GRP modulates conditioned fear, does indeed exist, however this pathway appears

exclusive to GRP at the level of the BLA in response to mPFC GRP activation. This suggests that an interaction between CRH and GRP does not exist, at least within the mPFC/BLA GRPergic pathway. It is possible that elevations in CRH at the level of the BLA may have been observed had CRH been the drug administered to the IL; the current study however, does not speak to this.

Thus, although the studies within this dissertation have shed more light on the functional interplay between CRH and GRP in terms of fear, both supporting previous literature in establishing a relationship between CRH and GRP, as well as illuminating inconsistencies in this relationship, it is yet unclear how and under what circumstances this relationship exists. It is possible that under some circumstances, GRP recruits the involvement of CRH in emotion-based processing, while under other circumstances CRH recruits GRP. It is equally possible that GRP mediates its effects through CRH (and vice versa), and finally, it is entirely possible that these two systems work in parallel to each other, possibly recruiting additional systems.

Other neurotransmitter/neuropeptide mechanisms

The evidence linking BLPs with additional neurotransmitters and/or neuropeptides, in terms of anxiety/stress or fear responses, is less revealing. There is evidence supporting the involvement of norepinephrine/epinephrine with BLP activation. For example, BB was reported to reduce norepinephrine-induced feeding [318] whereas central administration of BB potently increases circulating levels of NE [58, 59, 61]. Although no research exists confirming the activation of catecholamines in conjunction with BLP activation during the fear response, given the obvious role of epinephrine/norepinephrine in the stress response, flight or fight response and

its involvement in anxiety disorders, it seems likely that during learned fear, BLPs would recruit the catecholaminergic system; although the current research does not speak to this directly.

There is evidence, albeit limited, that supports the notion that GRP may exert some of its effects through other systems as well. For example, it was reported that RC-3095 blocked d-amphetamine-induced hyperlocomotion and increased nerve growth factor and brain-derived neurotrophic factor in a model of the manic phase of bipolar disorder [208]. It was also shown that RC-3095 blocked the effects of apomorphine, a dopamine agonist, on stereotyped behavior [289]. These findings suggest the recruitment of dopaminergic systems in response to GRP manipulations.

Roesler et al., [363] has also attempted to delineate the molecular pathways involved in GRP's modulation of emotional memory. For example, they found that the memory impairing effects of BB₂ receptor antagonists might be partially mediated by an inhibition in the function and/or expression of neuronal basic fibroblast growth factor (bFGF). When RC-3095 was administered to the CA1 region of the hippocampus, memory impairing effects were observed on inhibitory avoidance. Alternatively, bFGF enhanced recall of memory for this task. When RC-3095 and bFGF were administered concurrently, an otherwise ineffective dose of bFGF blocked the memory impairing effect of RC-3095. Thus, bFGF may be involved in the memory modulation effects of BB₂ receptors, at least where inhibitory avoidance learning is concerned.

Roesler et al., [392] also assessed the involvement of BB₂ receptor signaling with protein kinase pathways. The pre-training enhancement of inhibitory avoidance memory by intra-hippocampal injections of BB was prevented by pretraining infusions of RC-3095 or inhibitors of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and protein kinase A (PKA), but not by a neuromedin B receptor antagonist. Further, the BB-induced enhancement of

consolidation was potentiated by co-infusion of activators of the dopamine D1/D5 receptor/cAMP/PKA pathway and prevented by a PKA inhibitor. Thus, the authors conclude that memory modulation by hippocampal BB₂ receptors is mediated by PKC, MAPK, and PKA pathways.

Implications

The present findings that BLPs may be involved in the mediation and/or modulation of the response to conditioned fear, and that these effects may be mediated, at least in part through pathways located in, and between, the amygdala and mPFC have several possible neuroscientific and heuristic implications. The first is that the current findings support the contention that the BLP system may serve as a novel therapeutic target for anxiety disorders [389]. Current treatments for anxiety disorders are not without complications. For example, amongst the most widely prescribed treatments for this group of disorders are benzodiazepines, a class of drugs associated with such untoward side effects as sedation, memory impairments, habituation, dependence and sometimes lethal interactions with alcohol consumption [361].

Further, the current findings support the contention that BLPs play a modulatory role in emotional memory formation. Although the studies reported in this dissertation do not speak to memory modulation specifically, the observation that injection of BLP agonists elicit a reduction in fear upon recall testing suggests enhancement of extinction learning.

The current studies also lend support to the hypothesis that the mPFC and amygdala share a functional circuit where fear is concerned. We demonstrated that BLPs and CRH are involved at the level of the amygdala in conditioned fear, as both of these peptides are released in response to fear. And it is possible that the mPFC plays a role in ‘gating’ amygdala responses as administration of GRP to the mPFC leads to a subsequent release of GRP at the BLA. GRP

release at the amygdala may occur in an attempt to activate negative-feedback inhibitory processes and modulate fear through the recruitment of subsequent neurotransmitter and neuropeptide systems.

Future Directions

The findings from this thesis have provided a strong basis for future research. It would therefore be prudent to expand this line of research to include the following:

- 1) Studies should continue the attempt to identify the possible co-localization and/or interaction of BLPs and other peptides and/or transmitters known to be important in stress and fear-related physiology and behavior. For example, the linkages between BLPs with other neuropeptides such as CRH or neurotransmitters such as 5-HT, GABA, dopamine, norepinephrine and even glutamate might give insight on possible important interactions through which these peptides exert their actions and modulate the stress response and associated behaviors. Although some evidence exists to support a relationship between BLPs and CRH, 5-HT, GABA and dopamine, this evidence is inconclusive, and calls for further study.
- 2) It is important to take the functional relationship that we observed between the mPFC and the BLA one step further to more fully elucidate the GRP pathway contained between these two loci. Anatomical tract tracing, labeling and in situ hybridization studies could help to characterize this more fully.

- 3) The current set of studies focused solely on learned fear responses. It would be prudent to extend these findings by using animals models of unlearned fear, such as predator odor and/or predator exposure to determine if the effects of BLPs on the fear system are specific to the learning of cues and CS associations surrounding the aversive event, or if their role is more general to the fear response overall.
- 4) Leading from the above suggested direction, it is also important to delineate whether the observed findings in this dissertation were as a consequence of learning taking place, or whether these drugs are anxiolytic/anxiogenic in nature. For example, it is important to determine whether these peptides are involved in manipulating the physiological experience of the threat (i.e., fear and/or anxiety) or are they involved in modulating the memory trace, and by association, assembling the cues surrounding the event in order to properly assess the threat of the event.
- 5) Finally, the mPFC and amygdala were chosen targets to explore in this dissertation, however several other brain areas deserve equal attention, the most obvious being the hippocampus. The hippocampus is fundamental for contextual-based fear conditioning and several reports have linked BLP modification of fear-related processes at this site, thus it seems necessary to evaluate the role of BB-related peptides at the hippocampus in learning memory processes for aversive events.

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