

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

**ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600**

UMI[®]



Université d'Ottawa • University of Ottawa

**AN APPRAISAL OF THE ROLE OF CORTICOTROPIN-RELEASING
HORMONE AT THE AMYGDALA IN THE MEDIATION OF THE
STRESS RESPONSE: APPLICATION OF *IN VIVO* MICRODIALYSIS IN
THE ASSESSMENT OF NEUROPEPTIDE RELEASE**

A Doctoral Thesis Dissertation

By

David S. Michaud

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

University of Ottawa



**National Library
of Canada**

**Acquisitions and
Bibliographic Services**

**385 Wellington Street
Ottawa ON K1A 0N4
Canada**

**Bibliothèque nationale
du Canada**

**Acquisitions et
services bibliographiques**

**385, rue Wellington
Ottawa ON K1A 0N4
Canada**

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-76447-8

Canada

Abstract

Accumulating research suggest that an organism's response to psychological stressors includes an element of cognitive processing that is partially mediated through a change in the neurochemical milieu at the central nucleus of the amygdala (CeA). In this respect, corticotropin-releasing hormone (CRH) at the CeA may integrate the autonomic and behavioral effector systems of the stress response. Likewise, there is mounting evidence that bombesin-like peptides (BN-LPs) may act independently of, or through CRHergic systems to influence the stress response. Unfortunately, much of our knowledge regarding the precise role of CRH and BN-LPs has been disadvantaged by technical limitations that do not afford the direct assessment of neuropeptide release/utilization.

The initial experiments presented in this thesis demonstrate the feasibility of using a novel *in vivo* microdialysis approach combined with a highly sensitive radioimmunoassay to assess the release of CRH and BN-LPs in freely behaving animals. This research demonstrates that CRH and BN-LPs are released at the CeA in response to both aversive and appetitive stimuli, signifying that these peptides represent two of the neurochemical signals involved in assigning emotional salience to stimuli, regardless of its valence. These findings challenge the widely held view that CRH at the CeA orchestrates the behavioral changes associated with stressor exposure.

Based on the context and time-course of peptide release, it is suggested that CRH at the CeA might serve to maintain vigilance, direct attention, sub-serve memory formation, or represent a mechanism through which brain systems are sensitized to subsequent stressor exposure.

Acknowledgments

Without the uncompromising support and encouragement of my thesis advisor, mentor and friend, Dr. Zul Merali, none of this research would have been possible. I will be forever grateful to him for all he has taught me and the countless experiences that have come my way because of him. This goes beyond my scientific training and includes the strong family atmosphere he cultivated on a daily basis in the lab, and the many friendships I have made because of this.

Although this work bears only my name I cannot imagine having made it this far without the support of so many of my past and present lab mates. As a group we have shared each other's pain and happiness creating a lifetime of memories. I owe a great deal to Samir, Tania, Kelly, Anne, Lisa, Laurie, Tom, Scott, Shuye, Shara-Jane and especially Judy who continues to teach and inspire me more than she probably realizes. A very special thanks to Dr. Pamela Kent for all she has taught me over the years. Pam's patience and sense of "calmness" has always made working in the lab that much more enjoyable. Her feedback on this thesis was very much appreciated.

All who have gone before me will attest that being a student in the Merali Lab comes with a somewhat significant sacrifice of time that can be difficult on family. I am happy to finally be able to tell my family that I'm free this weekend! Each of them has been very supportive of me from the very beginning and I am grateful to them for this. The support from my brother Rob was without hesitation and seemed to have no limits. Special thanks to my mother and late father for their constant support of everything I've ever done, no matter how long it took me to finish.

I am very grateful that for most of my six years in the graduate program I have been able to share my experiences with my wife Line St-Pierre, who always understood exactly what I was going through, having done it herself not so long ago. The encouragement, patience and self-sacrifice that she has had for me since the day we met made this possible and my entire experience more enjoyable. Being with Line, meant that I was also fortunate to have the support and encouragement of her entire family and so a special thanks is extended to Lucy, Frank and Line's parents, Claude and Margo.

I remember thinking on my first day at the University of Ottawa that if I ever finished this chapter in my life I would make sure to thank my OAC chemistry teacher who probably doesn't realize the impact that he had on me before I even started university. Without his encouragement and support I don't think I would be writing this. Thank you Mr. Byers.

I would like to thank Dr. George Fouriezios who I was fortunate to work with near the end of my time in the lab. Our common interest in both science and bicycles made this experience very enjoyable. Special thanks to Sylvie Emond who made the long days of animal surgery so much easier, and Dr. Girodo for minimizing my pre-lecturing panic attacks. Many thanks to my thesis committee members Dr. Claude Messier, Dr. Hymie Anisman, and Dr. Hélène Plamondon for their guidance throughout this project. Finally, I would like to thank the University of Ottawa and OGS for their financial support.

Table of Contents

Abstract	ii
Acknowledgments	iii
Table of Contents	v
List of Figures	ix
List of Tables	xi
List of Abbreviations	xii
General Summary	xiii
General Introduction	1
Hans Selye's concept of stress	3
The prototypical stress response	5
<i>Activation of the pituitary-adrenal (HPA) axis</i>	5
<i>Activation of the autonomic nervous system (ANS)</i>	6
<i>The stress response: The body's homeostatic alarm</i>	8
<i>Activation of the hypothalamic-pituitary gonadal (HPG) axis</i>	11
<i>Activation of the hypothalamic-pituitary thyroid (HPT) axis</i>	12
The classical neurotransmitters and the stress response	14
<i>Dopamine</i>	15
<i>Epinephrine</i>	18
<i>Norepinephrine</i>	18
<i>Serotonin</i>	20
Neuropeptides implicated in the stress response	22
<i>Synthesis and release</i>	22
<i>Corticotropin-releasing hormone (CRH)</i>	23
<i>Arginine-vassopressin (AVP)</i>	29
<i>Bombesin-like peptides (BN-LPs)</i>	32
Role of the amygdaloid complex in the stress response	36

Assessing the role of neuropeptides in the stress response: methodological issues

<i>In situ hybridization</i>	43
<i>Immunohistochemistry</i>	45
<i>Micropunch</i>	45
<i>In vivo microdialysis and push-pull perfusion</i>	46

Thesis objectives	49
--------------------------	----

Preface to chapter 1	53
-----------------------------	----

Chapter I

Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala

Abstract	57
Introduction	58
Materials and methods	60
Results	70
Discussion	80

Preface to Chapter II	86
------------------------------	----

Chapter II

Differential impact of predator exposure, immobilization stressor, and sexual cues on central corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rats

Abstract	89
Introduction	91
Materials and methods	94
Results	108
Discussion	130

Preface to Chapter III	139
Chapter III	
Differential impact of audiogenic stressors on Lewis and Fischer rats: Behavioral, neurochemical and endocrine variations	
Abstract	143
Introduction	144
Materials and methods	146
Results	156
Discussion	172
Preface to Chapter IV	179
Chapter IV	
Diazepam reverses the behavioral effects of novel environment stressor but fails to block novelty-elicited release of corticotropin-releasing hormone at the central nucleus of the amygdala	
Abstract	182
Introduction	183
Materials and methods	185
Results	195
Discussion	204
Preface to Chapter V	210
Chapter V	
Stressor exposure elicits the release of corticotropin-releasing hormone at the peri-locus coeruleus: Possible activation through neurons arising from the central nucleus of the amygdala	
Abstract	212
Introduction	214

Materials and methods	216
Results	220
Discussion	223
General Discussion	228
Conclusions and future directions	245
Bibliography	251

List of Figures

Introduction

Fig. 1	Schematic representation of the prototypical stress response	10
Fig. 2	Schematic representation of the neuroanatomical projections of the amygdaloid complex	44
Fig. 3	Schematic representation depicting the relationship between in vivo microdialysis and push-pull perfusion combined with RIA	48

Chapter I

Fig.1	HPLC separation of CRH and BN-LP immunoreactivity in microdialysis samples	66
Fig. 2	Restraint-elicited release of CRH at the CeA	72
Fig. 3	Schematic illustration depicting histologically determined placement of microdialysis probes	73
Fig. 4	Restraint-elicited release of BN-LP at the CeA	74
Fig. 5	Meal-elicited release of CRH and BN-LP at the CeA	77
Fig. 6	Schematic illustration depicting histologically determined placement of microdialysis probes	78
Fig. 7	Effect of meal ingestion on plasma ACTH and corticosterone levels	79

Chapter II

Fig. 1	Photographic representation of the non-contact setup	102
Fig. 2	Effect of predator exposure and restraint on plasma ACTH and corticosterone in Fast and Slow rat line	110
Fig. 3	Effects of predator exposure and restraint on immunoreactive CRH levels at the PVN, Me/Arc, and Pit in Fast and Slow rat lines	113
Fig. 4	Effects of predator exposure on the time-course of plasma ACTH and corticosterone changes in Fast and Slow rat lines	118
Fig. 5	Effects of restraint on the time-course of plasma ACTH and corticosterone Changes in Fast and Slow rat lines	120
Fig. 6	<i>In vivo</i> release of CRH at the CeA following predator exposure in Fast and Slow rat lines	122
Fig. 7	<i>In vivo</i> release of CRH at the CeA following restraint in Fast and Slow rat lines	123
Fig. 8	<i>In vivo</i> release of CRH at the CeA following non-contact to estrous and non-estrous female cohorts in Fast and Slow rat lines	125
Fig. 9	Behavioral response of Fast and Slow rat lines towards estrous and non-estrous female cohorts under non-contact conditions	127
Fig. 10	Behavioral response of Fast and Slow rat lines towards estrous and non-estrous female cohorts under contact conditions	129

Chapter III

Fig. 1	Effects of acute noise exposure on changes in ACTH, corticosterone and prolactin 0- and 1-hr following exposure in Fischer and Lewis rats	157
Fig. 2	Effects of acute noise exposure on changes in CRH and BN-LP levels at the PVN, and Me/Arc 0- and 1-hr following exposure in Fischer and Lewis rats	159
Fig. 3	Effects of acute noise exposure on changes in CRH and BN-LP levels at the CeA, MeA, and BLA 0- and 1-hr following exposure in Fischer and Lewis rats	161
Fig. 4	Effects of acute noise exposure on changes in tissue levels of CRH and BN-LP at the locus coeruleus 0- and 1-hr following exposure in Fischer and Lewis rats	163
Fig. 5	<i>In vivo</i> release of CRH at the CeA following exposure to acute noise in Fisher and Lewis rats	167
Fig. 6	<i>In vivo</i> release of CRH and corticosterone at the anterior pituitary gland following acute noise in Fischer and Lewis rats	170
Fig. 7	Behavioral response to acute noise exposure in Fischer and Lewis rats	171

Chapter IV

Fig. 1	Photographic representation of the novel cage paradigm setup	188
Fig. 2	Changes in the consumption of a palatable snack in rats transferred from their home cage to a novel environment	198
Fig. 3	Diazepam-pretreatment dose-dependently reverses the behavioral effects of the novel cage	199
Fig. 4	<i>In vivo</i> release of CRH at the CeA in rats transferred from a home cage to a novel environment	203

Chapter V

Fig. 1	A schematic representation of a novel side-window push-pull probe used to collect the release of CRH at the peri-locus coeruleus	221
Fig. 2	<i>In vivo</i> release of CRH at the peri-locus coeruleus following restraint	222

List of Tables

Chapter II

Table 1.	Effect of predator exposure and restraint on regional changes in immunoreactive CRH tissue levels in Fast and Slow rat lines	114
Table 2.	Effect of predator exposure and restraint on regional changes in immunoreactive BN tissue levels in Fast and Slow rat lines	116

Chapter III

Table 1.	Effect of acute noise exposure on regional changes in immunoreactive CRH and BN-LP at 0- and 1-hr following noise exposure in Fischer and Lewis rats	164
----------	--	-----

Chapter IV

Table 1.	Effects of CP-154526 (CRH ₁ receptor antagonist) on novel environment induced suppression of snack consumption	201
Table 2.	Effects of anti Sauvagine-30 (CRH-type2 receptor antagonist) and CRH on snack consumption in a novel cage and home cage. respectively.	201

List of Abbreviations

ACTH, adrenocorticotropin-releasing hormone; **ANOVA**, analysis of variance; **AH**, anterior hypothalamus; **ANS**, autonomic nervous system; **AVP**, arginine vasopressin; **BA**, basal amygdala; **BLA**, basal lateral amygdala; **BN**, bombesin; **BN-LPs**, bombesin-like peptides; **BNST**, bed nucleus of the stria terminalis; **Cau**, caudate nucleus; **CeA**, central nucleus of the amygdala; **Cg Cx**, cingulate cortex; **CNS**, central nervous system; **CRF**, corticotropin-releasing factor; **CRH**, corticotropin-releasing hormone; **CRH-BP**, CRH-binding protein; **DH**, dorsomedial hypothalamus; **dHipp**, dorsal hippocampus; **FITC**, fluorescein-5-isothiocyanate; **fMRI**, functional magnetic resonance imaging; **FSH**, follicle-stimulating hormone; **GAS**, general adaptation syndrome; **GnRH**, gonadotropin releasing hormone; **GR**, glucocorticoid receptor; **GRP**, gastrin releasing peptide; **Hipp**, hippocampus; **HPA**, hypothalamic-pituitary adrenal; **HPLC**, high-performance liquid chromatography; **HPT**, hypothalamic-pituitary thyroid; **HPG**, hypothalamic-pituitary gonadal; **ICV**, intracerebroventricular; **IML**, intermediolateral; **ip**, intraperitoneal; **ir**, immunoreactive; **KRB**, Krebs's Ringer solution; **LC**, locus coeruleus; **LH**, luteinizing hormone; **M Cx**, motor cortex; **MeA**, medial amygdala; **Me/Arc**, median eminence arcuate complex; **mPFC**, medial prefrontal cortex; **MPOA**, medial preoptic area; **mRNA**, messenger ribonucleic acid; **nAcb**, nucleus accumbens; **NMB**, neuromedin B; **NMC**, neuromedin C; **NTS**, nucleus of the solitary tract; **OT**, olfactory tubercle; **PET**, positron emission tomography; **Pit**, pituitary gland; **PRL**, prolactin; **PV**, paraventricular thalamic nucleus; **PVN**, paraventricular nucleus of the hypothalamus; **S Cx**, somatosensory cortex; **T3**, triiodothyronine; **T4**, thyroxine; **TRH**, thyroid-releasing hormone; **TFA**, trifluoroacetic acid; **TMT**, trimethylthiazoline; **TSH**, thyroid-stimulating hormone; **VTA**, ventral tegmental area; **VMH**, ventral medial hypothalamus

General Summary

The prototypical stress response includes both an activation of the hypothalamic-pituitary adrenal (HPA) axis and sympathetic nervous system that together with stressor-associated behavioral change underscore the means through which an organism copes with threatening stimuli. This typically transient response is essential to an organism's survival albeit when chronically activated deleterious consequences on one's physical and mental health may ensue. In humans, the adverse health effects associated with chronic stressor exposure are readily apparent and range from depression and panic disorder to heart disease and cancer. Understanding the neurochemical and behavioral underpinnings of the stress response has facilitated our ability to develop behavioral and pharmaceutical treatments for stress-related disorders. More recent effort has been focused on the role of neuropeptides in both the stress response and etiopathogenesis of stress-related conditions. In this context, corticotropin-releasing hormone (CRH) of hypothalamic origin represents one of the most widely studied hormones mediating the activation of the HPA axis, in addition to playing a role in the behavioral changes commonly associated with stressor exposure. More recent pharmacokinetic advances have led to orally administrable CRH receptor antagonists that readily pass the blood brain barrier in efforts to treat stress-related conditions by targeting central CRH receptors. The assumption behind these developments is that these ailments include a hyper-secretion of CRH (or some other ligand) in the brain, prompting an abnormal activation of CRH receptors. Identifying the brain regions where the release of CRH and/or other neurochemicals might be imbalanced represents a major scientific undertaking. In many cases, both human and animal experiments suggest that the

amygdala, and in particular the central nucleus of the amygdala (CeA), represents a major brain region involved in processing stimuli that constitute processive/psychological stressors. Processive stressors activate the stress response through a higher level of cognitive processing (in comparison to physical/systemic stressors) and are analogous to situations commonly experienced by humans. Therefore, much can be gained by elucidating the neurochemical processes that occur at the CeA during stressful conditions. Continued progress in this area necessarily requires that we glean a better understanding of how CRH (and other hormones) are utilized in the brain across various behavioral states, such as stressor exposure, in freely behaving animals. In this context, the identification of novel stress-related neuropeptides, such as bombesin-like peptides (BN-LPs) increases the scope of potential therapeutic intervention.

Much of what we know about the classical neurotransmitters (dopamine, serotonin, norepinephrine and epinephrine) has been derived from studies assessing the *in vivo* release of these compounds in freely behaving animals using techniques such as microdialysis. Unfortunately, attempts to apply *in vivo* microdialysis to investigate the endogenous release of peptides such as CRH and BN-LPs have been stalled by poor recovery rates of the dialysis membrane and insufficient *ex vivo* quantification capabilities. As an alternative, indirect approaches such as *in situ* hybridization, post-mortem tissue content analyses, and exogenously administered compounds have been relied on to determine the extent to which CRH (and other peptides) are involved in the different aspects of the stress response, ranging from endocrine to behavioral changes.

As a result, our ability to ascribe specific roles to either CRH or BN-LPs during the stress response is difficult and will remain so until more direct techniques are developed.

The initial experiments presented in this thesis demonstrate the feasibility of using a novel *in vivo* microdialysis approach combined with a highly sensitive radioimmunoassay (RIA) to assess the release of CRH and BN-LPs in freely behaving animals. With this technique firmly established we were able to assess the release of CRH and BN-LPs before, during and following various behavioral conditions that had aversive (noise, restraint, predator and novelty) and appetitive (meal ingestion and exposure to sexual cues) attributes. A fundamental hypothesis addressed in this work was *if CRH at the CeA mediates the behavioral response associated with stressor exposure then:*

- 1) Experimental paradigms that elicit different behavioral responses in animals should evoke differential release patterns of amygdaloid CRH. Likewise.
- 2) Rat strains that display unique behavioral patterns towards stressors should show strain-dependent release dynamics of CRH at the CeA.

If on the other hand the role of CRH and/or BN-LPs goes beyond the stress response to include the attribution of emotional salience, despite the inherent quality of the stimulus, then:

- 3) Exposure to appetitive events might also evoke the release of these peptides at the CeA.

Taken together, the experiments presented in this thesis confirm that CRH and BN-LPs are released at the CeA in response to both aversive and appetitive stimuli. These findings suggest that these peptides represent two of the neurochemical signals involved in assigning emotional salience to stimuli, regardless of its inherent positive or negative attributes. Similar findings are reported for the release of CRH in response to sexually receptive female cohorts. Over the course of these experiments it was readily apparent that the release pattern of CRH at the CeA in response to several conditions (i.e. restraint, noise, novelty, predator, meal consumption, and sexual cues) was similar regardless of the pre-existing strain-dependent behavioral repertoire of the animals, and the nature of the stimulus (positive or negative). Indeed, the introduction of a stressor or appetitive stimulus consistently evoked a rise in the release of CRH at the CeA, which remained well after the stimulus was removed. Furthermore, we report that rats pretreated with diazepam display anxiolytic-like behavioral patterns *despite* a concomitant rise in the release of CRH at the CeA. In summary, the findings in this thesis strongly oppose the position that CRH at the CeA orchestrates the behavioral changes associated with stressor exposure. However, this research clearly demonstrates that stressor exposure, like appetitive stimuli, evokes a protracted release of CRH and BN-LPs at the CeA. This response pattern provides insight into the role of these peptides in general and at the CeA in particular. We discuss the sustained rise of CRH in the context of the experimental design in which it was observed and postulate that a protracted CRH release might serve to maintain vigilance, direct attention, sub-serve memory consolidation, neuroprotection, or represent a mechanism through which brain systems are sensitized to subsequent stressor exposure.

General Introduction

In Canada, after pregnancy, the major causes of hospitalization and death are stress-related conditions [179]. The wear and tear that life stressors impart on one's physical and mental health is profound and it is estimated that 64% of Canadians over the age of 18 report that they are chronically exposed to at least moderate levels of stress. Of these, a significant proportion (26%) report experiencing high levels of stress [179]. Stressful events (stressors) can range from the relatively minor everyday occurrences (lost wallet, traffic jam, and schedule demands) to the more major and often unexpected misfortunes like natural disaster, illness or death of a loved one. As will be discussed in some depth, the body's responses to stressors are designed to help one cope with stressors (fight or flight response). However, chronic exposure to stressors (of mild or severe intensity) can lead to perturbations of one's physiological and behavioral resources and promote the onset of a variety of ailments ranging from psychiatric [57] to immune [183] and cardiovascular disorders [52:339].

It is not surprising then that the mechanisms underlying stressor responses and stress-related disorders have become amongst the most investigated topics, spanning across several disciplines including sociology, psychology, endocrinology and cellular, molecular and behavioral neuroscience. Despite the widespread interest in this topic, the concept of *stress* remains, at best, loosely defined [452]. The difficulty in defining stress arises in part because it is a multidimensional concept that consists of an interaction between at least 1) the incoming stimuli (stressors), 2) the mechanisms involved in processing the stressor, and 3) one's physical, psychological and/or behavioral response

to the stressor. In the current thesis "stress" is viewed as the physical, psychological and/or behavioral response(s) in anticipation of, or in response to, a threatening stimulus (i.e. the stressor). This response may occur consciously or unconsciously. This umbrella definition attempts to incorporate views that have evolved over time. Without a doubt, much of our understanding of stress-response began with the pioneering work of Hans Selye, more than five decades ago [400].

In recognition of Selye's contribution to the field of stress research this introduction begins with a brief overview of Selye's work at McGill University. Although the underlying position held by Selye (that stress is a non-specific response) is no longer widely accepted, researchers do accept the concept that certain common physiological changes do represent the prototypical stress response. In this context, the second part of this introduction details the concomitant activation of the hypothalamic pituitary adrenal (HPA) axis and sympathetic nervous system as the benchmark signals underscoring the prototypical mammalian stress response. These changes are discussed in the perspective of how they might represent the body's defense against threats to homeostasis. Although the activation of these physiological changes represent the most widely studied stress responses, it is important to discuss them in the context of other stressor-reactive systems. Thus, this section includes a brief discussion of how stressors influence the hypothalamic pituitary thyroid (HPT) and gonadal (HPG) axes.

The next section reviews the neurochemical substrates implicated in the stress response beginning with a brief summary of how stressors influence the classical

neurotransmitter systems within the brain. However, because the central theme of this dissertation concerns the role of neuropeptides in the stress response, this section includes a detailed analysis of how peptidergic systems are affected by stressors. Similarly, central to the studies presented in this dissertation was an assessment of how the amygdala, particularly its central nucleus (CeA), might be involved in the animal's response to both aversive and appetitive events. As such, the final section to this introduction addresses the possibility that the CeA may play a central role in the integration of processes through which sensory stimuli gain motivational and emotional significance.

Hans Selye's concept of stress

As a young researcher at McGill University, Selye was not initially studying, nor was he particularly interested in, stress. In his effort to discover a new sex hormone, much of his time was dedicated towards injecting rats with placental and ovarian extracts and assessing whether they produced unique effects. To his initial amazement, Selye observed that his extract injections all produced a considerable enlargement of the adrenal cortex, accompanied by atrophy of the thymus, spleen and lymph nodes as well as the appearance of ulcers at various levels of the gastrointestinal tract [401]. These events all occurred concurrently and the magnitude of change appeared to be dependent upon the amount of the extract injected. Because none of the known sex hormones produced such a syndrome, Selye felt that the ovarian and placental extracts must contain an as yet undiscovered sex hormone(s). However, it was soon discovered that extracts from several different organs as well as toxins and environmental change, also produced

a similar set of symptoms. It was clear that the condition he observed was not unique to a potentially novel sex hormone, and he was urged by many of his contemporaries, to abandon this line of research and to return to traditional research in endocrinology.

In many ways the medical field today is indebted to Selye because he went against the advice of his colleagues, and continued his relentless pursuit to understand the basis for what he would soon coin the term "general adaptation syndrome" (G.A.S.) sometimes referred to as the *stress syndrome*. In Selye's first submission of a manuscript to *Nature* in 1936 [400] he used the word "stress" to describe the *alarm, resistance* and *exhaustion* response stages of the G.A.S. However, the use of this term outside the field of physics was met with so much resistance that the editors of the journal replaced it with "nocuous". It took Selye some time to convince the medical field that "stress" was a more suitable term and even longer to define it as "*...the state manifested by a specific syndrome which consists of all the nonspecifically induced changes within a biologic system*" [401] p.54. From then on, the medical use of the word "stress" grew in popularity, unbound by different languages around the world.

Selye recognized and characterized the specific physiological changes that occurred in response to stressful stimuli but did not consider this to be a part of the stress-syndrome. On the other hand, he felt it was the underlying *non-specific* changes that constituted the stress response and that this response should only be studied after the specific changes were subtracted out. In this regard, many researchers since Selye have

taken issue with the notion of non-specificity, and debates on this topic are common even today [327:328].

This summary of how Selye's research in stress unfolded is admittedly limited in scope, compared to the breadth of his accomplishments, but does underscore how his ideas and discoveries served as a springboard for much of the research that followed. Since the early discoveries of Selye, scientists have learned a great deal more about the underlying mechanisms that characterize the prototypical stress response.

The prototypical stress response

Activation of the hypothalamic-pituitary adrenal (HPA) axis

Ordinarily, the neurochemical signals that influence the HPA axis activity are maintained within a narrow range, fluctuating primarily in an ultradian rhythm and in response to deviations from a designated range [67]. However, when an organism anticipates, or confronts a threatening situation, a rapid (within seconds) mobilization of the HPA axis is elicited. This is characterized by a cascade of neurochemical changes leading to the release of corticotropin-releasing hormone (CRH) from the median eminence of the hypothalamus. CRH is then transported (via the portal blood system) to the anterior pituitary gland, where it binds to receptors on corticotropic cells. Corticotropic cells produce and release adrenocorticotropin-releasing hormone (ACTH), in response to activation by CRH and/or other secretagogues. Once released, ACTH is transported via general circulation to various organs including the adrenal cortex where it activates specific receptors to provoke the release of glucocorticoids, namely cortisol (in

primates) or corticosterone (in rodents). Release of ACTH and corticosterone (or cortisol) is evident within minutes of stressor exposure [67]. As will be discussed in subsequent sections, there are other peptides and non-peptide molecules that can act independently of or synergistically with CRH to influence the activation of the HPA axis.

Activation of the autonomic nervous system (ANS)

In addition to an activation of the HPA axis, stressors also activate the ANS [67]. Efferent neurons of the sympathetic nervous system innervate various tissues including the heart, blood vessels, smooth muscle, lymphoid tissues as well as endocrine and exocrine glands. Thus, a threat to homeostasis caused by either a change in the internal milieu or external environment activates a variety of neural circuits that regulate a wide range of bodily functions that include respiration, cardiovascular function, digestion, body temperature, salivation, metabolism, or immune function. Most organs receive input from both divisions of the ANS, namely the parasympathetic and sympathetic divisions [467]. Because these two systems can have antagonistic effects on various target organs, the functional status of the innervated organ/tissue depends on which system is dominant at any given time [467]. For instance, during stressor exposure the activity of the sympathetic nervous system becomes more dominant, facilitating pupillary dilation, thickening of saliva, piloerection, increased respiration, mobilization of blood glucose, acceleration of the heart rate, and increased blood pressure. This constellation of physiological change is due, in large part, to the release of epinephrine from the adrenal medulla. Adrenal activation is mediated through preganglionic fibers that arise out of the lower thoracic spinal nerves and pass through the chain ganglia to terminate directly upon

the adrenal glands. Together, norepinephrine and epinephrine enhance sympathetic activity by directly activating a number of biological systems that are also under neuronal control, and although this hormonal control is slower, it is more sustained.

Like the HPA axis, the activation of the sympathetic nervous system is under the control of the central nervous system (CNS), in particular, the nucleus of the solitary tract (NTS), medulla, parabrachial nuclei, paraventricular nucleus of the hypothalamus (PVN), amygdala and prefrontal cortex all project directly and/or indirectly to the sympathetic preganglionic neurons of the intermediolateral (IML) cell column located in the thoracic and lumbar segments of the spinal cord to determine sympathetic outflow [95:329]. The central coordinating locus appears to be the PVN, which responds to incoming impulses originating from the viscera and other cortical and sub-cortical brain regions that are sensitive to change in an organism's internal and/or external environment [467]. Signals relayed to the hypothalamus, in turn, activate autonomic efferent neurons in the brain stem shifting the balance between the parasympathetic and sympathetic nervous system in favor of sympathetic action [467]. Its dominance over the activation of the body's organs is critical for preparing the organism for the fight or flight response [467]. It is important to note that although the hypothalamus acts as a central regulator of both effector systems of the stress response, it clearly activates the HPA axis and the ANS through distinct mechanisms. The consequences of activating both systems serve a unique set of functions, which ultimately enhance the organism's ability to cope with the impending danger.

The stress response: The body's homeostatic alarm

In the early 1920's, Walter B. Cannon, a physiologist from Harvard University, coined the term "homeostasis" as an organism's ability and need to maintain an internal state of constancy despite changes around it [401]. In this context, the prototypical stress response can be regarded as an alarm signal to real or perceived threats against homeostasis. The sympathetic nervous system mediated changes that occur within seconds after stressor onset serve to 1) release and transport energy stores to areas of demand, inhibit energy storage, and induce gluconeogenesis (production of glucose); 2) increase cardiovascular output to facilitate the rapid delivery of oxygen and energy to muscles; 3) stimulate of the immune system via cell translocation (trafficking) to body tissues that may be wounded; 4) inhibit reproductive physiology and behavior; 5) suppress food intake and appetite; and 6) increase vigilance and cerebral glucose utilization [388;467]. The timing of these physiological changes (seconds) suggests that they are mediated by rapidly activated second messenger systems.

On the other hand, the end product of an activated HPA axis, namely elevated glucocorticoid levels, exerts most of their effects through genomic changes, so their effects are likely more slower in onset and protracted in duration. The secretion of glucocorticoids may serve several biological functions (reviewed in [388]), including limiting the activation of the immune system [15:67:372]. In addition, via an activation of hardwired negative feedback loops, glucocorticoids limit the duration and/or magnitude of the stress response, ensuring that the biologically costly consequences of HPA axis activation are short-lived [15]. Although the precise brain loci where

glucocorticoids exert their suppressive effects on the HPA axis are not completely elucidated. it is believed that type II (GR) receptors located at the hippocampus, PVN, and pituitary gland are among the likely targets [387:481]. In concert, the cascade of events described above not only prepares the organism for the fight or flight response [15], but returns the body to homeostasis. This normally transient stress response is a very adaptive way for the organism to survive and cope with impending danger (see Figure 1).

An inappropriate stress response (blunted, exaggerated, and/or protracted) can predispose one towards adverse states of well being [303]. Hypersecretion of glucocorticoids can promote the onset of systemic diseases that include colitis, asthma, hypertension as well as affective disorders such as depression, post-traumatic stress disorder, and advance neuronal degeneration [266:267]. On the other hand, a blunted secretion of glucocorticoids during stressor exposure may insufficiently limit immune responses, result in inadequate energy availability, decrease coping behaviors, impair memory formation, and in general, lead to an inadequate defense of homeostasis [260]. It has also been suggested that a blunted stress response might impair the organism's ability to respond to subsequent stressors [388]. As alluded to earlier, the activation of the HPA axis and the sympathetic nervous system should be viewed in context with other systems that are also activated in response to stressor exposure.

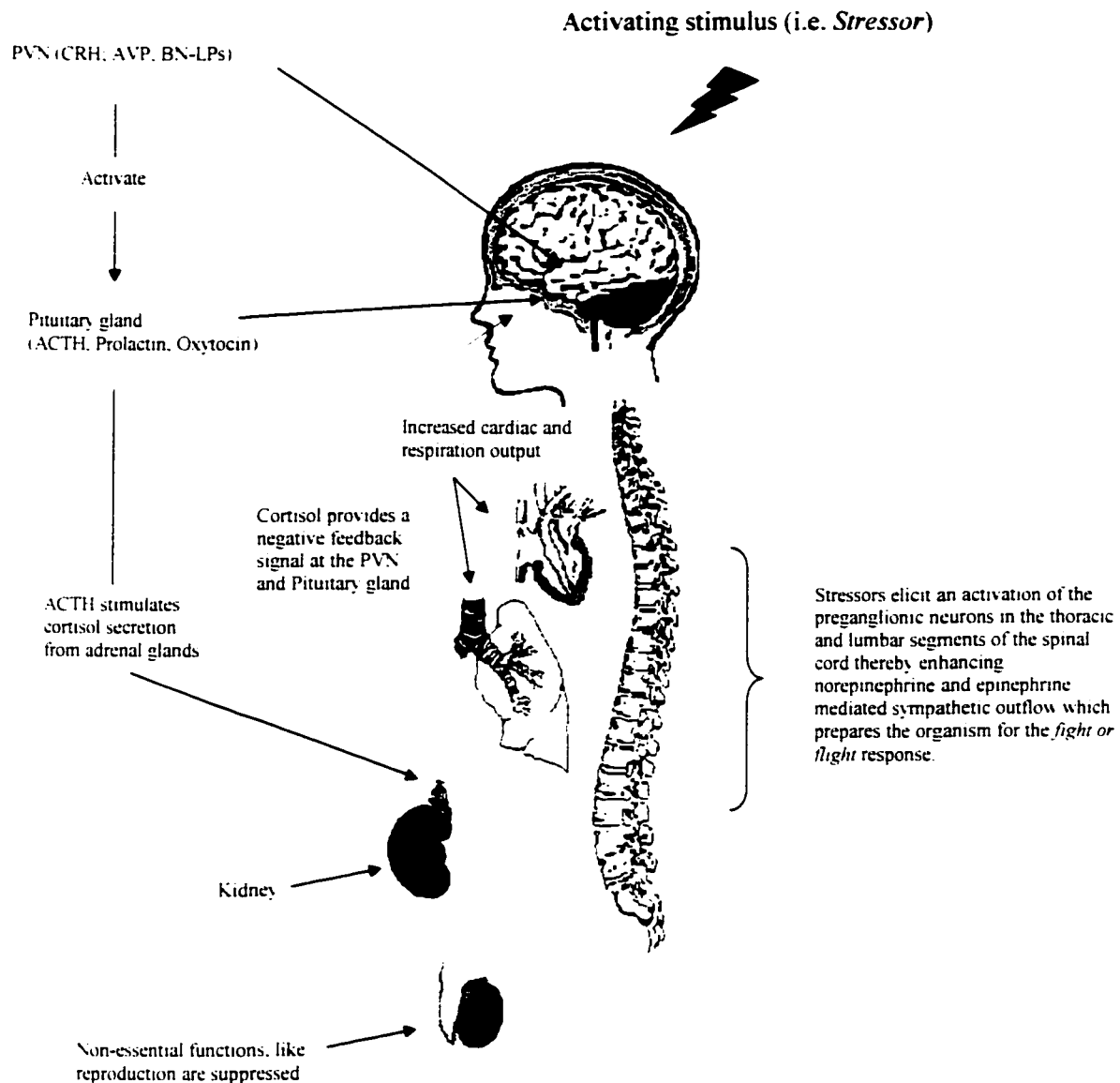


Figure 1. Schematic representation of the prototypical mammalian stress response which includes an activation of the HPA axis and sympathetic nervous system following exposure to (or in anticipation of) an activating stimulus (i.e. stressor). ACTH, adrenocorticotropin-releasing hormone; AVP, arginine-vasopressin; BN-LPs, bombesin-like peptides; CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus of the hypothalamus

Activation of the hypothalamic-pituitary gonadal (HPG) axis

The predominant female sex hormones, including estrogen and progesterone, are produced by the ovaries, whereas the predominant male sex hormone, testosterone, is primarily produced by the testes. The adrenal glands also produce testosterone (in both males and females) but to a much lesser extent than the testes. Gonadotropin releasing hormone (GnRH) cell bodies within the medial preoptic area (MPOA) of the hypothalamus play a major role in the regulation of sex hormones. The pulsatile release of GnRH from the median eminence into the portal blood system evokes the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. Interestingly, continual (rather than pulsatile) release of GnRH suppresses LH/FSH release [159]. Once released into the general circulatory system, LH and FSH travel to the gonads to stimulate the synthesis of both estrogen and progesterone or testosterone. Testosterone acts as a negative feedback signal at the level of the MPOA and pituitary gland by indirectly suppressing the firing rate of GnRH neurons and inhibiting further release of LH and FSH, respectively. The feedback signals transmitted by estrogen and progesterone either stimulate or inhibit FSH and LH, respectively, depending on the phase of the estrous cycle. Specifically, if the female is in diestrus the ovarian hormones have an inhibitory effect on the MPOA and pituitary gland, however this effect is reversed during the proestrus phase [217].

A wide variety of stressors can influence the activation of the HPG axis [7:94:252]. Like many other stress-responsive systems, the HPG axis activity is partially dependent on the duration and type of stressor, age and gender of the organism, as well as

the level of gonadal hormones at the time of stressor exposure [67]. Although chronic stressor exposure is commonly associated with a suppression of GnRH and LH, acute stressors can activate or suppress this response [67]. Accumulating research indicates that CRH and arginine vasopressin (AVP) are likely involved in the stressor-induced inhibition of the HPG axis, however it is not completely understood if the CRH/AVP-ergic control over the HPG axis arises from the PVN or some other brain site, like the MPOA where most of the GnRH synthesizing neurons reside [67:373].

Activation of the hypothalamic-pituitary thyroid (HPT) axis

Much less is known about how stressors influence the activation of the HPT axis compared to the HPA and HPG responses. In short, when thyroid-releasing hormone (TRH) (of hypothalamic origin) is released into the hypophyseal portal blood it binds to thyrotrophic cells at the anterior pituitary gland [198]. The binding of TRH to its receptors promotes the synthesis and release of thyroid-stimulating hormone (TSH) from the anterior pituitary gland [141:198]. Once released into circulation TSH binds to receptors located on epithelial cells in the thyroid gland where it promotes the synthesis and release of thyroxine (T4) and triiodothyronine (T3) [471]. Like other endocrine systems, the regulation of the HPT axis is under tight negative feedback control [233]. In this case when blood levels of thyroid hormones reach a certain level they inhibit the production of TRH and synthesis of pituitary TSH [214:233]. Conversely, a drop in circulating levels of T4 and T3 stimulates the production of TRH (and consequent secretion of TSH). It is thought that thyroid hormones influence most cells in the body [141:198]. The most widely studied effects of thyroid hormones have been related to

their ability to stimulate metabolic activity, increase lipid and carbohydrate metabolism, and influence processes related to growth and development [78:198]. In addition, thyroid hormones also augment cardiac contractility, increase vasodilation, and affect mental and reproductive functions [131:134:198:371].

The release or suppression of TSH from the anterior pituitary gland appears to be dependent on the type of stressor. For instance, restraint, ether as well as forced exercise suppress plasma TSH levels while noise, forced swim and cold exposure increase TSH plasma levels [67:219]. There has been some suggestion that the activity levels of the HPA and HPT axes oppose one another [319:479]. Not only do CRH and TRH neurons of the PVN form reciprocal connections [191], but also thyrotrophic cells at the anterior pituitary gland express AVP receptors [85] and injection of AVP into the hypothalamus rapidly suppress plasma TSH levels [265]. Furthermore, stressor-induced elevations in plasma corticosterone suppress the activation of the TRH messenger ribonucleic acid (mRNA) expression in the PVN [213]. To date there is not widespread agreement on how the HPT axis responds to stressors, specifically what types of stressors evoke its activation or suppression. It has, however, been suggested that the activation of this system may be unique to stressors which alter an organisms metabolic rate [67].

In conclusion, although the prototypical stress response is viewed as a concomitant activation of the HPA axis and sympathetic nervous system, it is important to understand this response in the context of other stressor responsive systems, like the HPG and HPT axes. Taken together, an organism's ability to survive in the face of real or

perceived danger is to a large part determined by successful mounting of appropriate and adequate endocrine, autonomic and behavioral responses, without exhausting resources during activation of such biologically costly adjustments. Inadequate responses can often be detrimental or fatal (e.g. falling victim to the predator or developing stress-related disorders).

The classical neurotransmitters and the stress response

Twenty years ago Anisman and Zacharko (1982) reviewed the evidence supporting an association between stressor-induced disruptions in neural transmission of brain circuits using classical neurotransmitters, including serotonin, norepinephrine and dopamine, and the onset of mood disorders, particularly depression [16]. Since then, accumulating research has continued to strengthen the contention that stressors exacerbate mood disorders. Indeed, several clinical treatments that target the brain monoaminergic systems alleviate many symptoms associated with stress-related ailments (i.e. anxiety, panic, depression, social phobia, and eating disorders) [281:427]. Although the focal interest of this dissertation is the role of neuropeptides in the coordination or mediation of the stress response, it should be borne in mind that the classical neurotransmitters (that are affected by stressors) may also influence peptidergic functioning. There is voluminous literature dealing with each of these monoaminergic transmitters in context of stress research, and will be reviewed only briefly.

Dopamine

The deployment of a successful coping response depends upon first being able to appropriately *identify* the stimulus as being potentially harmful, and then eliminating or minimizing exposure to it. This contrasts with responses to potentially pleasurable/beneficial stimuli, where the tendency is to approach them. Thus the propensity to approach or to avoid a stimulus is partly a consequence of emotional salience attributed to the stimulus [42:43:493]. The potential involvement of the dopaminergic system(s) in processes attributing emotional salience to various stimuli [42:43:47], makes this system(s) of particular interest in the context of the stress response.

Neuronal cell bodies synthesizing dopamine are well identified and appear to be localized predominantly at the ventral tegmental area (VTA) and substantia nigra. The nigrostriatal system consists of dopaminergic projections from the substantia nigra to the neostriatum and plays a role in the coordination of movement [76]. The mesolimbic and mesocortical systems comprise, respectively, of projections from the VTA to limbic structures (nucleus accumbens (nAcb), amygdala, hippocampus) and frontal cortex. These limbic projections have long been suspected to be involved in the genesis of the cognitive and behavioral response to stressors [138:310:443]. A fourth dopaminergic system is the tuberoinfundibular system, which projects from the hypothalamus to the pituitary, exerting an inhibitory action over prolactin secretion [36:37]. In context of the stress response the balance between dopaminergic activity at the cortex and limbic regions, seems particularly important.

While several environmental perturbations evoke the release of dopamine from terminal buttons at the medial prefrontal cortex (mPFC), no change, or a slight drop, in dopamine release at the nAcb typically accompanies elevated release at the cortex [138]. This heterogeneous response is presumably mediated through a dopamine-induced inhibition of glutamatergic transmission in pyramidal neurons projecting from the mPFC to the nAcb [126]. It is of interest to note that dopamine-depleting lesions restricted to the mPFC potentiate stressor-induced dopamine release at the shell of the nAcb [227]. This provides a neurochemical and neuroanatomical substratum for the observation that stressor exposure (both acute and chronic) interferes with an animal's propensity to engage in appetitive behaviors. For example, stressors are known to obstruct several aspects of copulatory behavior and reduce the ingestion of a palatable snack: both of which are influenced by dopaminergic systems [127:199:338].

Research in rodents and non-human primates suggests that it is not the release of dopamine at the mPFC *per se* but rather the *absolute* amount of dopamine released that influences an animal's cognitive assessment of environmental stimuli [315]. Thus, optimal cognitive functioning may be impeded by stressor-induced excessive dopamine release at the mPFC. Of note, in this regard, is the observation that exposure of a bystander to a foot shocked cohort selectively evokes the release of dopamine at the mPFC without affecting the release of serotonin or norepinephrine [338]. This, together with the evidence that stressor exposure can sensitize dopamine release to subsequent stressors (with long-term repercussions on behavioral outputs [41:165:361:501]), underscores the possibility that dopamine may play a functional role in the

etiopathogenesis of stress-related disorders in humans [138]. For instance, prolactin secretion from the anterior pituitary gland is under the tonic inhibition of tuberoinfundibular dopamine neurons in the arcuate nucleus, so that a stressor-induced suppression of these dopamine neurons (and subsequent disinhibition of prolactin secretion) might underscore conditions such as hyperprolactinemia [147]. Similarly, some authors have suggested that an interaction between the HPA axis and the dopaminergic systems may underlie features common to certain mental illnesses [395].

There is some evidence that the neurochemical mechanisms underlying stressor-induced exacerbation of mood disorders may arise from a change in the functioning of the HPA axis. Indeed, some animal research and clinical observations support the notion that hyperactivity within the HPA axis might augment dopamine transmission in the brain. For example, a transient activation of the HPA axis (by CRH and/or ACTH injections) consistently produces an increase in plasma homovanillic acid levels (a dopamine metabolite) the following day [358;359]. In rodents, glucocorticoid treatment evokes the release of dopamine at the nAcb, hypothalamus, and caudate nucleus, but not at the mPFC or the striatum [382;489].

In conclusion, it appears that there is functional cross talk between the HPA axis and dopaminergic neurotransmission, such that elevated HPA activity can produce a delayed elevation in dopamine turnover. These findings suggest that stressor-elicited HPA activation might affect dopaminergic responses, possibly impacting upon the etiology of psychiatric disorders.

Epinephrine

Stressor-elicited activation of the sympathetic nervous system provokes the secretion of adrenal medullary hormones, namely epinephrine and norepinephrine, into the general circulation. As outlined earlier, elevated epinephrine may serve many functions, including the preparation for the fight or flight response (see above). In the brain, catecholaminergic cell bodies are located in the ventrolateral medulla (i.e. C1 cell group), and project to the PVN and supraoptic hypothalamic cell bodies [97], and spinal cord where they innervate sympathetic preganglionic neurons in the IML cell column [380]. Adrenergic neurons are also located within the dorsomedial medulla (i.e. C2 cell bodies): where they give rise to axons that terminate at the PVN, lateral hypothalamus, and CeA [336]. Consistent with the fact that norepinephrine levels in the brain far exceed that of epinephrine, is the assertion that epinephrine in the brain plays a minor role in the stress response, at least compared to that of norepinephrine and that ascribed to its function during sympathetic activation [76]. Still, acute stressor exposure has been reported to produce a protracted (up to 24-hr) decrease in hypothalamic epinephrine levels, while a 21-day chronic stressor exposure was associated with increased hypothalamic tissue epinephrine content, an effect attributed to altered utilization and turnover [381].

Norepinephrine

Whereas epinephrine is the predominant catecholamine released from the adrenal medulla, the primary neurotransmitter of the post-ganglionic nerves of the sympathetic nervous system is norepinephrine. Its release is associated with many of the bodily

changes that are under the control of this branch of the stress response (see above discussion). In the brain, noradrenergic projections are extensive and have been implicated in the psychological, physiological and behavioral responses to stressor exposure [102:409:442]. The firing rate of the locus coeruleus (the major source of norepinephrine cell bodies) [104:431] is augmented in response to aversive stimuli [209:409]. Several lines of evidence indicate this elevated activity of the locus coeruleus evokes the release of norepinephrine across a wide range of brain regions including those implicated in the stress response, such as the PVN [103], amygdala [442], frontal cortex [391], and the hippocampus [331]. In several mammalian species the activity level of the locus coeruleus corresponds to the behavioral state of the animal. Thus, when the animal is most active, the noradrenergic neurons of the locus coeruleus are at their peak and quiescent when the animal is at rest or sleeping [1:20:21].

Stressor-elicited release of pituitary hormones (ACTH, TSH, and prolactin) may be under the control of noradrenergic tone at the PVN [103]. Indeed, the PVN is densely innervated by noradrenergic terminals [98] and receives a steady supply of norepinephrine [234:335]. Several *in vivo* microdialysis experiments have demonstrated that norepinephrine release is increased at the PVN during stressor exposure [325:447] and indirectly evokes an increase in plasma ACTH levels by stimulating adrenergic receptors located on CRH neurons ([111:234] and reviewed in [357]). Norepinephrine may also be associated with the stressor-induced increase in prolactin secretion through its ability to inhibit the activation of dopamine neurons in the hypothalamus (which, as discussed above, provide tonic inhibition over prolactin secretion) [36:37].

In addition to influencing the endocrine and autonomic aspects of the stress response, noradrenergic neurotransmission in the frontal cortex and hippocampus is likely involved in the integration of cognitive and memory aspects of the stress response [185:232:390]. This contention is supported by the finding that the activity of neurons at the locus coeruleus rapidly habituate to the repeated presentation of the same stimulus, suggesting that their firing rate is, at least in part, under the control of brain areas that are involved in the attribution of the novelty or meaning to a stimulus [391]. Moreover, this effect cannot be attributed to decreased neuronal sensitivity since the presentation of a heterotypic stressor following desensitization has been shown to evoke a robust increase in locus coeruleus activity [1:392]. In this regard, the finding that activation of the PFC inhibits the activity of the locus coeruleus is of particular interest [391]. The observation that cortical activation can suppress the response of the locus coeruleus underscores a pathway through which cognitive appraisal of an event might influence the stress response. Stressor exposure likely engages several aspects of memory including recall and/or consolidation [152:405-407]. The stressor-elicited increase in norepinephrine transmission at the hippocampus suggests that norepinephrine may be involved in these processes [232]. Likewise, cues previously paired with aversive stimuli, activate the locus coeruleus [389:392], and consequently regions innervated by its projections.

Serotonin

Serotonin in the brain is largely synthesized in regions located in the raphe nuclei of the midbrain, pons, and medulla [76] and serotonergic projections are widely diffused within the brain [76]. Based on stressor-induced activation of immediate early genes

within the serotonergic cell bodies [237:357], it would appear that the dorsal and medial sub-nuclei of the raphe are particularly sensitive to stressful stimuli [81]. Indeed, these areas project extensively to brain regions implicated in the stress response, including the frontal cortex, amygdala, hypothalamus, locus coeruleus, and hippocampus [81:348:409:410]. The activation of serotonin cell bodies in the dorsal and/or medial raphe nuclei evoke the release of serotonin from nerve terminals at these regions in response to a wide variety of stressors including restraint, cold/noise exposure, predator exposure, forced swim and immune challenge [2:212:220:264]. Also interesting is the possibility that some aspects of the HPA axis may influence the serotonin response to stressors.

It has been suggested that stressor exposure may elicit changes in the CRH-ergic systems in hypothalamic and limbic structures, which may then affect serotonin networks. For instance, stressor-elicited release of CRH may stimulate activity at the locus coeruleus [458:460]. In support of this contention, microinjections of CRH into the dorsal raphe nucleus alters neuronal firing rate in a dose-dependent manner. This is an inverted-U dose response curve such that at low doses of CRH inhibit neuronal output, while higher doses are associated with elevated neuronal discharge [230]. Moreover, serotonergic synaptic contacts at the PVN arising from the dorsal raphe have been shown to elicit an excitatory effect on the release of CRH into the hypophyseal portal blood system thereby providing a direct pathway through which serotonin could activate the HPA axis during stressor exposure [239]. In contrast to the well-established negative feedback signal provided by corticosterone on the HPA axis, there is no strong consensus

regarding such a modulatory role at the level of the raphé nuclei on serotonergic transmission [81]. Although some authors suggest a glucocorticoid mediated up regulation of serotonin receptors in the brain regions implicated in stress [241:242:441], this possibility remains equivocal [80:82]. Dysfunction in the brain serotonergic system(s) has thus been implicated in a variety of stress-related disorders including anorexia nervosa [178] depression, anxiety [228:229:264] and suicidal behavior [140:416:450].

In conclusion, there is ample evidence from both animal experimental and clinical research that dopamine, serotonin and norepinephrine are influenced by stressor exposure and that these changes might underlie mediation of the stress response. There is also the likelihood that they may be relevant in the etiology of certain stress-related disorders.

Neuropeptides implicated in the stress response

Synthesis and release

Neuropeptides are synthesized through a series of transcription and translation processes, whereby large precursor molecules are formed and packaged into storage vesicles in the soma of the neuron. Axoplasmic flow carries these vesicles down the nerve axon, during which time post-translational processes convert these larger prohormones into mature/active neuropeptides. At the nerve terminals, neuropeptides are stored in vesicles until specific stimuli trigger their release [67]. When released, neuropeptides can function as neurohormones (traveling through the blood to act upon distant organs/tissues), neuromodulators (released into the extracellular fluid in the brain

to influence a wide range of neurons) or as neurotransmitters (released from axon terminals to act on receptors within close vicinity) [222]. This discussion will be restricted to the role of neuropeptides in response to stressors.

Corticotropin-releasing hormone (CRH)

Although the existence of a secretagogue that could stimulate the release of pituitary ACTH had long been predicted [168], its identity remained a mystery for several years. In 1981, Dr. Wylie Vale and his colleagues successfully identified this “releasing factor” as being a 41 amino acid polypeptide, and initially called it corticotropin-releasing factor (CRF) [453]. Since then, the molecular biology, including the gene encoding this peptide has been characterized, as has its role as being the primary secretagogue for ACTH: as such it is more commonly referred to as corticotropin-releasing hormone (CRH) [29:45]. However, CRH and CRF continue to be used interchangeably in the literature.

Central administration of CRH dose-dependently produces endocrine, autonomic and behavioral changes which resemble those elicited by stressor exposure [129:326:393]. For instance, intracerebroventricular (ICV) administration of CRH elevates plasma ACTH and β -endorphin levels, while it suppresses the release of growth hormone [323]. Likewise, ICV CRH activates the sympathetic-mediated increase in plasma norepinephrine, epinephrine, glucose, and glucagon [95]. Behaviorally, the effects of CRH appear to be situation-dependent. Thus, the same dose of CRH administered to a rat in a familiar (presumably unstressful) environment will increase the

output of a variety of behaviors (grooming, exploration, rearing) but will decrease them in a novel (presumably stressful) environment [393:412]. It is interesting also that the same context-dependent activation of c-fos mRNA has been noted in the CeA in rats treated with cocaine or amphetamine in their home vs. novel cage [110]. Day and colleagues [110] showed that amphetamine pretreatment in animals exposed to a novel cage reduced c-fos activation at the CeA, compared to that observed in animals housed in their home cages [110]. The CRH-associated behavioral effects are likely mediated centrally since peripherally administered CRH antiserum, dexamethasone, or hypophysectomy did not alter these effects [55:132:301]. Conversely, all of the effects associated with exogenously administered CRH (endocrine, autonomic, and behavioral) are either attenuated or blocked by the central pretreatment with CRH antiserum or antagonists [56:58:374]. It should be borne in mind however that this effect does not unequivocally prove that the release of endogenous CRH mediates the responses to stressors. For instance, new evidence derived from genetically altered mice devoid of CRH-producing gene (resulting in a complete CRH deficiency) indicates that although the endocrine (ACTH and corticosterone) and certain autonomic (plasma epinephrine) responses to stressors are dramatically blunted in these animals, their behavioral response to stressors remains intact and comparable to that of wild-type control mice [313:475]. Although results obtained using genetic manipulation should be interpreted with caution, the possibility that CRH is not involved in the genesis of stressor-induced behaviors should be considered in light of these findings and subjected to further testing.

In order to assess the potential involvement of the CRH systems in the mediation or modulation of the stress response, several studies have assessed the stressor-associated fluctuations of this peptide at specific brain sites. To this end, numerous investigators have exploited the micropunch technique originally described by Palkovits and Brownstein [334] together with the radioimmunoassay (RIA) technique (to measure fluctuations in peptide content). In addition, *in situ* hybridization studies have been used to map the fluctuations in synthetic capacity following exposure to stressors [29:231:238]. Although these techniques only afford an indirect indication of peptide utilization (discussed below) other approaches, particularly *in vivo* techniques (that directly assess neurochemical release), have shown that aversive conditions are associated with enhanced release of immunoreactive (ir)-CRH at various stress-relevant brain regions (e.g. PVN, median eminence and amygdala) [204:297:351:369].

Neurons containing CRH are widely distributed within the CNS, the highest concentrations being present at the amygdala, hypothalamus, and bed nucleus of the stria terminalis (BNST) [50:300:434]. Tract-tracing studies have shown that CRH containing neurons of the parvocellular division of the PVN project to the external zone of the median eminence [6], where released CRH enters the portal blood system, eventually reaching the anterior pituitary. There, it stimulates the CRH receptors located on the corticotrophs, triggering the release of ACTH, which stimulates the release of glucocorticoids from the adrenal cortex: a cascade of neuroendocrine changes characteristic of the stress response (see above and Figure 1) [95]. In addition to its neurohormone properties, CRH is believed to serve as a neurotransmitter within the brain

[457]. In this context, several indirect approaches sustain the contention that CRH neurons at loci other than the PVN, serve non-endocrine functions. For instance, at the amygdala CRH is thought to mediate some of the behavioral and autonomic activation effects, the latter via projections to the locus coeruleus [349:458:459]. Similarly, *in situ* hybridization studies have localized CRH producing neurons in lower brain stem regions, like Barrington's nucleus. Furthermore, tract-tracing experiments have shown that these neurons project to the lumbosacral spinal cord, the periaqueductal gray, dorsal motor nucleus of the vagus, and locus coeruleus, where they presumably utilize CRH as a neurotransmitter to influence the autonomic and behavioral responses to stressor exposure [456]. Indeed, assessing the possibility that endogenous CRH is released at the locus coeruleus during stressor exposure was one of the objectives addressed in this thesis.

The receptors through which CRH mediates its effects has been the focus of several investigations. Thus far, two major forms or subtypes of this receptor have been identified, namely CRH-type1 and CRH-type2. Their heterogeneous distribution in the brain suggests a different role for each receptor subtype [463:492]. Centrally administered CRH appears to induce c-fos mRNA activation at brain regions that predominantly express a CRH-type1 receptor. This is consistent with *in vitro* binding studies showing that CRH has a greater affinity for this receptor over the CRH-type2 form [46]. The anterior pituitary predominantly expresses CRH-type1 receptor, suggesting that the CRH-induced activation of the endocrine arm of the HPA axis is mediated through these receptors [95]. The same receptor primacy is observed in other brain structures.

most notably the cortex, olfactory bulbs, as well as several midbrain and brain stem nuclei (reviewed in [46]). The CRH-type2 receptor has been identified in four different forms (α , α -tr, β , δ). The CRH-type2 α receptor is most similar to CRH-type1 (71% amino acid homology) and is synthesized primarily in the lateral septum and hypothalamus [262], while CRH-type2 β receptor is located mostly in the periphery with moderate levels being expressed in the choroid plexus [79:261]. To date, the CRH-type2 δ receptor has been cloned exclusively in human brain tissue and does not appear to exist in the rodent [236]. The most recent receptor variant to be cloned in the rat brain is the short splice form of the CRH-type2 α receptor, denoted CRH-type2 α -tr [305]. This receptor subtype has been located in brain structures that do not express the α subtype, suggesting a possible unique pharmacology for this receptor: however this is at present unknown [393].

In addition to these receptors, CRH also binds with an equal or greater affinity to CRH binding protein (CRH-BP) [68:399:451]. The distribution of CRH-BP throughout the brain overlaps to a large extent with brain regions rich in both CRH- and CRH-receptors [258:360:399]. Because CRH bound to its binding protein loses its affinity for the functional CRH receptors [232], it is believed that the induction of CRH-BP and its interaction with CRH serves to modulate the activity of the CRH-ergic system: modulating the bio-availability of free (or bioactive) levels of the peptide [232:399]. For example, the *in vitro* administration of CRH-BP to pituitary tissue cultures blocks CRH-elicited increases in ACTH release [89:346] and aversive conditions like restraint, cytokine loading, and food deprivation all increase the expression of CRH-BP mRNA

and immunoreactive CRH-BP in brain regions involved in maintaining homeostasis of the HPA axis during the stress response [284:399]. In essence, CRH-BP can be thought of as an endogenous CRH antagonist.

It is important to mention that in addition to CRH, other mammalian CRH-like peptides, including the 40-amino acid polypeptide urocortin (the mammalian counterpart to fish urotensin), may act in concert with or independent of CRH, on CRH receptors and likewise be regulated by CRH-BP. In this respect, urocortin is of particular interest because it has a greater affinity for both CRH receptors (especially CRH-type2) and CRH-BP, compared to CRH itself [385]. However, because the synthesis of urocortin is relatively restricted to the Edinger-Westphal nucleus in the midbrain, if it does function similar to CRH in response to stressor exposure it must do so through an as yet unidentified pathway(s) [476]. On the other hand, there has been some indication that urocortin might be synthesized in the anterior pituitary where it could bind to CRH-type1 receptors to elicit ACTH release. This could conceivably mediate the endocrine response to stressors and/or modulate the HPA axis in the absence of CRH [490]. Despite the uncertainty surrounding the role of urocortin in the stress response, evidence suggests that CRH may be the primary ACTH secretagogue, however, other peptides (such as AVP) may work synergistically with, or independently of CRH to influence ACTH release [341:497].

Arginine-Vasopressin (AVP)

Arginine-vasopressin was synthesized and characterized long before the discovery of CRH and was thought at one time to be the unknown hypothalamic factor controlling the stimulation of ACTH release [280:283]. Although research has assigned this role to CRH, there is still a great deal of interest in AVP as a stressor-responsive neuropeptide. Like CRH, AVP mRNA and protein is widely distributed throughout hypothalamic and extra-hypothalamic brain regions [87:174:184] and exerts its effects in the brain and pituitary by activating AVP_{1A} or AVP_{1B} receptors, respectively. While the distribution of AVP_{1A} receptors is diffuse throughout the CNS (olfactory bulb, hypothalamus, BNST, NTS, amygdala, locus coeruleus and spinal cord) [324], AVP_{1B} receptors are primarily expressed at the pituitary gland [17] although some reports now indicate AVP_{1B} mRNA is expressed beyond the pituitary corticotrophs [257:383]. Therefore, while both receptors may be involved in the behavioral and autonomic stress responses: it appears that AVP induces an activation of ACTH release strictly through AVP_{1B} receptors.

Even though AVP is produced to a great extent in magnocellular neurons of the PVN, these neurons (by way of the internal zone of the median eminence) activate the release of hormones from the posterior lobe of the pituitary gland in response to fluid and electrolyte imbalances [222]. Of particular interest in the context of stress are the AVP synthesizing neurons of the parvocellular division of the PVN [469]. At this level, these neurons send their axons primarily to the external zone of the median eminence [6], and like CRH, give rise to the secretion of AVP into the hypophyseal portal blood system [184] where it can act independently, or in synergy with CRH, to stimulate ACTH release

[18:156:478]. On its own AVP has at best, a very weak effect on ACTH release, compared to CRH [239] but acts synergistically with CRH to provoke ACTH release. Thus AVP may be especially important in *modulating* the HPA axis in response to stressful stimuli. For example, stressors not only stimulate AVP mRNA in the parvocellular PVN neurons that co-localize CRH mRNA, but they also increase the concentration of the peptide in CRH terminal fields at the median eminence [29]. The increase in AVP levels at the median eminence following chronic stressor exposure contrasts the reduction in CRH at this region, suggesting that AVP may act to maintain the integrity of the HPA axis during periods of excessive demand when CRH stores may be depleted [29]. In addition to its ability to influence the endocrine arm of the stress response, AVP appears to be involved in stressor-elicited autonomic activation.

In freely moving rodents, centrally injected AVP dose-dependently increases sympathetically driven changes, reminiscent of those induced by stressor exposure (i.e. elevated heart rate, blood pressure, and plasma norepinephrine/epinephrine) [376:499]. These responses may be mediated, in part, through the AVP-ergic neurons of the parvocellular PVN. Indeed, intra-PVN injection of glutamate evokes sympathetic activation that is blocked by intrathecally administered AVP_{1A/B} antagonist [275:278]. Other brain regions like the NTS and dorsal vagal complex are likely involved in sympathetic outflow since pretreatment with AVP_{1A/B} receptor antagonists in these regions prevents the cardiac changes in response to PVN stimulation [95]. As has been speculated for CRH, the AVP-evoked cardiovascular changes may be mediated via changes in locus coeruleus neuronal discharge. Indeed, AVP microinjected into the locus

coeruleus increases mean arterial blood pressure and heart rate while these effects are blocked in animals pretreated with an AVP_{1A/B} antagonist in the locus coeruleus [38]. The evidence that AVP is implicated in the genesis of stressor-induced behavioral changes is also growing.

As mentioned earlier, memory system(s) play an important role in the launching of adequate responses to threatening stimuli, especially if the coping strategies deployed were successful during the initial exposure to that (or a similar) stressor. In this context, the effects of AVP are interesting, as its administration prior to, or immediately following a learning task facilitates the consolidation of information processing in a conditioned avoidance paradigm [117]. Similarly, the Brattleboro rat (which lacks AVP due to a defective AVP producing gene) [271] displays deficits in active/passive avoidance tests [118] and conditioned freezing [423:424]. In addition to learning, AVP appears to be involved in stressor-induced grooming [69] and locomotion in a familiar environment [123]. Moreover, these behavioral responses to AVP are blocked by pretreatment with AVP_{1A/B} receptor antagonists, including measures of anxiety assessed on the elevated plus maze [246:256].

At the beginning of this section it was highlighted that neuropeptides are multifunctional neurochemicals that mediate several functions beyond the stress response. This is true of both CRH and AVP and equally apparent for bombesin (BN)-like peptides (BN-LPs), which have been well characterized as a satiety peptide [294:354:356]. More recently, however, we have come to realize that this family of

peptides may also be involved in the mediation of stressor-related brain signals [222:223:274:297].

Bombesin-like peptides (BN-LPs)

Bombesin is an amphibian-expressed tetradecapeptide originally isolated in 1971 [9]. In mammals, both peripherally and centrally injected BN evoke a robust dose-dependent suppression of food intake [154:155:289] and intravenous injections in humans produce similar satiety-like effects [316]. While BN itself does not exist in mammals, there are at least two BN-LPs that appear to be the mammalian counterparts to amphibian BN. In the context of the mammalian stress response, the most relevant BN-LPs appear to be the structurally related neuropeptides, gastrin releasing peptide (GRP) (GRP₁₋₂₇ and its truncated form, GRP₁₈₋₂₇; sometimes called neuromedin C (NMC)), and neuromedin B (NMB) which is also expressed in two forms (NMB₁₋₃₂ and NMB₂₃₋₃₂) [79]. Neurons expressing BN-LPs are extensively distributed within the CNS [223] [448]. The fact that these peptides are present within various stress-relevant regions, including the PVN, MPOA, amygdaloid nuclei, arcuate nucleus, median eminence, pituitary, NTS, hippocampus, locus coeruleus, and olfactory bulbs, supports the notion that they might be involved in the mediation or expression of the stress response [61:62:91:356].

The molecular mapping studies tracing the distribution of mRNA for BN-LPs reveal that GRP synthesizing cell bodies are dominant at the parvocellular PVN, suprachiasmatic and supraoptic nuclei, BNST, amygdaloid nuclei, and hippocampus

while NMB mRNA appears to be predominantly expressed at the anterior pituitary gland with traces in other brain regions, including the dorsal raphe [196].

In terms of receptors for this family of peptides, four distinct receptor subtypes have thus far been identified and cloned in mammals. According to the new standardized nomenclature, these are identified as BB₁, BB₂, BB₃ and BB₄. Gastrin-releasing peptide and NMB likely exert their effects by activating either the BB₁ (NMB) and/or BB₂ (GRP) receptors. The BB₃ and BB₄ receptors are designated as orphan receptors, as their natural ligands remain unidentified to date [277:475]. Using appropriate molecular probes, the BB₁ and BB₂ receptors have been localized within anatomical loci constituting the HPA axis (i.e. PVN, median eminence, and pituitary), as well as other extra-hypothalamic brain regions such as the locus coeruleus, NTS, BNST, hippocampus and amygdala [491]. The ratio of BB₁:BB₂ receptors appears to match the relative preponderance of NMB and GRP, respectively (see above) and suggests that endogenously released BN-LPs may generate some of the endocrine, autonomic, and behavioral changes that occur during stressor exposure [491].

It has been suggested that the BN-LPs might share membership in the class of so called "stress peptides" [223-225:297]. In keeping with this notion, a wide variety of stressors have been reported to affect changes in tissue levels of BN-LPs as well as in the density of their receptors, particularly at some of the stress relevant brain regions [223:295:448]. In addition, centrally applied BN 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 [63:65:223]. Mapping studies have suggested that the NTS is particularly sensitive to BN-induced sympathetic activation [77]. The NTS gives rise to fibers projecting to the dorsal vagal complex (where BN-LP terminals have been localized [91]) and retrograde tract tracing experiments combined with radiolabeling reveal that the source of BN-LPs identified at the dorsal vagal complex originates from BN-LP synthesizing neurons at the parvocellular PVN [91]. Thus, stimuli that elicit an activation of the sympathetic nervous system may do so, in part, through BN-LPs at the PVN (similar to that described for CRH and AVP). In this regard, there is evidence to suggest that BN-LPs may exert their central effects, at least in part, through an activation of CRH receptors [224:225]. Specifically, BN's ability to dose-dependently activate the HPA axis and sympathetic nervous system is attenuated in animals pretreated with α -helical CRH (a non-selective CRH receptor antagonist) [223]. Similarly, Garrido et al. (1998) demonstrated that α -helical CRH pretreatment blocked GRP-induced plasma ACTH and corticosterone [153]. Also, Olsen et al. (1992) reported that GRP pretreatment potentiated CRH-induced ACTH secretion [322]. More direct studies have shown that mild stressors (e.g. air puff stressor) is associated with an increased availability of GRP, NMB, CRH and AVP in perfusates collected from the anterior pituitary gland [222]. Similarly, microinfusion of BN into the 4th ventricle has been shown to evoke an increase in the availability of CRH and AVP at the median eminence and the anterior pituitary gland (detected by push-pull perfusion) [222]. Taken together, these results support the notion that BN-LPs (along with other well-characterized stress peptides) may be important in the genesis of the endocrine and autonomic responses to stressors. Furthermore, exogenously

administered BN also evokes behavioral changes reminiscent of those observed following stressor exposure. These include the suppression of food intake, increased grooming, increased exploration in a familiar environment, and decreased exploration in a novel environment [91:240:299]. Indeed, some of these effects (e.g. grooming and satiety) are suppressed in animals pretreated with CRH antagonists. Although this research is still in its infancy, there is mounting evidence indicating that there exists a functional interplay between BN-LPs and CRH in stressor-related responses [224].

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 neuropeptides are involved in orchestrating the endocrine, behavioral and autonomic aspects of the stress response.

A consistent change in a neuropeptidergic system (reflected by mRNA or tissue peptide levels, peptide release or changes in the receptor affinity or density), in response to a range of stressors, implicate that system in the stress response. Although this review focused primarily on CRH, AVP and BN-LPs, it does not preclude the potential role of other peptides like oxytocin, somatostatin, prolactin, growth hormone, neurotensin, substance P, cholecystokinin, and opioid peptides, in the mediation of the stress response. Most of these neuropeptides have been localized in stress relevant brain regions and respond to certain stressors and in some cases, modulate the responses of one another (reviewed in [45]). Indeed, many of these neuropeptides are sometimes co-localized

within the same nerve terminals with other peptides and/or monoamines, where they can interact in a neuromodulatory manner.

Role of the amygdaloid complex in the stress response

The amygdaloid complex is located within the medial aspect of the temporal lobes, and forms part of the mammalian limbic system. Neuroanatomical investigations have revealed that the amygdala is intricately connected to brain regions involved in the orchestration of the stress response. More notably are the reciprocal projections to and from the mPFC, PVN, hippocampus, parabrachial nucleus, VTA, locus coeruleus, NTS and dorsal vagal complex that have been well characterized [8:106:163:248]. These studies provide anatomical support to the concept that this nuclear complex likely plays a role in the cognitive, endocrine, and autonomic responses to stressors. In addition to the vast afferent/efferent projections, there are also complex inter-amygdaloid connections [432]. Structurally, the amygdala consists of a heterogeneous group of at least 17 different sub-nuclei containing unique afferent and efferent projections that appear to serve different functions [130:249:432:466]. The four most widely investigated amygdaloid nuclei are the medial (MeA), basolateral (BLA), basal (BA), and central (CeA) nuclei. A plethora of studies, involving human and animal subjects, suggest that the amygdala plays an important role in emotional responses [318:344].

In humans, electrical stimulation of the amygdaloid complex produces subjective feelings of fear, which do not occur when electrical stimulation is restricted to the hypothalamus [76]. Similarly, the normal emotional reaction that accompanies aversive

stimuli is absent in people with damage to the amygdala [344]. Likewise, these subjects also fail to acquire a conditioned emotional response, so that previously neutral tones paired with aversive stimuli do not by themselves generate an emotional response in subjects with damage to the amygdala, as they do in control subjects [344]. Of note, however, in these studies, amygdaloid damage did not alter unconditioned reactions. Finally, visual imaging techniques, like positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) consistently reveal enhanced neuronal activity at the amygdala in people undergoing conditioned fear acquisition, recalling violent events, or during subjective feelings of frustration [70:244:397]. These studies demonstrate the importance of the amygdaloid complex in emotional responding in humans, but preclude strong conclusions regarding the role of the specific sub-nuclei, or the underlying neurochemical signals involved in amygdaloid function.

Clinical findings together with diverse animal experimental studies have led to a better understanding of the neurochemical underpinnings of the stress response. Several of these approaches have focused on the role of specific amygdaloid nuclei, in the stress response. Likewise, considerable advances have been made towards identifying the neurochemical substrates at the amygdaloid complex and how stressors influence these systems. Indeed, electrical and/or chemical stimulation of the CeA evokes behavioral, endocrine and autonomic changes reminiscent of those that occur during stressor exposure. These responses include behavioral changes, increased cortical, HPA and sympathetic activity [33:71:130:421]. Conversely, lesioning, or temporarily cooling this structure attenuates or prevents stressors from eliciting all of these parameters

[31:144:284:500] and blocks the acquisition of amphetamine-induced conditioned place preference [134].

The CeA is heavily innervated by extraneous terminals as well as interneurons, and contains various neurotransmitters and/or neuromodulators. Thus, present at the CeA are locally synthesized and innervating neuropeptides (somatostatin, neurotensin, BN-IPs, CRH, and AVP), incoming monoaminergic signals (serotonin, dopamine, norepinephrine) and locally produced and released excitatory (glutamate) and inhibitory (GABA) transmitters [49:162:470:485]. Many of these signaling molecules are colocalized in nerve terminals at the CeA where they may interact to influence the net impact on the receiving neurons [31:124:332:337]. Data continues to accumulate in support of the contention that some of the behavioral, endocrine, and/or autonomic responses associated with amygdaloid manipulations may be directly/indirectly mediated through changes in CRH activity.

It is well established that the endocrine response to stressors is mediated by CRH at the PVN. However, it is thought that CRH may also orchestrate the autonomic and some of the behavioral responses to stressors. However, such effects of CRH are likely mediated via extrahypothalamic brain regions. Several studies have attempted to localize the site(s) of such CRH mediated effects. In this context, CRH, CRH mRNA and all three known CRH binding sites (i.e. CRH-type1&2 receptors & CRH-BP) are prominent at the CeA [258:360:437:495]. Localized microinjection of CRH not only provokes neuronal activation of the CeA [46] but also dose-dependently elicits behavioral.

endocrine, and autonomic responses reminiscent of those provoked by stressors [171:482:484]. Moreover, localized administration of CRH antagonists at the CeA attenuates or blocks many of the responses to CRH and/or stressor exposure [171:255:437]. Finally, aversive conditions, including withdrawal from cocaine, alcohol or cannabinoids are associated with elevated CRH release at the CeA as measured by *in vivo* microdialysis [351:369:370:375]. In these latter studies, however, it should be noted that the perfusion medium contained a CRH antibody and may have exaggerated the CRH gradient across the microdialysis membrane, confounding the "normal" release dynamics of this peptide. Taken together, these results provide strong evidence in support of the contention that CRH released locally at the CeA and/or at CeA target sites, may play an important role in orchestrating the stress response. Contrary to this perspective, however, some of the other studies have failed to observe the expected changes [112:113]. Upon careful analyses, some of the discrepant findings seem to be attributable to factors that influence the stress response, namely the stressor type, stressor regimen or the rat strain. Herman and Cullinan (1997) have suggested that different neurocircuits may be involved in the mediation or modulation of the effects of different stressor types [185]. Such a concept could explain why the CeA (or CRH for that matter) appears to be more involved in certain types of stressors and not others. Several factors determine an organism's response to a stressor, and the consequence of this response. These factors include the type of stressor (e.g. bereavement, bacterial infection, or public speaking), the stressor regimen (e.g. acute, chronic and/or intermittent), and the psychological/physiological makeup of the organism (e.g. ontogenetic and/or genetic factors) [15]. In general, the type of stressor can be classified as either "systemic" or

"processive" [185]. Systemic stressors (sometimes denoted *physical*) represent immediate challenges to the physiological makeup of an organism, such as ether exposure or oxygen deprivation. As such, they represent an eminent threat to survival and are characterized by a rapid, and direct, activation of the HPA axis via the PVN. Stressors of this nature do not require cognitive assessment prior to the activation of the stress response. This contrasts with the so-called processive stressors (sometimes referred to as *psychological* stressors), which do not directly alter homeostasis but are *perceived* to be (or potentially are) stressful based on previous experience (such as immobilization or noise exposure). Included in this category may be stimuli that activate neurocircuitry that has developed through evolution to enhance the survival of a species (e.g. sight of a snake, predator odors). These stimuli typically elicit a "hard-wired" species-typical *fight or flight* response that does not necessarily require prior experience; however, this response can over time be influenced by cognitive assessment (e.g. domesticated rodent no longer evades the presence of the house dog). Stressors of a processive nature are believed to activate brain centers that are involved in the attribution of emotional salience to an event (either positive or negative) before activating circuits that mediate the behavioral and/or neuroendocrine response [185]. In this respect, the CeA is thought to mediate the physiological and behavioral responses to processive/psychological stressors [162:297] and play less of a role in the response to systemic stressors.

When various stressors are differentiated according the categories outlined by Herman and Cullinan (1997) and viewed along side stress-markers activated (i.e. c-fos, mRNA, tissue levels, *in vivo* release), one does not necessarily end up with a reconciled

picture. On the contrary, a complex relationship emerges. For example, some authors have shown that noise, restraint, forced swim, predator-cues, and cytokine loading activates CRH neurons at the CeA [197:258] while others limit these results to only certain stressors [72:73:329] and sometimes do not include changes in CRH-ergic signals [329]. Similar inconsistencies are found when comparing the stressor-induced CRH mRNA changes at the CeA. Thus, while Pacák et al. (1996) showed that 3-hr of immobilization failed to alter CRH mRNA levels at the CeA [330], others [197:216:276] reported a robust increase in central amygdaloid CRH expression immediately following 1- and 2-hr restraint intervals. Lesion studies also fail to provide consistent conclusions regarding the role of the CeA in the stress response. Beaulieu and colleagues (1987) reported that restraint-induced ACTH release was attenuated in rats with bilateral lesions to the CeA [32] but Dayas et al. (1999) found that the lesions restricted to the CeA had no impact on restraint-induced ACTH release [113].

What is consistent in the literature are the reports concerning the efferent projections originating from the CeA to the parvocellular division of the PVN (either directly or via the BNST) [163], which in turn, provide reciprocal neuronal connections [162:163]. In addition, neurons emanating from the CeA directly innervate the locus coeruleus, NTS and dorsal vagal complex, potentially influencing the output of these structures [106:164:249] (see Figure 2). In this capacity, it is notable that CRH has been detected at all of these structures [448]. Even with the above-mentioned inconsistent findings, there is strong evidence (in both humans and experimental animals) that the amygdala, and more specifically the CeA, is involved in integrating the response to

aversive stimuli. In addition, there is mounting evidence that the CeA may play a role in emotional reactions to appetitive events as well.

As referred to earlier, some authors contend that the amygdala might play a role in the attribution of emotional salience to an event. The observation that lesions to the amygdala interfere only with conditioned (but not unconditioned) stress responses supports this notion [160]. That is, denoting meaning (i.e. salience) to the conditioned stimulus appears to require an intact CeA. Using PET scans, Childress et al. (1999) reported elevated cerebral blood flow to the amygdala in detoxified cocaine users observing cues that induced subjective reports of craving [84]. More recently, Parkinson and colleagues (2000) have shown that lesions to the CeA, but not other amygdaloid nuclei, impair appetitive Pavlovian conditioning in rats [340]. Furthermore, elevated CeA activity has been reported in male rats exposed to sexually receptive females [114]. These studies provide some evidence that the CeA is involved in the integration of both aversive and appetitive events, however, the role that CRH might play in this response remains to be clearly elaborated. It is of interest, however, that low doses of CRH into the amygdala enhance memory retention for both appetitive and aversive events [250]. In this context, one of the major objectives of the research presented in this thesis was to assess the extent to which amygdaloid CRH is involved in aversive and appetitive events.

Assessing the role of neuropeptides in the stress response: methodological issues

There is no doubt that the advent of novel techniques in molecular biology has afforded neuroscientists a greater ability to study the role of neuropeptides in the stress

response. At the same time, some techniques provide potentially contradictory results and this highlights the need to understand the strengths and limitations of each approach.

In situ hybridization

All proteins and neuropeptides are produced when the gene within the nucleus from which they are derived is activated. This activation causes the gene to produce the mRNA for the particular protein. When mRNA migrates out of the nucleus it attaches itself to ribosomes where a variety of translational promoting and/or inhibiting factors influence the ultimate synthesis of the protein. When the sequence of the nucleotides that constitutes the mRNA is known, technological advances permit scientists to create a complementary RNA strand of nucleotides that will bind to the mRNA when applied to the tissue. Commonly, complementary RNA is bound with a radioactive isotope before it is applied to the tissue so that exposing the tissue to x-ray films reveals the location of the cell bodies that produce the peptide of interest. The density of the radioactive signal provides a quantitative assessment of peptide synthesis. Of note, however, translation inhibiting factors can prevent mRNA from being translated into protein [5] and so elevated mRNA signals do not necessarily reflect enhanced peptide production, but rather should be regarded as the *potential* signal for peptide production. Likewise, even if the peptide is produced it may not be released into the synaptic cleft [295], so at best, *in situ* hybridization offers an indirect inference of peptide utilization at the time the tissue was collected.

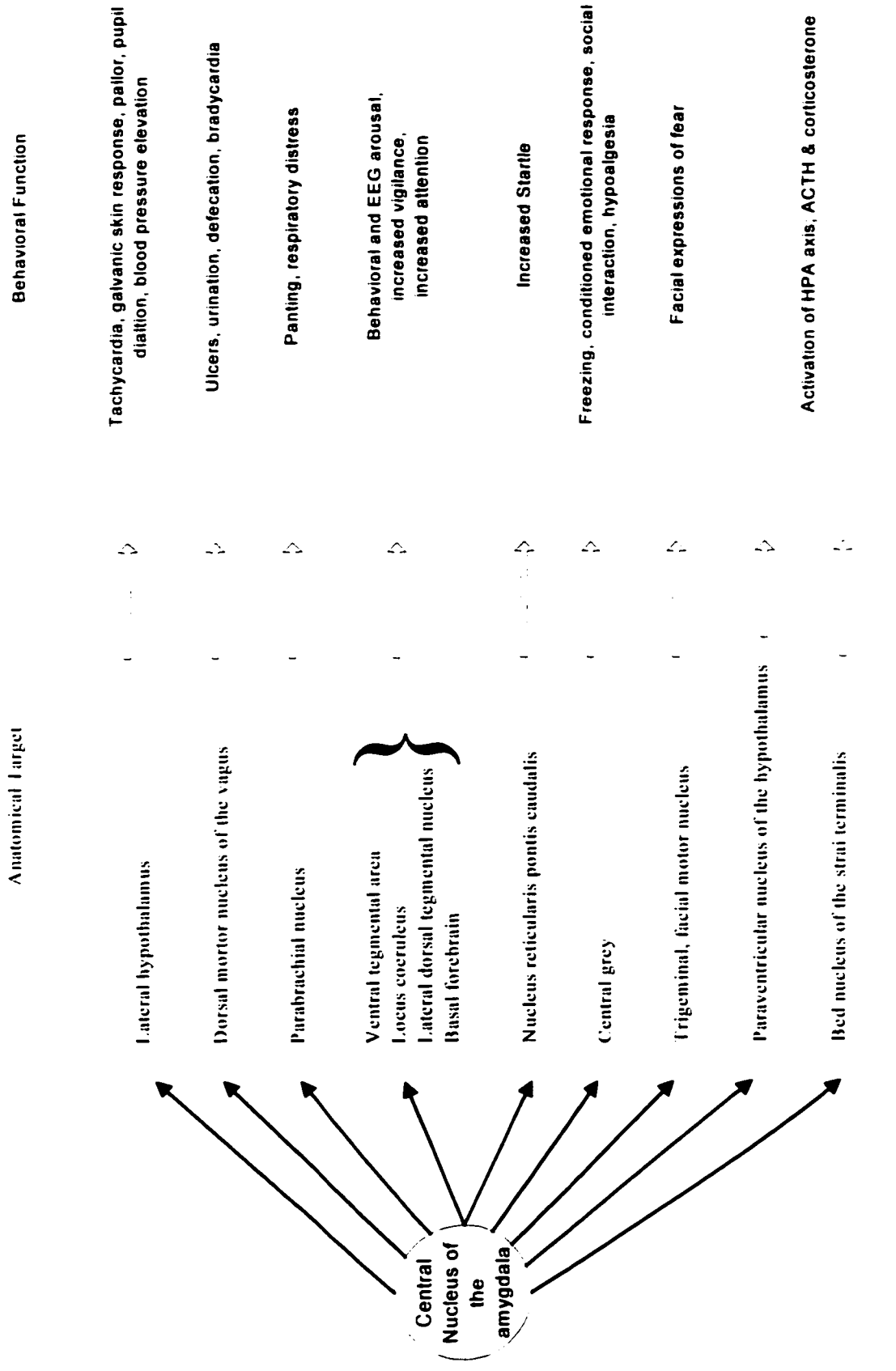


Figure 2. A schematic representation of the major neuroanatomical projections arising from the central nucleus of the amygdala and which behavior these projections may sub-serve. *Adapted with permission from Davis and Whalen, 2001.*

Immunohistochemistry

This approach is very similar to that of *in situ* hybridization except that instead of applying a radiolabeled complementary RNA probe to the tissue, an antibody against the *actual* protein (or protein-antibody complex) is used. This antibody can be directed against receptors, enzymes, or peptides and because it is labeled with a fluorescent molecule it will (under specific wavelengths) emit fluorescence from the protein to which it is bound. The application of two or more antibodies bound with different fluorescent molecules (i.e. excitable at different wavelengths) permits one to investigate the expression of multiple proteins within the same tissue section. The intensity of the emitted fluorescence observed under microscopic examination can be used to quantify changes in protein expression. Like *in situ* hybridization, interpretations of any changes are limited to the time at which the tissue was collected and do not permit the direct assessment of underlying contributing factors. Thus, for the same reasons discussed above, elevated peptide signals could imply increased, decrease or no change in peptide utilization.

Micropunch

In order to map regional fluctuations in peptide levels associated with physiological or pharmacological processes, investigators need to dissect out various brain nuclei or regions, which is accomplished by freehand dissection or using precision micropunch technique of Palkovits and Brownstein [334]. The tissue peptide content can then be determined using RIA and/or high-pressure liquid chromatography (HPLC). Such techniques allow one to map potentially important peptidergic changes in brain

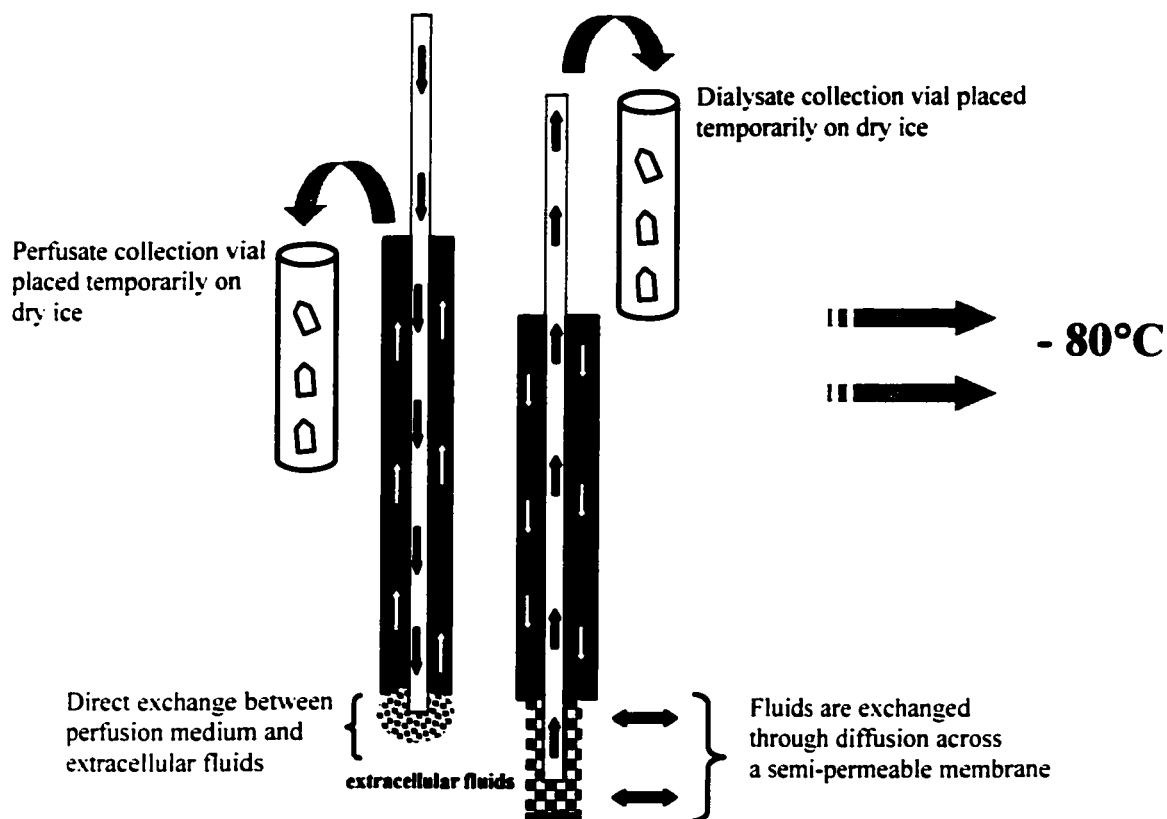
regions under various conditions, including following stressor exposure. However, one of the limitations of this approach is the anatomical precision of the micropunch itself, and is limited to the time the brain was obtained (i.e. "snap shot" reflection during the brief window of assessment). The interpretation of the observed changes, particularly in context of the stress response, is often not clear-cut. This is because altered peptide levels could be interpreted as being due to altered rate of synthesis, release or metabolism of the peptide(s) in question. Furthermore, when tissue peptide levels do not change in response to an event, this may not necessarily reflect the lack of peptidergic involvement, because altered peptide utilization can easily be masked if, for instance, the rate of peptide release is balanced by a compensatory change in the rate of peptide synthesis [295] or degradation. Thus interpretation of tissue peptidergic changes (or lack thereof) should be done with caution.

In vivo microdialysis and push-pull perfusion

More than nine decades ago, Legendre and Piéron demonstrated that 'sleep factors' could be collected from the cerebral ventricles, an event that initiated an era where scientists began to focus on monitoring the release of endogenous neurochemicals from the brain during a physiological event [317]. Early efforts were restricted to analyzing neurochemical changes in the fluid collected from the large ventricular spaces or in fluids collected through miniature cups placed on the surface of the cortex [304]. However, today relatively small push-pull perfusion cannulae and microdialysis probes permit the *in vivo* collection of neuropeptide released at specific brain regions. Push-pull perfusion consists of an open-flow (i.e. direct exchange) between the perfusion medium

and the extracellular fluids. Thus, this technique can be used to sample a variety of neurochemicals, because unlike in the case of microdialysis technique, the molecular weight of the analyte is of no consequence: albeit, this advantage is offset by the fact that peptide-degrading enzymes are likewise collected. The nature of the push-pull perfusion design also entails the high risk of causing tissue damage when the rates of fluid infusion and withdrawal are not matched exactly (e.g. due to tissue obstruction). Microdialysis, on the other hand, is similar to push-pull perfusion but utilizes a semi-permeable membrane at the tip of probe (i.e. the active membrane). Compounds inside (perfusion medium) and outside (extracellular fluids) diffuse from high concentrations to low concentrations across the membrane. However, only molecules with a molecular weight below the cut-off of the semi-permeable membrane will diffuse through the membrane. Thus, whereas the recovery of the extracellular contents is reduced relative to the push-pull approach, the microdialysis approach circumvents some of the technical problems associated with the open flow design of the push-pull cannula.

When these techniques are combined with a highly sensitive RIA they can provide invaluable insights into the actual release/utilization of neuropeptides before, during and following a stressor exposure. As such, the subject serves as its own control, making it possible to assess individual differences and help minimize the number of animals required for each experiment. A drawing depicting the relationship between *in vivo* push-pull perfusion and microdialysis in combination with RIA is shown in Figure 3.



Basic principles of solid-phase radioimmunoassay

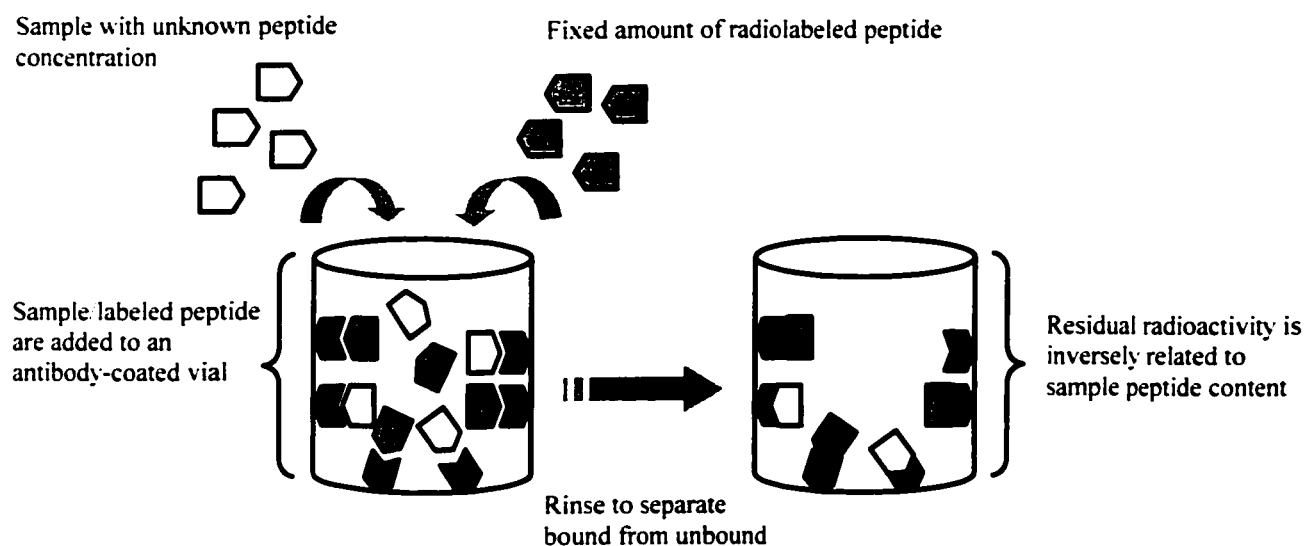


Figure 3. A schematic illustration of combining the use of *in vivo* microdialysis/push-pull perfusion and solid phase radioimmunoassay for the collection and detection of endogenously released peptides, respectively.

Thesis objectives

Since Selye's pioneering efforts, significant progress has been made in the characterization of the endocrine, behavioral and autonomic changes that accompany an organism's reaction to stressors. As indicated earlier, rigorous scientific inquiry has revealed some of the neurochemical and neuroanatomical underpinnings of the stress response, and continues to provide information of novel factors that may be involved in this response. There has been a concurrent evolution in the appreciation of the variables influencing the stress response. For instance, it is well known that the genetic makeup of an organism may have a bearing on the endocrine, behavioral and/or autonomic reactions to stressors [14:15:19:86:121:426]. In addition, evidence supports the contention that the neurocircuitry activated is partially dependent upon the nature of the stimulus [15:185:327:329]. A stressor-dependent activation of brain systems might likewise imply a stimulus-dependent activation of different peptidergic circuits. It was with an appreciation of these influencing factors that the work in this thesis was undertaken. We assessed the role of CRH and BN-LPs using several different rat strains exposed to biologically meaningful stimuli of inherently unique attributes. Four major objectives formed the framework for this dissertation, which ultimately represents an effort to further our understanding of the role of neuropeptides in the stress response in general, with specific focus on CRH and BN-LPs at the amygdala.

1. Only a handful of laboratories have attempted to assess the dynamics of peptide release in behaving animals. In the few that have utilized *in vivo* microdialysis to assess CRH release, most have included CRH antiserum in the perfusing medium, to enhance peptide recovery (and detection), which may perturb the gradient and

confound the results. In addition, with the exception of studies carried out in our laboratory, there have been no studies evaluating the potential role of BN-LPs in the stress-response. Our first objective therefore was to assess the feasibility of using *in vivo* microdialysis with an antibody-free perfusion medium in the assessment of neuropeptide release from targeted brain regions of behaving animals.

2. The next objective was to utilize this approach to assess the endogenous release patterns of CRH and/or BN-LPs at the CeA in freely behaving animals exposed to certain processive stressors. One of the caveats of the RIA procedure is that the antibodies used in such assays may display affinity not only for the targeted peptide(s) but also for other structurally related endogenous ligands. Thus one needs to authenticate the identity of the immunoreactive peptide(s) detected, using alternate techniques, such as HPLC.
3. As discussed earlier, the CeA appears to be involved not only in aversive conditions (those which an animal avoids), but also under appetitive conditions (those which an animal readily approaches) as well. With the notion that environmental stimuli can be classified as falling within a continuum that ranges from aversive at one end, to appetitive at the other, we wanted to assess the release patterns of CRH and BN-LPs at the CeA along this continuum. It was hypothesized that if CRH differentially mediated the appetitive and aversive responses, then this would be reflected by opposite release patterns. If these peptides are involved in the attribution of

emotional salience to biologically important stimuli, then the release patterns might change in the same direction for both the appetitive and aversive conditions.

4. The recent development of genetically tailored transgenic strains has provided scientists with the ability to enhance or knock out the capacity of the organism to produce specific proteins or peptides. Although the data collected from such mouse models can be very revealing, caution needs to be exercised in interpretation of the results insofar as these manipulations likely recruit redundant and/or compensatory brain mechanisms during development. In this respect, it is particularly interesting that mice that have a complete deficit in CRH show baseline and stressor-induced behavioral changes that are similar to their wild-type cohorts [313]. Such findings could argue against the contention that CRH integrates behavioral changes that occur in response to stressors. We studied the role of CRH in the genesis of stressor-induced behavioral changes by assessing the release of CRH in different strains of rats or different rat lines within the same strain that exhibit differential behavioral responses to a variety of stressors. If CRH at the CeA mediates stressor-related behavioral responses, then its release should vary in a predictable manner in rat lines or strains that display differential responses to stressors. Furthermore, by employing stressor paradigms with "anxiogenic" properties, as reflected by specific anxiety-like behavioral changes (i.e. novelty-induced suppression of snack intake), we could determine the extent to which the behavioral changes were reflected by changes in the release of CRH at the CeA, by simultaneously assessing the release of CRH during this paradigm. In this context, if we could manipulate the behavioral response to the

novel environment, then CRH release at the CeA should be affected if it plays a specific role in the genesis of the observed behavior.

It is important to mention at the outset that in some studies the concentration of BN-LPs in the dialysates was below the level of detection in the RIA. As such, the general discussion at the end of the thesis pertains mostly to the release of CRH at the CeA in response to biologically salient stimuli in general, and the extent to which this peptide mediates the behavioral response in particular. Some of the chapters have been published as multi-authored manuscripts and where I am not the primary author my specific contribution is defined at the outset of each chapter. When appropriate, some chapters contain multiple papers for the sake of continuity and brevity.

Preface to Chapter I

CRH is believed to play a role in the genesis of several aspects of the stress response. The major impetus for this contention has stemmed from the fact that stressor exposure is almost always associated with elevated corticosterone levels and that CRH is the primary mediator of ACTH and corticosterone response. However, the support for role of CRH in the genesis of behavioral and autonomic responses to stressors is more tenuous as it is based on either pharmacological studies or indirect physiological measures. Specifically, assessment of peptide utilization using *in situ* hybridization [238], immunohistochemistry [445], or post-mortem tissue level analyses [396], only indirectly reflect actual peptide release, and that too at static time-points. There have also been a few more direct studies, using *in vivo* microdialysis to monitor the release of CRH during various aversive conditions [351:369:370:375].

In terms of the neuroanatomical substrates involved in the mediation or coordination of the stress response, the amygdala (particularly the CeA) is thought to play a salient role. However, its role appears to be dependent upon several factors, including the nature of the stressor: that is whether it is a processive (i.e. psychological), physical or physiological (i.e. systemic) type of stressor [112:113:329]. In this regard, the findings are controversial, as some authors report that processive stressors do not activate the CeA [73:237:329] while others report a robust induction of c-fos following exposure to such stressors [34:96:279]. Similarly, stressor-related changes in CRH mRNA at this region have also been inconsistent [216:276:330]. These inconsistencies may be attributable to differences in the stressor protocols used. In any case, a direct assessment of CRH

release at the CeA during restraint may help clarify some of the conflicting reports in the literature.

Thus, our first objective was to establish an *in vivo* microdialysis procedure to monitor the release of endogenous CRH and BN-LPs at the CeA, before, during and following restraint in freely moving animals.

Evidence from our laboratory [13:223-225] and those of others [54:61:62:66:139] suggests that BN-LPs may also be involved in the stress response. Interestingly, there appears to be some cross talk between CRH and BN-LPs in this regard [224:225:299]. Using the *in vivo* microdialysis approach, we concomitantly assessed the release of BN-LPs at the CeA prior to, during, and following restraint in freely moving animals.

As alluded to earlier, there is some data from both clinical and the animal experimental realms suggesting that the CeA may respond to the salience (or meaning) of a stimulus, as opposed to simply its positive or negative attributes [84:160:340]. We evaluated this contention in the current experiment by monitoring the release of both CRH and BN-LPs at the CeA in response to an aversive as well as an appetitive event. We consider the distinction between an aversive and an appetitive event to be that which an animal avoids (restraint) versus one that an animal readily approaches (in this case a meal), respectively.

In addition to providing a direct assessment of peptide release, *in vivo* microdialysis also permits each animal to serve as its own control. This not only cuts down the number of animals required for each experiment but also permits an assessment of individual differences that may occur over the course of a treatment regimen. Despite these advantages, one must consider the possibility that the insertion of the probe *in itself* may affect the release of (in this case) CRH and BN-LPs. It was thus important to determine in these initial experiments, if potential irritation caused by the insertion affected the release of CRH and BN-LPs at the CeA. To this end, we implanted probes into the CeA to determine the release profiles of CRH and BN-LPs in undisturbed freely behaving animals over extended periods of time.

Finally, we recognized the possibility that the antibodies used in the RIAs (for CRH and BN-LPs) may have an affinity for not only the peptides of interest, but also other structurally related peptides; in other words, immunoreactive signals may represent various forms of CRH and/or BN-LPs. Thus, our final objective in this set of experiments was to verify the authenticity of the immunoreactive signals gleaned through the RIAs.

Note: The data presented in this chapter have been published as a multi-authored manuscript [297]. My specific contributions to this work included some of the conceptualization/development of the methodology for the authentication of the ir-peptides collected in the microdialysis study, providing assistance in carrying out some of the microdialysis experiments, including the design and manufacture of the microdialysis pedestals, tethering system and implementation of microdialysis procedure. My primary interest in this study had to do with the responses to stressors.

CHAPTER I

**Aversive and appetitive events evoke the release of corticotropin-releasing hormone
and bombesin-like peptides at the central nucleus of the amygdala**

Abstract

There is wide agreement that corticotropin-releasing hormone (CRH) system(s) within the brain is (are) activated by stressful stimuli. There is also mounting evidence for the role of bombesin (BN)-like peptides (BN-LPs) in the mediation of the stress response. To date, however, the extent to which other stimuli increase the activity of these peptidergic systems has received little attention. In the present investigation we validated and used *in vivo* microdialysis sampling followed by *ex vivo* radioimmunoassays (RIAs), to monitor the release of CRH and BN-LPs during appetitive (food intake) and stressful (restraint) events. It is demonstrated for the first time that the *in vivo* release of CRH and BN-LPs at the central nucleus of the amygdala (CeA) was markedly increased by both stressor exposure and by food ingestion. In fact, the meal-elicited rise of CRH release was as great as that associated with 20-min of restraint stress. Paralleling these findings, circulating adrenocorticotropin-releasing hormone (ACTH) and corticosterone levels were also increased in response to both food intake and restraint. Contrary to the current views, these results indicate that either food ingestion is interpreted as a "stressful" event by certain neural circuits involving the central amygdaloid nucleus, or that the CRH and/or BN-related peptidergic system(s) may serve a much broader role than previously envisioned. Rather than evoking feelings of fear and anxiety, these systems may serve to draw attention to events or cues of biological significance, such as those associated with food availability as well as those posing a threat to survival.

Introduction

There is considerable evidence suggesting that CRH, a hypophysiotropic peptide comprising 41 amino acids [453], plays a fundamental role in stress reactivity. In particular, stressors reliably enhance the expression of CRH mRNA in hypothalamus [29:231:238:394:451], and central administration of CRH elicits a constellation of behavioral, physiological, and endocrinological changes similar to those produced by stressors [129]. Conversely, CRH antagonists attenuate the behavioral effects of CRH, as well as those elicited by stressors [162:180]. In addition to hypothalamic CRH, it seems that central amygdaloid CRH changes are also intricately involved in orchestrating the response to stressors. In this respect, amygdaloid CRH manipulations predictably affect behaviors indicative of anxiety [129:216:436], although these effects may be more closely aligned with a fear response than nonspecific anxiety [251]. Moreover, stressors have been shown to affect CRH mRNA levels and the levels of CRH within the amygdala [351]. In this context, various aversive conditions (such as withdrawal from alcohol, cocaine or cannabinoids) influence the levels and/or turnover of CRH at the CeA [351:369:370:375].

The hypothalamic-pituitary-adrenal (HPA) axis activation associated with stressors, is an exceedingly robust phenomenon, so much so that glucocorticoid changes have been taken to reflect the presence of stressors. Contrary to this dogma, however, there is reason to believe that alterations of HPA activity may be influenced by appetitive stimuli, just as aversive events elicit such effects. For example, Dallman et al. [105], Schwartz et al. [398], and Shiraishi et al. [404] have reported that the HPA

responsiveness to stressors was influenced by whether the animals were food deprived or sated. Furthermore, food consumption itself, as well as other rewarding stimuli also promotes glucocorticoid secretion [350]. In the case of humans, cortisol response to food occurs prior to the food actually being absorbed from the gastrointestinal tract [4:145:218]. As well, electrophysiological evidence supporting the view that information processing involving the cortex-amygdala-lateral hypothalamus contributes to the control of feeding behaviors. In particular, it was demonstrated that some amygdala neurons responded to cues associated with food and that the degree of responsiveness varied with the apparent affective significance of the stimulus [148]. The suggestion has indeed been offered that the amygdala contributes to the processes by which sensory stimuli gain motivational and emotional significance [150:211:502]. In effect, neurons of the CeA may react to the salience or significance of emotional stimuli, rather than simply the negative or positive attributes of these stimuli.

Like CRH, the BN-family of peptides may be part of a constellation of responses to both stressors and to feeding. For instance, endogenous levels of BN-LPs in several brain regions vary during the course of a meal [294:356], and exogenous administration of BN suppresses food intake [155:298]. Interestingly, BN administration markedly elevates circulating ACTH levels and this effect can be blocked by pretreatment with a CRH receptor antagonist [225:299]. Moreover, stressors influence BN-like immunoreactivity in several brain regions, particularly the hypothalamus [223]. Thus, there is reason to believe that BN-LPs modulate the HPA response to both appetitive and aversive stimuli.

In the present investigation we demonstrate that in response to food intake, as in response to stressors, plasma ACTH and corticosterone levels are increased. Moreover, it is shown for the first time that the release of CRH and BN-LPs at the CeA, as assessed by *in vivo* microdialysis, was markedly increased by both stressor exposure and feeding. It is suggested that the CeA, and particularly CRH and/or BN-LP neurons within this region, are fundamental in mediating the response to emotionally laden events, irrespective of whether they are negatively or positively charged.

Materials and Methods

Subjects and surgical procedures

Male Sprague-Dawley rats weighing approximately 350–450 g ($n = 50$) were individually housed in standard clear plastic cages, and were maintained on a 12-hr light-dark cycle (light phase: 0630–1830-hr). Animals had free access to Purina™ lab chow and tap water. All experimental procedures followed the guidelines of the Canadian Council on Animal Care, and were approved by the Research Ethics Committee of the University of Ottawa. Rats were anesthetized (60 mg/kg pentobarbital i.p.) and stereotaxically implanted with a 20-gauge guide cannula containing a removable 24-gauge obturator, aimed at the CeA. The placement coordinates (obtained from Paxinos and Watson, 1982) [342] were A/P = -2.3 mm, D/V = -7.0 mm, L = $\pm$ 4.2 mm, with level skull. The guide cannula protruding from a custom manufactured Delrin[®] pedestal was secured to the skull with 4 screws and dental cement. After surgical recovery (a minimum of 7 days), animals were transferred to individual testing chambers and allowed to acclimate for 48-hr prior to testing. The testing chambers comprised of Plexiglas cages

(25 x 35 x 34 cm) with a stainless steel grid floor. Food (powdered Purina™ lab chow) was available through a 3 cm hole centered in a short tunnel (6.5 x 6.5 x 10 cm) protruding from the cage. A food cup located beneath the hole was positioned atop an electronic scale, permitting continuous monitoring of food consumption.

Carotid cannulation for blood sampling

For some experiments, rats were implanted with carotid arterial catheters under aseptic conditions. The carotid artery was exposed by blunt dissection, and a small incision was made using a 23-gauge needle. The catheter, consisting of silicone tubing (Dow Corning) and polyethylene (PE-50) tubing, was inserted into the artery, ligated to the vessel, tunneled subcutaneously, and exteriorized at the neck level. During sampling, the cannula was connected to remote syringe, using a tethering jacket and a swivel assembly that permitted animals to move about freely in their cages during the experiment.

In vivo microdialysis

Two hours prior to testing, rats were briefly (~ 3-min) anaesthetized with halothane, and the obturator within the guide cannula was replaced with a microdialysis probe. The concentric microdialysis probe had 2.5 mm active membrane (250 µm O.D.) of regenerated cellulose (6000 MW cut-off; Spectrum Medical Industries, CA), which protruded into the CeA. Each probe was secured with a retaining screw and connected via a PE tubing to a liquid swivel and a 2.5 ml glass gas-tight infusion syringe attached to a pump (Harvard, model 22). Microdialysis probes were perfused at the rate of 2 µl/min.

with filtered Kreb's Ringer Phosphate (KRB) solution consisting of (in mM) 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻, 0.05 ascorbate, pH 7.4 [306] and BSA (0.1%). Upon collection, each sample (40-60 μ l) was immediately frozen on dry ice and stored at -80°C until RIA analyses. The efficiency of the microdialysis probes in terms of peptide recovery, was assessed *in vitro* as follows: probes were first submerged in a plain KRB solution and flushed with perfusion medium (KRB with 0.1% BSA) at the rate of 2 μ l/min for 1-hr. They were then switched to tubes containing either [¹²⁵I-Tyr⁰] CRF or [¹²⁵I-Tyr⁴] BN in KRB. The percent recovery between individual probes was 3.3 ± 0.6 % for CRH and 9.2 ± 0.15 % for BN, over 5 successive sampling periods. When the solution bathing the probes was changed from one containing one of the ¹²⁵I-labeled peptides to that of plain KRB, there was only a slight carry-over effect on the first sample (< 0.6%) and then dropping to an average of 0.15%. Reinsertion of the probes into the solution of iodinated peptide was accompanied by recovery of counts in the perfusate to their level originally seen with that probe, within the first sampling period.

RIAs

The detection and quantification of CRH was achieved through a solid-phase high sensitivity adaptation/modification [270] of the double antibody liquid phase RIA originally described by Vale and colleagues (1983) [454]. BN-LPs was detected using a similar solid-phase RIA [356]. Briefly, protein A/G (Calbiochem-Novabiochem Corp., La Jolla, CA) coated Immulon[®]-4 wells (Dynatech Laboratories, Inc., Chantilly, VA) were incubated with anti-CRH serum (rC70 kindly provided by W. Vale) or anti-BN serum (α -BN₂ kindly provided by Dr. T. Moody) for 2-hr at 20°C. Samples, standards

(reconstituted in the KRB solution, ranging from 0.05 to 250 fmol/well) or blanks were incubated for 24-hr at 4°C. Next, 25 µl assay buffer containing 5000 - 6000 CPM [125 I-Tyr⁰] rCRF (Amersham Canada Ltd., Oakville, ON) or [125 I-Tyr⁴] BN (iodinated in house, as per [386]) was added to each well and incubated for an additional 24-hr period at 4°C. Finally, the wells were rinsed, separated and their residual radioactivity counted in a gamma-counter (Cobra[®] II Auto-gamma, Meriden, CT). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically about 0.1 and 2 fmol/well for CRH and BN, respectively.

The specific anti-CRH serum used in the study recognized CRH₁₋₄₁ and displayed negligible cross-reactivity with other related peptides [454] including urotensin I and urocortin (data not shown). The BN antibody used in the RIAs recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with amphibian BN (100%) and certain mammalian BN-LPs including gastrin releasing peptide (GRP)₁₋₂₇ (110%), and GRP₁₈₋₂₇ (i.e. NMC (82%)), but only weakly with GRP₁₋₁₆, neuromedin B (NMB)-10, NMB-32 or substance P ($\leq 0.1\%$) [309]. We have shown in the past that the major source of BN-like immunoreactivity from the hypothalamus is attributable to GRP [294].

In order to verify the identity of the immunoreactive CRH (ir-CRH) material detected in the dialysates, probes positioned within the CeA (n = 3) were perfused at the rate of 2 µl/min for 8-hr. The total dialysate volume (960 µl) collected over ice was split in two aliquots (480 µl each) and lyophilized for two distinct high-pressure liquid

chromatography (HPLC) analyses. The lyophilized samples were reconstituted and separated by a reverse-phase HPLC system consisting of Spectra-Physics gradient pump (P-2000; Spectra-Physics Analytical, San Jose, CA) and a C-18 Vydac[®] column (model 218TP54, 250 x 4.6 mm, 5 μ , C18, 300-Å pore size). The column was equilibrated with mobile phase "A" (10% acetonitrile with 0.1% trifluoroacetic acid (TFA), in H₂O) and peptides eluted with mobile phase "B" (90% acetonitrile with 0.1% TFA, in H₂O), using a linear gradient (from "A" = 100% to "B" = 100%, over 50-min), at a flow rate of 1 ml/min. Authentic CRH standard (rat/human CRH₁₋₄₁, Peninsula laboratories, CA) was run under identical conditions and the absorbance of the elutant monitored at 214 nm (Applied Biosystems absorbance detector model 783A). The peak elution time for synthetic CRH was 31.8-min, and that of ir-CRH material contained within the dialysate eluted in fractions collected over 31-32-min, coinciding with the elution time of authentic CRH (Figure 1, upper panel). The major peak of ir-BN material eluted in fractions collected over 17-18-min and coincided with the elution time of synthetic GRP₁₋₂₇ (Peninsula laboratories, CA) of 17.4-min, whereas, the smaller peak (preceding or shouldering the major peak) that eluted with fractions collected over 14-16-min, corresponded with elution time of synthetic GRP₁₈₋₂₇ (see Figure 1, lower panel).

The identity of eluting peptides was replicated and verified using a) the same HPLC conditions but a different microdialysis sample and b) a second, distinct set of HPLC conditions. This alternate HPLC procedure utilized a different C18 column (Jupiter[®] 250 x 4.6 mm, 5 μ , C18, 300-Å pore size; Phenomenex, Torrance, CA) and the following elution conditions: The system was equilibrated with mobile phase "A" (10%

acetonitrile with 0.1% TFA in H₂O) and peptides eluted with mobile phase "B" (90% acetonitrile with 0.1% TFA in H₂O), using a linear gradient (from 100% "A" to 100% "B" in 100-min) at a flow rate of 1 ml/min. The eluting fractions were collected every 30-sec and lyophilized for subsequent RIA analyses. Under this set of conditions, synthetic CRH eluted at 50.3-min and the major peak of endogenous CRH material from the dialysate was contained within fractions collected over 50-51-min. Immunoreactive-BN from the sample eluted at 26.5-min (minor peak) and 29-min (major peak), once again corresponding to retention times of synthetic GRP₁₈₋₂₇ (25.9-min) and GRP₁₋₂₇ (29.2-min), respectively (data not shown). These results are consistent with the assertion that the ir-CRH material in the amygdaloid dialysate represents authentic CRH whereas ir-BN material represents GRP₁₋₂₇ and/or GRP₁₈₋₂₇.

Plasma ACTH and corticosterone measurements

Corticosterone and ACTH levels were measured using commercial RIA kits (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

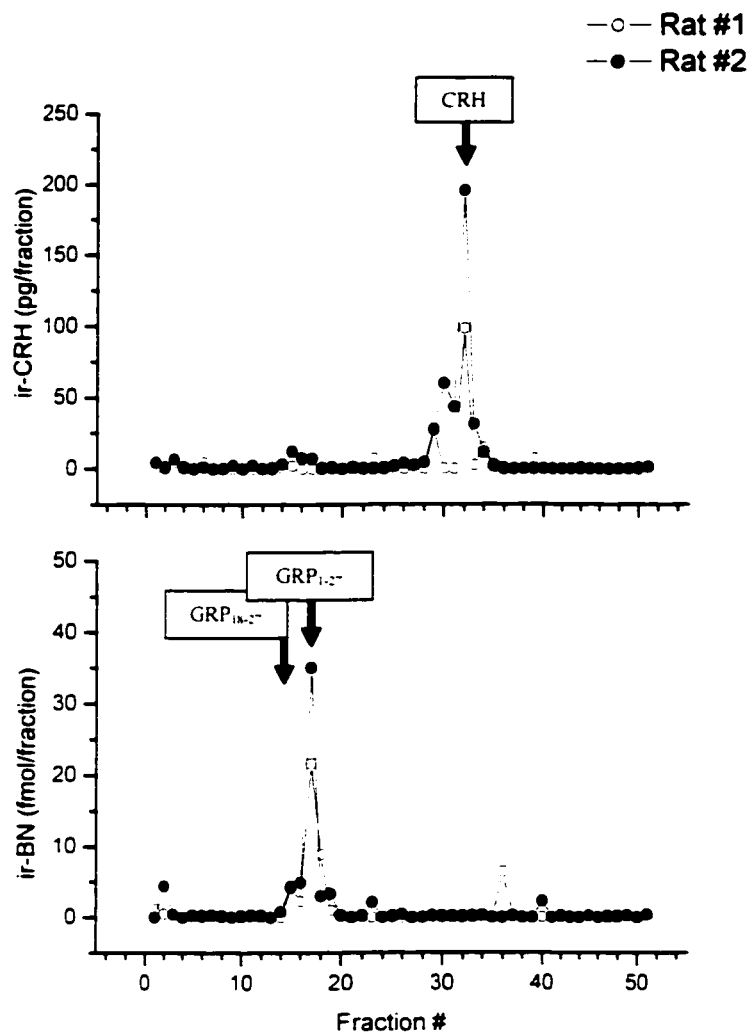


Figure 1 HPLC separation of endogenous CRH and bombesin-like immunoreactivity in microdialysis samples collected and pooled from two separate rats, open and closed symbols, respectively. Boxed arrows indicate elution time for synthetic CRH (upper panel) and GRP₁₋₂₇ and GRP₁₈₋₂₇ (lower panel) using UV detection. High-pressure liquid chromatography (HPLC), corticotropin-releasing hormone (CRH), bombesin (BN), gastrin releasing peptide (GRP), immunoreactivity (ir), ultraviolet (UV).

Experimental design and procedures

A total of 30 rats with probes aimed at the CeA participated in the initial experiment (stress study). The probes were continually perfused with KRB, and dialysates pooled every 30-min. throughout the experiment. After collection of 5 baseline samples, rats in the “stress” group ($n = 20$) were manually restrained for 20-min. The restraint procedure consisted of a rat, situated on the floor of the test chamber, being lightly grasped about the shoulder/forelimbs by an experimenter’s gloved hand. Thus, the ability of the animals to move about was prevented. Dialysate samples continued to be collected (every 30-min) for 2.5-hr. after which the animals were again restrained (for 20-min) and dialysates collected for an additional 2.5-hr. The control or “no stress” group ($n = 10$) underwent identical collection procedure, however they were not stressed and remained undisturbed in the test cages throughout the experiment. In addition to the dialysate samples, blood (100 μ l) was collected from a tail nick immediately prior to restraint, just prior to release from restraint, and again just prior to the end of the second restraint period. Plasma samples were stored at -80°C for subsequent ACTH and corticosterone analyses.

A second experiment (feeding study) using 10 rats assessed central amygdaloid CRH and BN-LP fluctuations associated with various spontaneous ingestive states in animals with *ad libitum* access to food (powdered Purina™ lab chow). Probes were inserted into the guide cannula at 1400-hr (4-hr before dark onset) and were continually perfused with KRB solution. Commencing 2-hr after probe insertion, dialysates were pooled over every 30-min period (over 5-hr). The food cup was positioned atop an

electronic scale, thus permitting continuous monitoring of food consumption. When an animal initiated a meal the 30-min dialysate sampling period began anew. A meal was defined as consumption of a minimum of 0.3 g during the 30-min sampling period. The meals ranged in size from 0.3 to 3.1 g (mean $\pm$ S.E.M. = 1.26 ± 0.12 g) and the first meal usually occurred within 90-min of dark onset. The 30-min period prior to meal initiation was considered as the preprandial period, and the 30-min interval preceding this was considered as the baseline. The postprandial period was the 30-min period following meal termination, during which no food was consumed. If during this time a new meal was initiated, then the postprandial period was considered as the subsequent 30-min period.

While the preceding experiment assessed peptide and endocrine variations in the context of spontaneous meals, a separate experiment evaluated plasma ACTH and corticosterone levels before and after presentation of a palatable snack (Graham Wafers). This experiment was conducted during the light-phase of the diurnal cycle (1000-1200-hr) among animals that had previously experienced this particular type of snack (on 4 occasions, to reduce any neophobic responses). Rats ($n = 8$) equipped with a carotid artery catheter, had blood samples drawn at 20-, 10- and 0-min prior to food presentation. Rats were then presented with the familiar palatable food (Graham Wafer: 16 g) for a 10-min period, during which all animals readily consumed some food (mean $\pm$ S.E.M. = 3.40 ± 0.84 g). Blood samples were drawn 5-min after meal initiation (i.e. during the 10-min meal) and then again at 15-, 30- and 60-min after meal presentation/initiation. Plasma samples were stored at -80°C for subsequent ACTH and corticosterone determinations.

Histology

At the end of each experiment, animals were perfused under heavy sodium pentobarbital anesthesia and the brains extracted, sectioned and stained for histological examination.

Statistical analyses

In all microdialysis experiments, only data from correctly positioned probes (verified histologically) were included for statistical analyses (i.e. excluding values from animals with misaligned or “off site” probes). From animals included in the statistical analyses, there were occasional missing values, due to accidental sample loss, assay error or flow problems, contributing to variations in sample size. As in the case of most microdialysis studies assessing other central neurotransmitters (such as dopamine), appreciable inter-individual and inter-experiment variability was noted in the present experiments with respect to baseline interstitial CRH levels. This variability may have stemmed from genuine differences between animals, as well as technical aspects related to the microdialysis procedure, including variability in the relative peptide recovery of individual microdialysis probes, subtle differences in probe placements, as well as the variability associated with individual RIAs. Accordingly, in the present investigation, the baseline values of each subject were averaged and defined as 100%. All values were then expressed as a percentage of the average baseline values. Repeated measures analysis of variance (ANOVA) with sample blocks (baseline, post-stress 1, post-stress 2) and samples nested within each block treated as within measures, was performed independently for both CRH and BN-LPs. Post-hoc comparisons were conducted using

Tukey's tests. In the feeding study (Experiment 2) all values were expressed as a percentage of baseline. For some animals, multiple values were available for particular feeding state(s), and these values were averaged, prior to repeated measures ANOVA analysis. The baseline, preprandial, prandial and postprandial periods were treated as a within factor.

Plasma concentrations of ACTH and corticosterone values were derived from all animals (irrespective of probe position) prior to and following both stressor presentations, and were analyzed by repeated measures ANOVA. In the food intake study, values from all animals with patent blood sampling cannula, were subjected to repeated measures ANOVA to compare both the ACTH and corticosterone concentrations at various times prior to food presentation (20-, 10- or 0-min) and at the various times during or following food presentation (5-, 15-, 30- or 60-min).

Results

Restraint stress-induced release of CRH and BN-LPs at the CeA

Data from animals with correctly positioned probes ($n = 10$) revealed that the interstitial CRH varied as a function of the Stress condition (restraint or no stress) x Blocks (baseline, stress1 and stress 2) x Samples (time) interaction $F_{8, 144} = 4.72, p < 0.01$. Levels of BN-LPs varied as a function of Stress condition x Samples interaction ($F_{4, 56} = 9.37, p < 0.01$). The comparisons of the means of the simple effects comprising these interactions revealed that among non-stressed rats the both CRH and BN-LP levels were stable throughout the session. The initial stressor application resulted in an immediate

(within the initial 30-min) and sustained (over 2.5-hr) rise in the interstitial levels of CRH (see Figure 2). Upon the second stressor application, a further increase in the release of CRH was evident, which continued to rise for the duration of the test period.

In a parallel control “no stress” group, animals with probes correctly positioned at CeA ($n = 7$), CRH release did not fluctuate significantly over time. The stress effect appeared to be specific to the CeA, as there was no significant change in CRH release from “off site” probes with detectable levels of the peptide ($n = 5$ from total of 10 “off site” probes, data not shown). See Figure 3 for location of the microdialysis probes.

The stressor also promoted an immediate rise in the release of BN-LPs: compared to CRH release, this response was slower in onset and less pronounced, but continued to increase progressively over the entire session (see Figure 4). Upon stressor re-exposure, a further increase was noted, which persisted to end of the test period (2.5-hr). As in the case of CRH, this response was stressor related, as non-restrained animals did not show significant variations in the interstitial levels of BN-LPs. Yet, in non-stressed animals a modest, non-significant increase on BN-LPs was evident near the end of the session, a few hours prior to dark onset, raising the possibility that circadian factors may also have contributed the rise in BN-LPs in stressed animals. It is of interest to note that the fluctuations of BN-LPs were not as site-specific as those of CRH, in that some “off site” probes with detectable ir-BN levels ($n = 6$), showed some elevation in the release of ir-BN, but not until much later (1.5-hr after second stressor application) (data not shown).

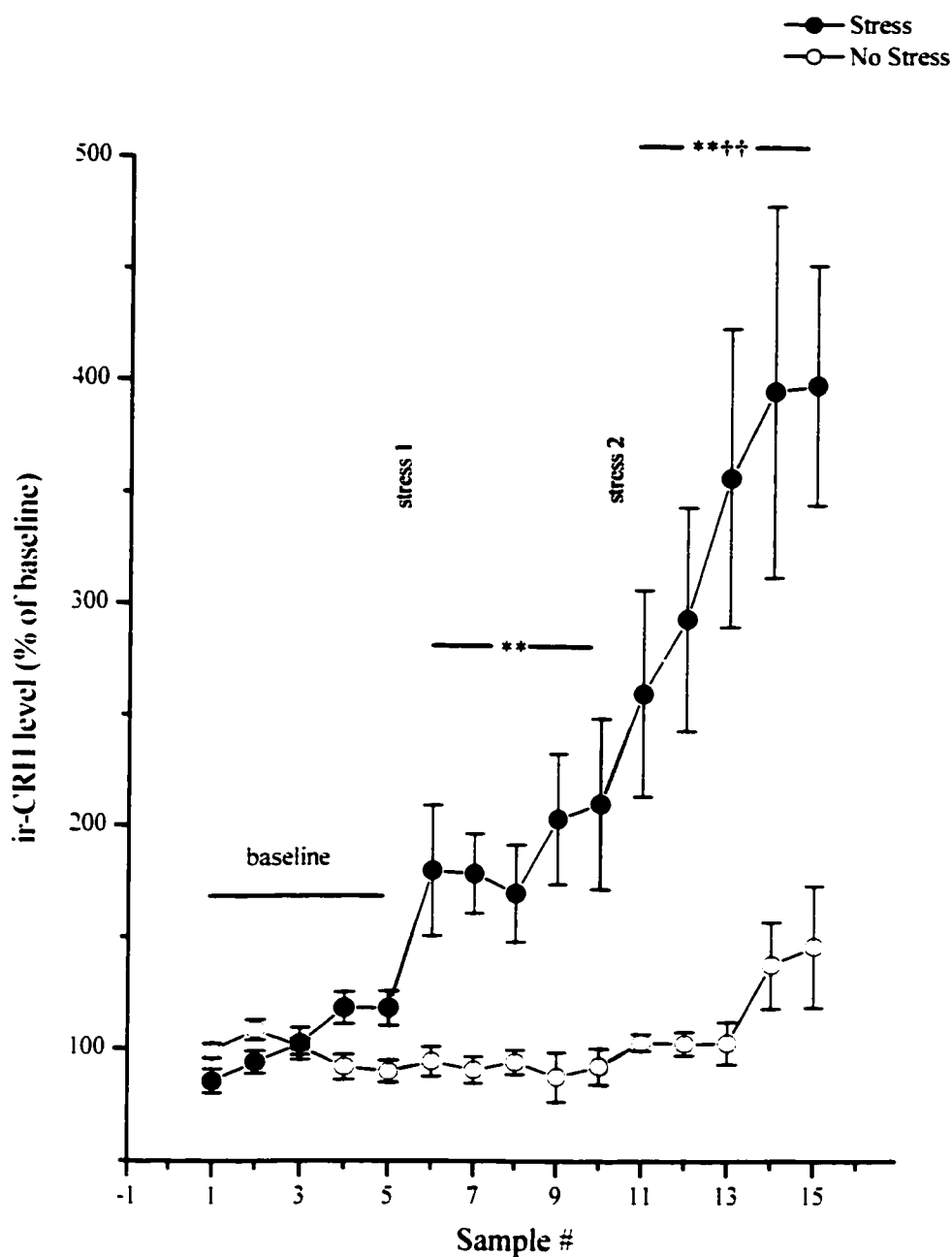


Figure 2 Restraint-elicited release of ir-CRH at the central nucleus of the amygdala as measured by *in vivo* microdialysis in samples collected every 30-min. After collecting five baseline samples, rats in the stress group ($n = 8-10$) were hand-restrained (filled circles) for 20-min episodes on two occasions (indicated by the shaded vertical bars, stress1 and stress2). The rats in the no stress group ($n = 5-7$) were left undisturbed throughout (open circles). The five baseline values from each of the subjects were averaged and defined as 100%. All values were then expressed as a percent of that baseline. Basal CRH values for the no stress and stress groups were 2.77 ± 0.11 and 2.31 ± 0.39 fmol/sample, respectively. Corticotropin-releasing hormone (CRH), immunoreactive (ir). ** Significantly different from baseline ($p < 0.01$). +++ Significantly different from stress1 ($p < 0.01$).

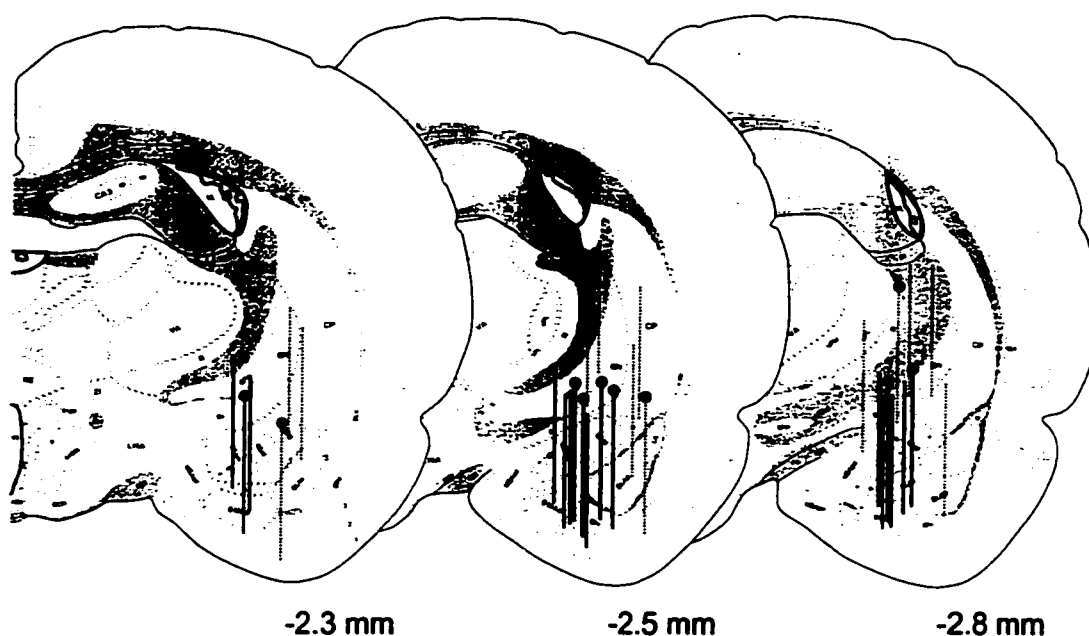


Figure 3. Anatomical localization of the microdialysis membranes aimed at the central nucleus of the amygdala in the stress study. A series of consecutive brain sections bearing the trace of microdialysis probes were stained, the most ventral location of the probe tip was determined, and a 2.5 mm line was drawn vertically, tracing the estimated location of the active region of the probe. Solid vertical lines with pinheads represent probes of animals included in the no-stress control group. The off-site probes (broken vertical line with pinhead) were excluded from analysis. The probe placements of animals included in the stress group are identified with solid vertical lines, whereas those considered off-site and excluded from analysis are depicted by broken vertical lines.

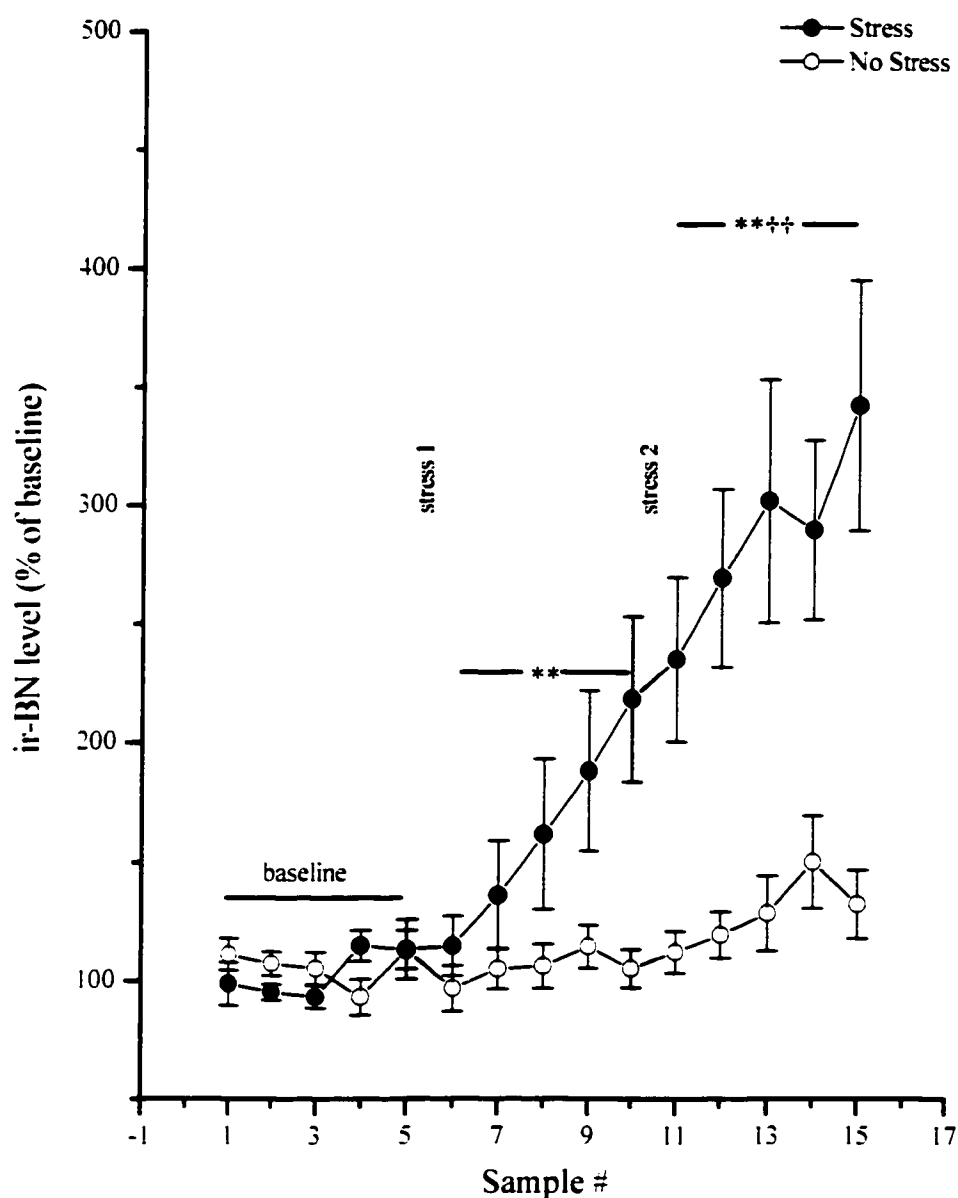


Figure 4. Restraint-elicited release of Bombesin-like peptides from the central nucleus of the amygdala as measured by in vivo microdialysis in samples collected every 30-min. After collection of five baseline samples, rats in the stress group ($n = 10$) were hand-restrained (filled circles) for 20-min episodes on two occasions (shaded vertical bars, stress1 and stress2). The rats in the no stress group ($n = 7$) were left undisturbed (open circles). The five baseline samples from each of the subjects were averaged and defined as 100%. All values were then expressed as a percent of that baseline. Basal values of ir-BN were 3.01 ± 0.42 and 3.28 ± 0.33 fmol/sample for the stress and no stress groups, respectively. Bombesin (BN), immunoreactive (ir). ** Significantly different from baseline ($p < 0.01$). ++ Significantly different from stress1 ($p < 0.01$).

See Figure 3 for location of the microdialysis probes.

Effect of acute restraint episodes on circulating ACTH and corticosterone levels

As expected, the plasma ACTH and corticosterone concentrations were significantly elevated by the stressor treatment ($F_{9,23} = 29.88$ and $F_{9,22} = 100.85$, respectively) $p < 0.01$. The multiple comparisons indicated that relative to basal ACTH concentrations (50.1 ± 9.6 pg/ml) elevations of the hormone were seen immediately after the first (395.4 ± 63.9 pg/ml) and second stressor exposure (274.3 ± 96.9 pg/ml). Likewise, compared to baseline (9.5 ± 2.3 μ g/100 ml), plasma corticosterone levels were increased both after the first and the second stressor exposures (25.8 ± 2.6 and 23.5 ± 1.8 μ g/100 ml, respectively). It is noteworthy that the levels of ACTH and corticosterone observed after the first and second restraint periods were comparable to one another, whereas the interstitial levels of CRH and BN-LPs were significantly higher following the second stressor, compared to the first. As a baseline blood sample was not taken immediately prior to the second stress period, the relative magnitude of the hormonal changes cannot be determined.

Meal-elicited release of CRH and BN-LPs at the CeA

Figure 5 shows the interstitial ir-CRH (upper panel) and ir-BN (lower panel) levels as a percent of baseline, prior to, during and following ingestion of a spontaneous meal. Both peptides varied significantly over the test session ($F_{6,18} = 21.62$, $p < 0.0001$ for CRH and $F_{6,18} = 9.11$, $p < 0.007$ for BN). The multiple comparisons revealed that during the preprandial period, neither peptide varied from baseline. However, during ingestion and during the postprandial period, interstitial levels of both CRH and BN-LPs

were markedly increased. Indeed, the meal-elicited rise of CRH was as great as that associated with the first restraint period (compare Figures 2 and 4).

The positions of probes on target as well as those misaligned (and thus excluded from analyses) are depicted in Figure 6. This response appeared to be specific to the CeA as the "off site" probes failed to show meal-related fluctuations in release of CRH and/or BN-LPs (data not shown).

Plasma ACTH and corticosterone, from samples taken before and after palatable food consumption (mean = 3.7 ± 0.7 g) were both found to vary over the course of the session. ($F_{6,36} = 3.91$, $p < 0.01$ and $F_{6,30} = 6.74$, $p < 0.01$, respectively). The multiple comparisons confirmed that ACTH levels were significantly increased during ingestion and 15-min after initiation of consumption, and declined thereafter (see Figure 7). The rise of corticosterone was slower than that of ACTH, and was significant at 15- and 30-min after meal initiation, and then declined at 60-min. It is noteworthy that while the extent of the CRH and BN-LP increase induced by food ingestion was comparable to that noted during a similar period following restraint exposure, the meal-elicited rise of ACTH was of a smaller magnitude than that elicited by the stressor, while both treatments provoked comparable elevations of corticosterone.

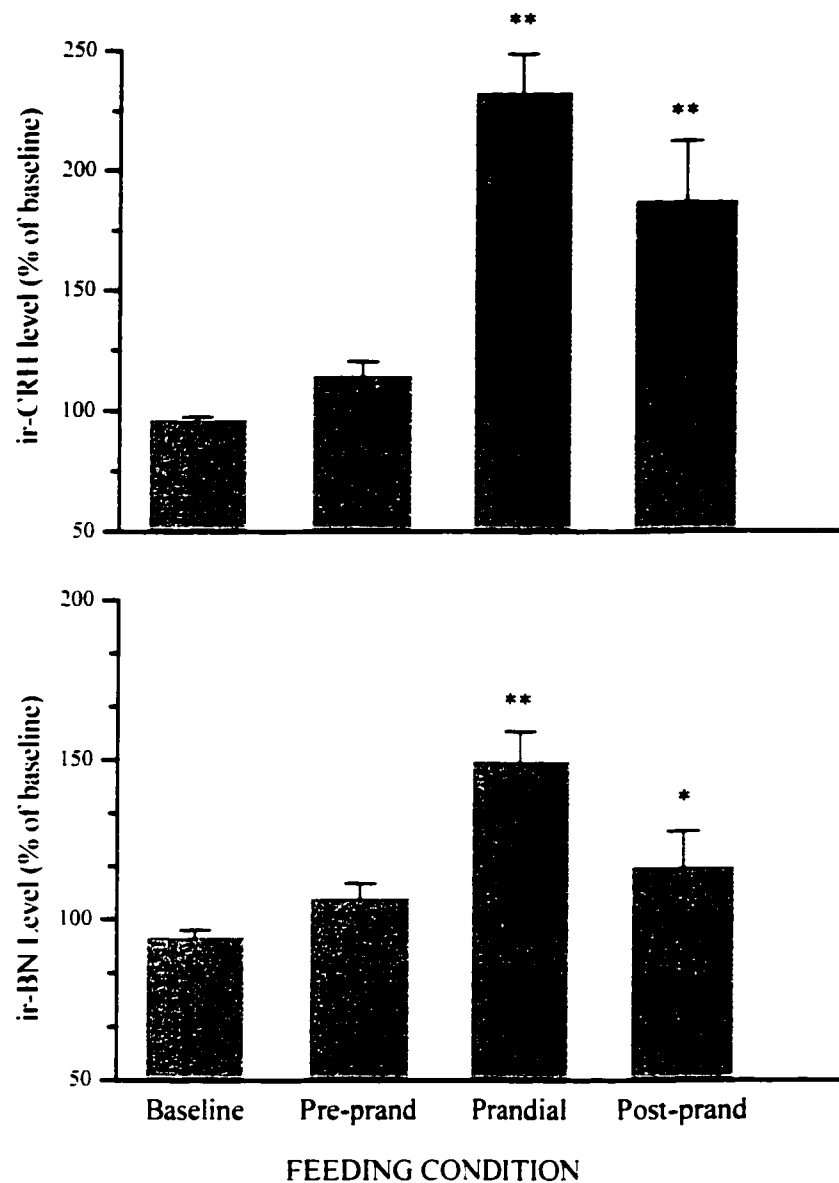


Figure 5. Meal-elicited release of ir-CRH (upper panel) and ir-BN (lower panel) at the central nucleus of the amygdala as measured by *in vivo* microdialysis in samples collected every 30-min for 5-hr. The quantity of food ingested during each 30-min bin was noted. The 30-min period before meal initiation was considered the preprandial period, and the 30-min sample preceding that was considered the baseline. The postprandial period was the 30-min period after meal termination. The baseline value was defined as 100%, and all other values were expressed as a percentage of this value. The basal values for ir-CRH and ir-BN were 0.95 ± 0.12 and 0.77 ± 0.07 fmol/sample, respectively. Corticotropin-releasing hormone (CRH), Bombesin (BN), immunoreactive (ir). *,** Significantly different from baseline at $p < 0.05$ and $p < 0.01$, respectively.

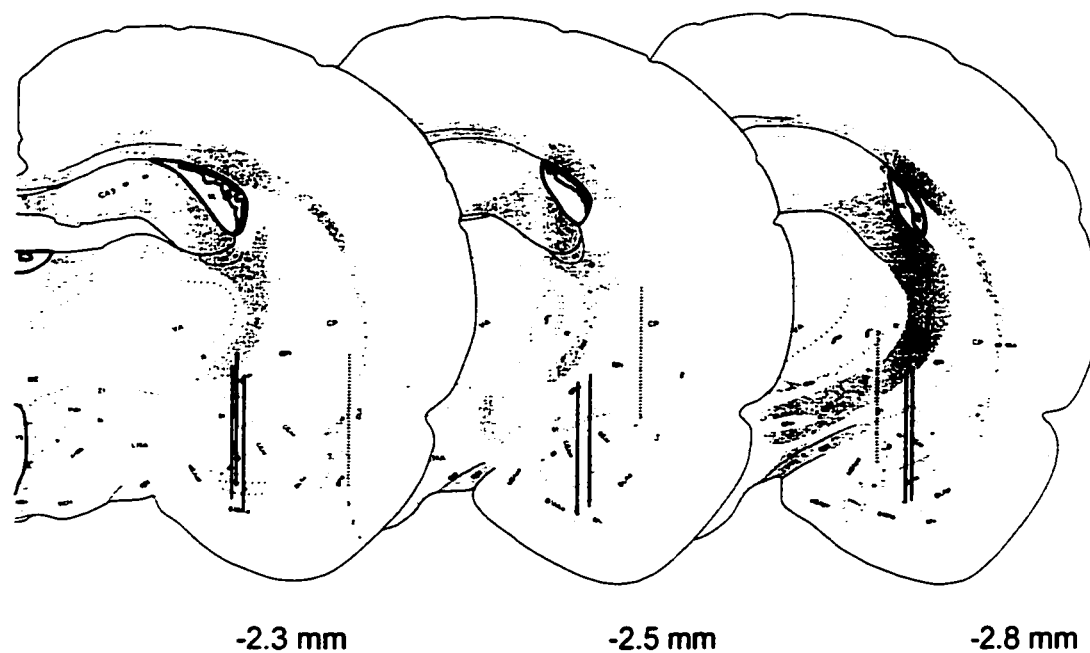


Figure 6. Anatomical localization of the microdialysis membranes aimed at the central nucleus of the amygdala in animals involved in the feeding study. Probe placements were identified and represented as described in Figure 3. Probe placements of animals included in the analysis ($n = 7$) are identified with solid vertical lines, whereas those of animals excluded from analysis attributable to misalignment (off-site, $n = 3$) are depicted by broken vertical lines.

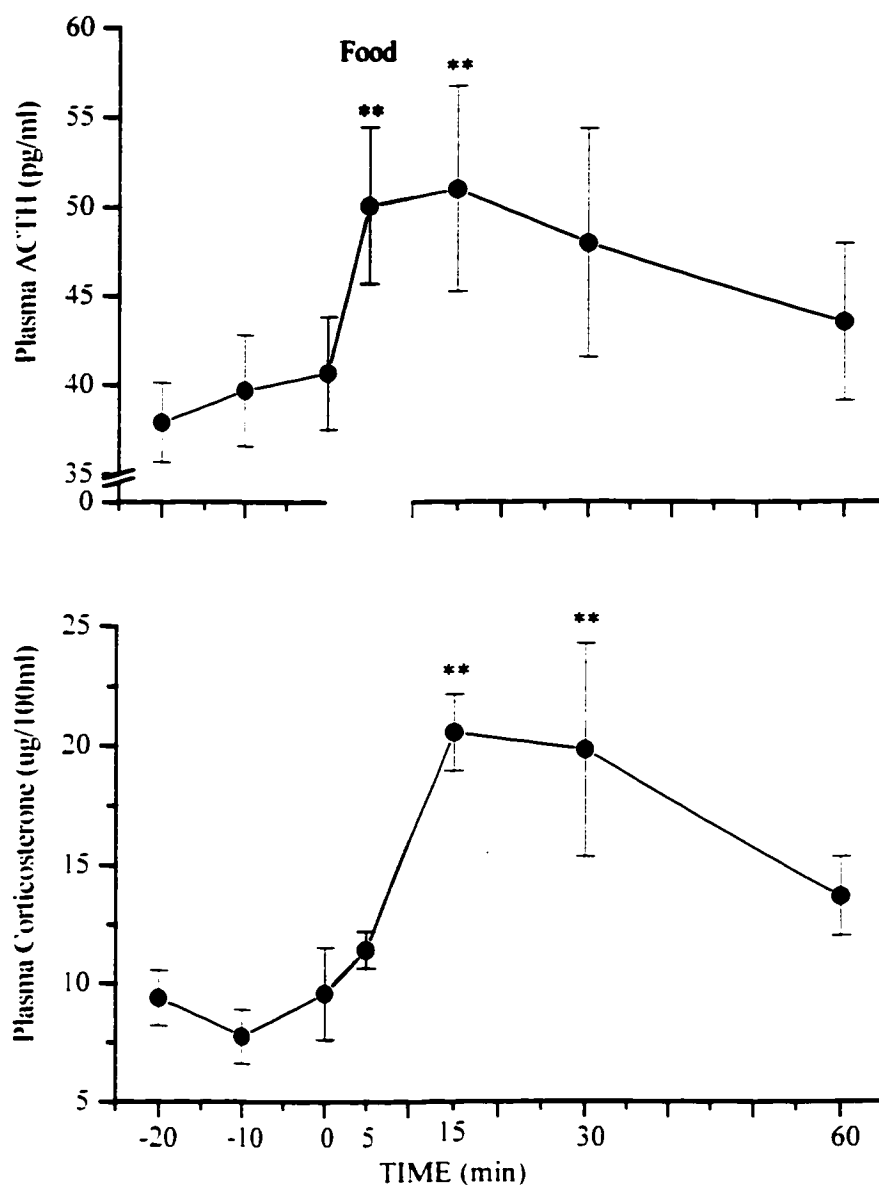


Figure 7. Effect of food ingestion on plasma ACTH (upper panel) and corticosterone (lower panel) levels. Rats equipped with carotid cannula were used in this study. Blood samples (400 μ l) were drawn remotely at 20-, 10-, and 0-min (before food presentation). Rats were then offered graham wafers for 10-min (shaded bar). Blood samples were collected at 5-, 15-, 30-, and 60-min after food presentation. Adrenocorticotropin-releasing hormone (ACTH). ** Significantly different from sample taken immediately before food presentation ($p < 0.01$).

Discussion

Most studies that have assessed the effects of stressors on central CRH have done so using relatively indirect techniques, such as measurement of mRNA expression, immunohistochemistry or postmortem tissue level analyses [238;396;445]. The former two approaches are indicative of potential variations of CRH, but do not necessarily reflect actual peptide release. Moreover, these techniques, like postmortem analyses of CRH content, do not permit evaluation of dynamic within subject variations that may occur over the course of a treatment regimen. In the present investigation, we demonstrate the feasibility of *in vivo* microdialysis, combined with highly sensitive solid-phase RIAs, in the measurement of interstitial levels of CRH and BN-LPs. Furthermore, using HPLC fractionation of brain dialysates and external standards, in combination with RIA and UV detection, respectively, we demonstrate that the ir-CRH within the dialysates corresponds to authentic CRH. In addition, through similar fractionation and analyses we identified the ir-BN-LP material in the dialysate to represent GRP₁₋₂₇ and/or GRP₁₈₋₂₇, the mammalian counterparts of amphibian BN [468]. In both instances, this was further confirmed through the use of two different columns and gradient conditions, wherein the variations of the retention times for the respective peptide standards was associated with a corresponding shift in immunoreactive peaks eluting from the dialysate samples.

Stressor exposure in the present investigation produced a pronounced and sustained increase in the release of CRH at the CeA. This increase was evident soon after stressor onset and was still pronounced and stable 2.5-hr afterwards. Earlier studies had

demonstrated that *in vivo* CRH release was elevated by stressors such as restraint or drug (alcohol or cannabinoid) withdrawal [351:375]. In these experiments, the detection and quantification of CRH involved incorporation of anti-CRH serum directly into the medium perfusing the microdialysis probes. In the present investigation, CRH changes were observed using a simpler, more direct method that did not require spiking the perfusion medium with the anti-CRH serum, and yielded comparable basal interstitial levels of CRH. In both the Pich et al. (1995) [351] and in the present study marked CRH elevations were evident in response to 20-min of restraint stress. While the magnitude of the effects reported by Pich and colleagues were more pronounced, they also were more transient (40-min vs. 2.5-hr in the present study). These differences may have been related to several factors. In their study the inclusion of the CRH antibody into the dialyzing medium may have created a greater positive gradient for CRH from the interstitial fluid, resulting in a sharpened, temporally limited CRH peak. Alternatively, the dynamics of CRH may have been influenced by the cues associated with the stressor. Specifically, in the Pich et al. report, the restraint was applied in a novel cage (thus the “stressful environment” comprised both the novel situation coupled with restraint) after which the rat was returned to its home cage (“safe environment”). In contrast, in the present study, the entire procedure was conducted in the rat’s home cage. Thus it is possible that the otherwise neutral home cage cues may have taken on secondary aversive (or “stressful”) qualities, hence leading to more protracted peptide variations. This supposition is consistent with the view proposed by Lee and Davis (1997) [251], who suggested that the amygdala plays a fundamental role in the fear response (being elicited by an identifiable stimulus and subsiding with the offset of this stimulus), whereas the

bed nucleus of the stria terminalis (BNST), a primary target of the amygdaloid projections, is more closely aligned with more generalized anxiety states. In effect, applying the stressor in the animal's home cage resulted in the sustained CRH release even when the primary stressor was terminated.

Paralleling the CRH changes, release of BN-LPs was also induced by the stressor, a finding commensurate with our earlier postmortem results [223]. The changes in BN-LPs developed relatively slowly over the post-stress period. Since interstitial ir-BN-LP levels increased progressively throughout the 2.5-hr following the initial stressor, it is unclear whether the further rise following the second stressor actually reflects the consequences of the second restraint episode or a sustained effect of the initial restraint period. Nonetheless, the fact that increased release of BN-LPs continued over time following termination of restraint, while the elevations of CRH release were relatively stable, raises the possibility that BN-LPs may be important in subserving sustained emotional changes associated with a stressor experience. Of course, as in the case of CRH changes, the possibility should not be dismissed that environmental cues associated with the stressor and the peptide alterations are related to one another. In fact, since BN promotes ACTH release and this effect is prevented by pretreatment with CRH antagonists [225:299], the possibility ought to be considered that BN-LPs provoke ACTH elevations by stimulating CRH release. The potential physiological role of BN-LPs at the CeA, particularly with respect to CRH release, remains to be elucidated. It is noteworthy that while the variations in ACTH, corticosterone and BN-LPs were evident within 30-min of stressor initiation, the increase in ir-BN was not evident until approximately 1-hr

after stressor onset. Accordingly, it is not likely that the plasma ACTH and/or corticosterone changes were causally related to variations of BN-LPs within the amygdala.

It has been suggested that the activation of HPA neurons may be a fundamental response to stressors. In this context, limbic regions, particularly the amygdala may be essential in determining the response to 'processive' stressors (requiring interpretation by higher brain structures) whereas 'systemic' stressors (involving immediate physiologic threat) may affect HPA activity through non-amygdaloid mechanisms [185]. Indeed it has been suggested that the amygdala may be important in the formation, consolidation and expression of those events that have been associated with aversive stimuli [108], and may activate the HPA axis via amygdaloid inputs to the hypothalamus [163:291]. While not discounting these views, the present results make it clear that appetitive stimuli also influence not only HPA activity but also the release of CRH and BN-LPs at the CeA. While restraint was clearly more effective in provoking the ACTH changes, the two treatments were roughly comparable in elevating circulating corticosterone levels. Similar prandial and/or postprandial ACTH and/or cortisol elevations have also been reported in human studies [4:145:218]. Moreover, indirect analyses of CRH, vasopressin and oxytocin, indicated that among deprived animals, the presentation of water might provoke increased release of these peptides from the median eminence [377]. Interestingly, in these animals, the frustration of being presented with empty water bottles also had similar effects. Thus, these peptidergic changes may reflect general arousal rather than the response to aversive stimuli.

While CeA has long been considered to contribute to emotional responses, the view has also been expressed that this brain region plays an essential role in complex processes such as attention, secondary reinforcement, reward, and social behavior [108]. In fact, in the present investigation *in vivo* ir-CRH and ir-BN changes were as pronounced following an appetitive stimulus as those elicited by the aversive event. Clearly, the amygdala is not uniquely responsive to aversive stimuli, and CRH and BN-LPs are released in response to appetitive events as well. This observation is reminiscent of the catecholaminergic responses noted at the prefrontal cortex and/or nucleus accumbens. Although once believed to be stress-specific, the catecholaminergic systems were subsequently found to be activated by stimuli with positive valence as well, including reinforcing drugs [488], food [187:368:438] or sex [477]. This led to the suggestions that catecholaminergic signals contribute to specific cognitive functions and/or arousal [368], or to processes related to attention and learning [438:480]. It is of interest to note that glucocorticoids too are secreted in response to stressful as well as rewarding events, and have been suggested to represent a biological substrate of reward. According to this contention, glucocorticoids may play a role of counteracting the aversive effects of external threats or insults, allowing for a better coping [350]. As in the case of brain dopamine circuits, we believe that the CRH system(s) may serve a much broader role than previously envisioned. Rather than evoking feelings of fear and anxiety, this system may serve to draw attention to biologically significant events (or cues) such as those associated with food availability as well as those posing physical threat.

The curious observation that an aversive event (such as acute restraint) as well as an apparently appetitive circumstance (such as meal ingestion) would both enhance the release of “stress” peptides (CRH and BN-LPs) at the amygdala and “stress hormones” (ACTH and corticosterone) into general circulation, may have an alternate explanation. It could be argued that at some central level, meal ingestion may be interpreted as a “stressful” event. Although, at first blush this may appear counterintuitive, observation of various species suggests that feeding related activities can indeed be threatening to the organism, often requiring vigilance and/or aggression. For instance, acquisition of a meal by many carnivorous species may require hunting of the prey, a physically challenging and dangerous situation. Furthermore, during ingestion, the organism may need to aggressively protect its food from others (e.g. a growling dog protecting his steak bone), or protect itself from attack during this vulnerable time (e.g. a bird at a feeder eating cautiously, vigilant about a potential attack from cohorts or birds of prey). Finally, the ingestion of a meal may signal imminent flooding of the system with nutrients and/or toxins that may threaten homeostasis. Thus, any or all of the events associated with feeding can potentially be deemed stressful. In the case of humans, the relatively plentiful conditions and structured social environment may have resulted in the brain evolving effective mechanism(s) to suppress and/or modulate the perception of stress associated with food ingestion. According to such a model, it is conceivable that disruption of the mechanism(s) balancing the positive and negative (stressful) attributes of food ingestion may be associated with disorders affecting food intake such as anorexia nervosa, bulimia nervosa, depression or obesity.

Preface to Chapter II

In the preceding series of experiments *in vivo* microdialysis combined with a highly sensitive RIA was used to demonstrate that both aversive and appetitive events elicited the release of CRH and BN-LPs at the CeA in freely moving Sprague-Dawley rats. These peptidergic changes were treatment-specific, as the release patterns of either of the peptides was fairly stable in undisturbed freely behaving animals. In addition, implantation or insertion of the probe only temporarily perturbed the peptide release, which rapidly reached a stable basal release rate. Furthermore, the basal release patterns of CRH or BN-LPs at the CeA did not have a discernable ultradian release pattern.

As discussed in the introduction, several variables can influence an animal's endocrine, physiological and autonomic response to stressors. Several lines of evidence suggest that these factors include the nature of the stressor (i.e. acute, chronic, intermittent), type of stressor (i.e. systemic, processive) as well as organismic variables that include gender, age, previous experience and genetic makeup [15]. Thus, potentially important mechanistic information could be gleaned from investigating the differential stress responses in genetically distinct rat lines. In the next set of experiments, we used two lines of male Long Evans rats previously selected according to their sensitivity (Fast) vs. resistance (Slow) to amygdaloid-stimulated kindling [12].

In addition to their rat line-dependent kindling proneness, they also showed distinct behavioral responses to various stimuli. For example, in response to predator (i.e. ferret) exposure, the Slow rats exhibit an exaggerated behavioral reactivity, relative to the

Fast rats, whereas in response to restraint the Slow rats are more docile relative to the Fast rats (that show greater resistance). Moreover, we serendipitously discovered that the Fast and Slow rats display very distinct behavioral responses to sexually receptive females (an appetitive stimulus). In general, the Fast rats show vigorous excitation (indicative of arousal) combined with audible vocalizations, while the Slow rats appear relatively tranquil both in terms of behavioral activation and vocalization patterns. Paradoxically, the Slow rats are the *more* successful breeders. In sum, these rats provide a unique opportunity to investigate the extent to which peptidergic transmission in the brain in general, and the release of CRH and BN-LPs at the CeA in particular, might underscore some of the rat line-dependent behavioral responses to both aversive and appetitive events.

It should be underscored that it was not our intention to directly compare the response to stressors and/or appetitive events of the Fast and Slow rats to that of the Sprague-Dawley rats (in Chapter I), but rather, we wanted to determine whether the stressor-elicited changes in release of CRH and BN-LPs noted in the Sprague-Dawley rats was generalizable to rats with different genetic underpinnings. Similarly, we wanted to assess the extent to which the release of peptides in response to food ingestion observed in Chapter I could be extended to other appetitive events, such as cues associated with sexual behavior.

Note: A portion of this chapter (predator cues and restraint) has been published as a multi-authored manuscript [296]. My contribution to this published manuscript included conceptualization of the experimental design, technical developments and the assessment of peptide release in response to stressor exposure. The second part of this chapter (peptide release in response to sexual cues) was an individual effort.

CHAPTER II

Differential impact of predator exposure, immobilization stressor, and sexual cues on central corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rats

Abstract

Lines of rats selectively bred for amygdaloid excitability, as reflected by kindling rates in response to electrical stimulation, also exhibit differences in tests of anxiety. Inasmuch as corticotropin-releasing hormone (CRH) and bombesin-like peptides (BN-LPs) have been associated with anxiety, regional levels and release of these peptides, as well as plasma adrenocorticotropin-releasing hormone (ACTH) and corticosterone, were assessed in Slow and Fast seizing rats following predator (i.e. ferret) exposure or immobilization. Ferret exposure elicited a greater increase of plasma ACTH and corticosterone concentrations in the Slow than in the Fast rats. In contrast, immobilization provoked a greater rise of plasma ACTH levels in the Fast rats, paralleling the vigorous struggling observed in this line. In Slow rats, stressor exposure elicited increased levels of immunoreactive (ir)-BN at the anterior hypothalamus, and increased ir-CRH at the median eminence/arcuate nucleus (Me/Arc), paraventricular nucleus of the hypothalamus (PVN) and pituitary (Pit), whereas decreased levels of ir-BN were found at the nucleus of the solitary tract (NTS). Fast rats likewise showed decreased ir-BN at the NTS, but unlike the Slow rats, ir-CRH was reduced in the Me/Arc, PVN and Pit in response to both stressors. *In vivo* microdialysis experiments revealed that in response to ferret exposure, only the Slow rats showed elevated CRH release at the central nucleus of the amygdala (CeA). In contrast, immobilization elicited the release of CRH in only the Fast rat line. In response to an appetitive stimulus (i.e. non-contact exposure to a female cohort) the release of CRH at the CeA increased in the Fast rats only in response to an estrous female, while in the Slow rats elevated CRH release was only observed in response to the non-estrous females. The release pattern of CRH in response

to the two different females did not appear to be associated with the non-discriminatory behavioral response among the Fast rats towards the females or the greater behavioral activation among the Slow rats in response to an estrous female. Taken together, the results of the present study demonstrate that these two lines of rats show differential endocrinological, neurochemical and behavioral response patterns to both aversive and appetitive stimuli. The role of CRH at the CeA in the genesis of these rat line-dependent responses is discussed.

Introduction

One of the most prominent effects of stressors is the release of the 41 amino acid peptide, CRH, as reflected by increased ir-CRH and induction of CRH messenger ribonucleic acid (mRNA) in various brain regions [28:83:96:201:333:451]. Parenthetically, some investigators have reported decreased ir-CRH levels shortly after stressor exposure, particularly at the Me/Arc and medial preoptic area (MPOA). However, this effect appears to be attributable to increased release of the peptide [83]. While the role of CRH at the HPA functioning is quintessential, there is accumulating evidence pointing to its involvement at other brain regions and functions. Indeed, *in vivo* microdialysis studies also indicated release of CRH from the CeA in response to stressors [297:351]. Supporting the contention that this peptide may contribute to numerous responses to stressors, central CRH administration produces endocrine, autonomic and behavioral changes that resemble those elicited by stressors [129:326] and these effects are blocked by pretreatment with CRH antiserum or antagonists [56:64:195:374].

In addition to CRH, it appears likely that BN-LPs are also involved in the mediation and/or modulation of the response to stressors. In this context, acute immobilization stressor induced site-specific alterations of endogenous levels of BN-LP, as well as the density of BN binding sites [223]. Moreover, like stressor exposure, central administration of BN and related peptides (gastrin-releasing peptide (GRP) and neuromedin B (NMB)) potently activate both the HPA axis and the sympathetic division of the autonomic nervous system [60:77:153:170:322]. Further, application of BN-LPs directly into the brain elicits a variety of behavioral responses that resemble those

induced by stressors, including suppression of food intake, enhanced grooming, decreased locomotor activity in a novel environment, and increased exploratory behavior in a familiar environment [154:203:240:345].

It has been argued that HPA activation evoked by different types of stressors may involve distinct neuroanatomical pathways. Herman and Cullinan [185] suggested that an immediate physiological threat to survival (systemic stressor) would necessarily require little sensory processing and therefore activate brain stem nuclei that project directly to the PVN where activation of the HPA axis would be initiated. In contrast, stressors requiring emotional appraisal through higher order sensory processing (processive stressors) via limbic brain structures, such as the hippocampus, amygdala, and prefrontal cortex, lead to indirect or 'filtered' HPA activation. It is important to note that the central effects observed might vary as a function of the specific processive stressor to which an animal is exposed [96]. Likewise, it has been suggested that learned and innate stressful stimuli (e.g., fear conditioning vs. predator odors) may differentially influence central "fear arousal" pathways [311]. The predator odor, trimethylthiazoline (TMT), displays a unique, stress-like pattern of dopaminergic and endocrinological activation in the rat.

The physiological changes evoked by stressors depend not only upon the nature of the stimuli, but also on the inherent individual differences of the organisms being assessed [210]. Predictably, the response to stressors also varies across different strains of mice and rats [13:120:121:285]. These strain differences are not only of heuristic

interest, but can also be used to identify the mechanism(s) contributing to specific stressor-elicited neurochemical alterations and the behavioral changes engendered by the treatment. In this context, we have assessed stressor responses in two rat lines selectively bred for differences of amygdaloid excitability, as realized by either Fast or Slow seizure development following mild electrical amygdaloid stimulation (kindling) [286:364]. Fast and Slow seizing rats differ over days in the number of stimulation trials necessary to produce a kindled state. Interestingly, these rat lines showed very different behavioral profiles in response to different aversive as well as appetitive stimuli. Upon being immobilized, the Fast rats exhibited protracted struggling (i.e. resistance) and a more pronounced endocrine response than did the Slow rats, which remained relatively still. In contrast, in response to a ferret exposure, the Slow rats showed greater reactivity and a greater endocrine response relative to the Fast rats [14:307]. Likewise, we serendipitously discovered that these rats elicit a robust rat line-dependent behavioral response to a sexually receptive female cohort. This is marked by a robust behavioral activation (indicative of arousal) combined with numerous audible vocalizations among the Fast rats compared to the relatively quiescent Slow rats. Paradoxically, the Slow rats are the *more* successful breeders.

In the current study, we further characterized the endocrine and neurochemical changes in the Fast and Slow rat lines in response to either predator exposure or to immobilization. In particular, these studies assessed the effects of the stressors on levels of circulating ACTH and corticosterone, and ir-CRH and ir-BN-LPs across a number of discrete brain regions (using brain micropunch technique). One of the caveats of

postmortem analyses is that the alterations may not reflect the dynamic changes associated with stressor application. Accordingly, a second more direct experiment was carried out to assess the dynamics of *in vivo* release of ACTH, corticosterone and amygdaloid CRH (through *in vivo* microdialysis) in response to the physical immobilization and predator exposure stressors.

The CeA was selected for detailed examination as it plays an important role in the animals' response to stressors [31:162:163:192], has been associated with *in vivo* CRH release in response to stressors [297] and is a distinguishing nucleus between the Fast and Slow rat lines in terms of kindling susceptibility [128:307]. Furthermore, in light of our previous demonstration that an appetitive event (like food ingestion) evoked the release of CRH at the CeA it was of interest to establish whether a vigorous appetitive event (like exposure to a female cohort) would likewise evoke the release of CRH at the CeA in rat lines with a different genetic backdrop than the Sprague-Dawley rats. In addition, given the implication that amygdaloid CRH may underlie the behavioral response to stressors, it was of particular interest to assess the extent to which the noted rat line-dependent behavioral responses to both aversive and appetitive events was reflected in the release of CRH at the CeA.

Materials and methods

Animals

The Fast and Slow lines of rats were selectively bred, without brother x sister mating, from an original parent population comprising Wistar and Long-Evans Hooded

rats. These lines were initially established at McMaster University (Hamilton, Ontario) [364] and, after F₁₁ generations, they were transferred to Carleton University, Institute of Neuroscience, where their kindling differences persisted despite relaxation of the selection procedure. The rats used in the present investigation were from the 42nd and 43rd generations of the two lines. All rats were male and at least 4 months of age, and allowed free access to food and water. Fast and Slow rats were of similar weights at 4 months (~450-500 g). The rats were maintained on a 12-hr light-dark cycle (lights on at 0600-hr), in a temperature (23°C) and humidity (60%) controlled room, and were housed as pairs in standard opaque plastic cages (32 x 22 x 20 cm). All testing was conducted between 0800-1200-hr to minimize the influence of endocrine and central neurotransmitter variations attributable to diurnal rhythms. The experiments met the guidelines set out by the Canadian Council of Animal Care with respect to the care and use of animals for experimental procedures, and received ethical approval from the Carleton University Animal Care Committee.

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

Fast and Slow rats were randomly assigned to 3 conditions (n = 10/group) and were subjected to no stress (control), 15-min of non-contact ferret exposure, or 15-min of immobilization. The procedures for restraint and predator exposure have been described elsewhere [14]. Briefly, immobilization involved placing a rat into a cone shaped plastic bag with an opening at the narrow end to permit the rat's snout to protrude. Once completely inside, the bag was taped shut around the rat's tail and the rat was placed on a

tabletop for 15-min. The bag provided a snug fit, which restrained both lateral and forward movements, and limited paw and head movement. The procedure for predator exposure comprised placing a rat in a typical shoebox clear polycarbonate cage (26 x 48 x 20 cm) with a stainless steel lid. The cage containing the rat was then placed inside a larger ferret cage (60 x 60 x 75 cm) containing a single ferret. The 15-min of ferret exposure permitted an exchange of olfactory and visual cues, but the ferret could not make physical contact with the rat. However, the ferret could sit atop the rat cage, and attempt to push the cage about (albeit to a limited extent). Typically, upon placing the rat cage into the ferret cage, the ferret would explore the top and sides of the rat cage, and make obvious attempts to gain access to the rat. In order to maintain a ferret's interest in the inaccessible rat, exposure of rats was alternated between four ferrets, and ferrets were only exposed to two rats on any given day. Since the ferrets differed in their effort to gain access to the rats, the ferrets were counterbalanced between the two rat lines. Immediately following stressor exposure, rats were transported, while in their cages, to a necropsy area where they were sacrificed 1-min later by decapitation. Trunk blood was collected for ACTH and corticosterone analysis by radioimmunoassay (RIA), while the brains were rapidly extracted, frozen on dry ice, and stored at -80°C for subsequent neuropeptide determinations.

Plasma ACTH, corticosterone and testosterone

Trunk blood was collected in tubes containing EDTA, centrifuged and the plasma was frozen and stored at -80°C. Plasma ACTH and testosterone levels were determined in duplicate using commercial RIA kits (ICN Pharmaceuticals, CA), as was

corticosterone (R&D Biochemicals). These assays yielded intra- and inter-assay variations of less than 10%.

Tissue Preparation for RIA

Using a cryostat, the brains were sectioned coronally (600 μ m thick) and the following 15 discrete nuclei were dissected from the sections, using a modification of the micropunch method described by Palkovits and Brownstein [334]: the median eminence/arcuate nucleus (Me/Arc), paraventricular nucleus of the hypothalamus (PVN), lateral (LH), anterior (AH), ventromedial (VMH) and dorsomedial (DM) hypothalamic nuclei, central nucleus of the amygdala (CeA), hippocampus (Hipp), locus coeruleus (LC), olfactory tubercle (OT), nucleus accumbens (nAcb), caudate (Cau), cingulate cortex (Cg Cx), somatosensory cortex (S Cx) and motor cortex (M Cx). A 16th region, the nucleus of the solitary tract (NTS), was micropunched from a horizontal section (600 μ m thick) of the brain stem, while the pituitary (Pit) was taken as a whole at the time of brain extraction. All nuclei were derived bilaterally (Micro Punch MP-600, ASI instruments, MI) using a 1 mm diameter punch, except for the Me/Arc, which was obtained using a single medial 1 mm diameter punch.

Areas that have been found to be stressor-responsive as reflected by stressor-related alterations of c-fos expression and/or other neurochemical changes (e.g. CRH, AVP, monoamine activity) include Me/Arc, LC, DM, VMH, CeA, PVN, LH, NTS, AH, nAcb, Hipp and Pit [96:279:290]. As a positive control, several other brain regions (OT, M Cx, S Cx, Cg Cx and Cau) were also included.

The tissue punches were placed in microcentrifuge tubes containing 200 μ l of 0.2 M acetic acid and heated to 80°C for 1-hr. The tissue was then homogenized (Kontes Micro-ultrasonic Cell Disruptor, Kontes, NJ) and 40 μ l of the homogenate was removed for protein content analysis (BCA Protein Analysis kit: Chromatographic Specialties, Canada) using a PC colorimeter (Model 800, Brinkmann, NY). The remainder of the homogenate was centrifuged at 10 000 x g for 4-min and the supernatant of each region was then divided into two 75 μ l aliquots, lyophilized and stored at -20°C for 1-3 weeks.

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

In view of the rat line-specific effects of immobilization and ferret exposure on ACTH concentrations in the preceding experiment, it was expected that in these rat lines the ACTH secretagogues, ir-CRH and ir-BN, within loci constituting the HPA axis, would likewise be differentially affected by the stressors. Fast and Slow rats were anesthetized with halothane and implanted with indwelling arterial catheters (PE 50 tubing) *via* the pulmonary artery. The saline-filled catheter was passed subcutaneously to the nape region and exteriorized. Rats were then fitted with harnesses around their front paws, which attached to a tether (to enclose the exteriorized catheter) at the base of the neck. Each rat was then placed individually into a cage and the catheters connected to a peristaltic pump (to permit continuous infusion of saline containing heparin through the catheter) *via* polyethylene tubing and a swivel. A three-way valve permitted blood sampling on demand.

Following a five-day recovery period, testing began with collection of a baseline blood sample (control condition). Rats in their cages were then taken to another room where they were exposed to the predator or were immobilized for 15-min ($n = 10/\text{group}$). Immobilization stressor involved manual immobilization (technically restraint as some movement was permitted) of the rat for 15-min. Predator exposure involved placing the rat (in its cage) into a 4.4 x 5.2 m room containing a single ferret. As in the first experiment, the ferret could move freely around the rat's cage, but could not make direct contact with the rat. Blood samples were taken at baseline, and again 5-, 15-, 30- and 60-min afterward.

Experiment 3. Effects of predator exposure and immobilization on the in vivo release of CRH at the CeA in Fast and Slow rats

Male Fast and Slow rats ($n = 13-14/\text{group}$) were anesthetized (60 mg/kg pentobarbital, i.p.) and stereotactically implanted with unilateral 20 gauge guide cannulae containing removable 24 gauge obturators aimed at the dorsal aspect of the CeA. The placement coordinates [342] with level skull were A/P, -2.1 mm; D/V, -7.0 mm; and L/M, ± 4.2 mm. The guide cannulae, protruding from a custom-manufactured Delrin[®] pedestal, were secured to the skull with four stainless steel screws and dental cement. After a minimum 7-day surgical recovery, animals were transferred to individual testing chambers and allowed to acclimate for 48-hr before testing. The testing chambers comprised of Plexiglas cages (25 x 35 x 34 cm) with stainless steel grid floors. Food (Purina[™] lab chow) and tap water were available *ad libitum*.

Experiment 4a. Effects of non-contact exposure to estrous or non-estrous females on the release of CRH at the CeA in male Fast and Slow rats

Sexually mature Male Fast and Slow rats ($n = 10/\text{rat line}$) weighing ~350–450 g were anesthetized (60 mg/kg pentobarbital, i.p.) and stereotaxically implanted with unilateral 20 gauge guide cannulae containing removable 24 gauge obturators aimed at the dorsal aspect of the CeA (see above coordinates). This study was carried out during the light cycle (0900–1300-hr). Following a minimum 7-day post-surgical recovery rats were transferred to the testing cages that comprised the same dimensions as those above with the addition of three 1 x 32 cm ventilation slits at the rear bottom of the cage. Rats acclimatized to the testing cage for at least 48-hr prior to testing. On the day prior to testing, one rat from each line was taken in their respective testing cages and placed in the testing room atop a table containing a non-contact chamber used to house the females that was comprised of Plexiglas (6 x 6 x 80 cm) with three 1 x 76 cm ventilation slits that aligned with those in the male's testing cages permitting the free exchange of olfactory cues (see Figure 1).

Determination of female sexual receptivity

The females used in this experiment were intact Fast and/or Slow ($n = 8/\text{rat line}$) and deemed to be in estrous if under 40 X magnification large keratinized or cornified epithelial cells were present. For Experiment 4b, ovariectomized Long Evans females (Charles Rivers, Canada) were brought into estrous with a subcutaneous injection of estradiol benzoate (20 μg) in 0.5 ml peanut oil 48-hr prior to male exposure (Loraine et

al., 1997) [259]. Random vaginal smears confirmed the efficacy of the estradiol injections to induce estrous.



Figure 1. Photographic representation of the non-contact behavioral testing paradigm. Separate testing cages house a single Fast and Slow rat with the non-contact chamber housing two estrous or non-estrous females (designated chambers). Note ventilation slots along the bottom of each male cage align with matching slots in non-contact chamber to permit the exchange of olfactory cues. Behavioral observation was made prior to during and following the presentation of the females into the non-contact chamber.

In vivo microdialysis

In all microdialysis experiments probes were implanted at least 120-min prior to baseline collection, a time frame determined by previous experience to be more than adequate to attain stable samples (see Chapter I). Rats were briefly anesthetized with halothane (~ 3-min), and the obturator within the guide cannula was replaced with a microdialysis probe, which extended 2.5 mm (includes 0.5 mm glue tip) beyond the end of the cannula. The concentric microdialysis probe had 2.0 mm of active membrane (250 μ m outer diameter) of regenerated cellulose (6000 MW cut-off: Spectrum Medical Industries) that protruded into the CeA. Each probe was secured with a retaining screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel and a 2.5 ml gas-tight infusion syringe attached to a pump (Harvard, model 22). Microdialysis probes were perfused at 3 μ l/min with filtered Kreb's-Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻, 0.05% ascorbate, pH 7.4 [386], and BSA (0.1%). On collection, each sample (90 μ l) was immediately frozen on dry ice and stored at -80°C until radioimmunoassay (RIA) analyses. The efficiency of the microdialysis probes was assessed in vitro (details described in Chapter I) with the average peptide recovery for CRH being $3.3 \pm 0.6\%$.

In all microdialysis experiments, 6 baseline samples were collected prior to treatment conditions (ferret, immobilization or female (estrous vs. non-estrous counterbalanced presentation) followed by one sample during each treatment and 6 post-treatment samples. Upon collection, dialysates were placed on dry ice and stored at -80°C until RIA (~2-3 weeks).

Experiment 4b. Behavioral response of Fast and Slow males in the presence of estrous or non-estrous female rats

Pre-exposure to sexually receptive female. In the current experiment male rats (n = 10/rat line) were exposed to a hormone-primed sexually receptive female in cages identical to the testing cages 24-hr prior to testing. Contact with the sexually receptive female was permitted to occur for up to 90-min. during which time the latency to mount the female was noted.

On testing day we assessed the behavioral response of the sexually experienced males (n = 10/rat line) towards non-injected and hormone-primed ovariectomized females under three different contact conditions: baseline (i.e. no female present), non-contact (i.e. 2 females (both estrous or non-estrous are placed in a designated (estrous or non-estrous) non-contact chamber), and contact (i.e. 1 female is placed into a Fast and Slow testing cage permitting direct physical contact). One hour following the removal of the females from the male's cages the process was repeated with the 2nd set of females (either estrous or nonestrous) and the order of female presentation was counterbalanced throughout the study. To permit the direct exchange of olfactory cues between the females and the males the same procedure was followed as described in Experiment 4a. To assess the behavioral response towards the females Fast and Slow males were videotaped so that they could later be observed every 5-sec for 20-min intervals during baseline, non-contact and contact conditions.

In the baseline and non-contact condition the males were monitored for the following behaviors: *resting*: whereby the animal is still, possibly sleeping; *rearing*: animal is standing using his rear legs; *exploring*: the animal actively examines an area of the cage; *face body grooming*: the forelimbs are wiped over the face or ventral regions of abdomen and thorax; *genital grooming*: male licks/washes his genital region; *sniffing other*: the nose and head of a stationary animal vibrate non-specifically within the cage; *sniffing non-contact chamber*: sniffing is directed towards the non-contact chamber. During the contact condition males were observed for the following behaviors: *sniffing female*: sniffing is directed at the female; *ano-genital sniffing*: male rat directs sniffing towards the ano-genital region of the female; *mounts*: male rat mounts female with or without intromission; *attempted mounts*: male rat makes unsuccessful attempts to mount female; and *pursuit behavior*: the male rat actively pursues female around the cage.

Histology

At the end of the experiment, the brains were extracted, rapidly frozen on dry ice, sectioned, and stained for histological examination of the microdialysis probe placements. Only those probes confirmed by an independent observer to be in the CeA were used in the data analysis. At the time of sacrifice, trunk blood was collected (n = 10/rat line), for the determination of baseline morning circulating testosterone levels.

Radioimmunoassays (RIAs)

The detection and quantification of CRH was achieved through a solid-phase high sensitivity adaptation/modification of the double antibody liquid phase RIA originally

described by Vale and colleagues [454]. BN-LP was detected using a similar solid-phase RIA [297]. Briefly, protein A/G (Calbiochem-Novabiochem Corp., La Jolla, CA) coated Immulon[®]-4 wells (Dynatech Laboratories, Inc., Chantilly, VA) were incubated with anti-CRH serum (rC70 kindly provided by W. Vale) or anti-BN serum (α -BN₂ kindly provided by Dr. T. Moody) for 2-hr at 20°C. Samples, standards (reconstituted in the KRB solution, ranging from 0.05 to 250 fmol/well) or blanks were incubated for 24-hr at 4°C. Next, 25 μ l assay buffer containing 5000 - 6000 CPM [¹²⁵I-Tyr⁰]rCRF (Amersham Canada Ltd., Oakville, ON) or [¹²⁵I-Tyr⁴]BN (iodinated in house, as per [386]) was added to each well and incubated for an additional 24-hr period at 4°C. Finally, the wells were rinsed, separated and their residual radioactivity counted in a gamma-counter (Cobra[®] II Auto-gamma, Meriden, CT). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically about 0.1 and 2 fmol/well for CRH and BN, respectively.

The specific anti-CRH serum used in the study recognized CRH₁₋₄₁ and displayed negligible cross-reactivity with other related peptides including urotensin 1 and urocortin [465]. The BN antibody used in the RIAs recognized the C-terminal fragment of BN and has been demonstrated to strongly cross-react with amphibian BN (100%) and certain mammalian BN-LPs including gastrin releasing peptide (GRP)₁₋₂₇ (110%), and GRP₁₈₋₂₇ (i.e. NMC (82%)), but only weakly with GRP₁₋₁₆, neuromedin B (NMB)-10, NMB-32 or substance P ($\leq 0.1\%$) [309]. We have shown in the past that the major source of BN-like immunoreactivity from the hypothalamus is attributable to GRP [294], also see Chapter I.

Statistical analyses

All results are expressed as means $\pm$ S.E.M. In Experiment 1, the effects of the stressor treatments on plasma ACTH or corticosterone, as well as ir-CRH and ir-BN at the various brain regions, were analyzed by two factor (Rat line $\times$ Treatment) analyses of variance (ANOVAs) followed by t-tests with Bonferroni correction. For the purpose of the Bonferroni correction, family-wise error was defined independently for each brain region, as specific predictions had been made concerning those regions in which stressor effects had been predicted, namely the nuclei constituting the HPA axis (PVN, Me/Arc. pituitary) as well as the CeA. In Experiment 2, the effects of ferret exposure on plasma ACTH and corticosterone values were analyzed separately by mixed measures ANOVA (Rat line $\times$ Time). Post hoc comparisons were again conducted using t-tests with Bonferroni correction in which post-stressor hormone levels were compared relative to non-stressed animals and between the rat lines. Microdialysis data from the ferret exposure and immobilization stressor in Experiment 3 were analyzed by first averaging the baseline samples (denoted as 100%) with subsequent samples expressed as a percentage of that baseline. The changes of ir-CRH levels in the ferret and immobilization studies were analyzed using mixed measures ANOVAs with Rat lines as the between-groups factor (Fast vs. Slow) and Time (two baseline samples and four post stressor samples) as the within-subjects factor. Similarly, in Experiment 4a the changes in ir-CRH release were analyzed using mixed measures ANOVAs with Rat lines (Fast vs. Slow) and female condition (estrous vs. non-estrous) as the between-groups factors and Time (two baseline samples and 6 post-female samples) as the within-subjects factor. In each of the experiments analyzed by repeated measures ANOVAs the rat lines were

considered independently in determining family-wise error for the Bonferroni corrections. Morning plasma testosterone levels were compared between the two rat lines using a pair-wise t-test.

Results

Experiment 1

Effects of predator exposure and immobilization on plasma ACTH and corticosterone levels

Plasma ACTH and corticosterone levels in Fast and Slow rats as a function of the stressor treatment are shown in Figure 2. The concentrations of ACTH varied as a function of the Stressor treatment x Rat line interaction, $F_{2,42} = 8.00$, $p < 0.01$. Subsequent t-tests with Bonferroni correction revealed that in the absence of a stressor, levels of ACTH were comparable in the two rat lines. Relative to non-stressed rats, both ferret exposure and immobilization significantly increased ACTH in the two lines. However, in response to ferret exposure, the increase was greater in the Slow rats, exceeding that evident in the Fast line. Although following immobilization the ACTH increase among the Slow rats was comparable to that elicited by ferret exposure, the increase was considerably greater in Fast rats, and hence the hormone levels significantly exceeded that of Slow rats.

Like ACTH, plasma corticosterone levels were altered by the stressor manipulation, $F_{2,52} = 25.89$, $p < 0.01$, but the interaction between the Stressor and the Rat lines was not statistically significant. The multiple comparisons indicated that ferret

exposure and immobilization increased corticosterone levels to a comparable extent in both rat lines.

Effects of predator exposure and immobilization on regional ir-CRH levels

Figure 3 shows ir-CRH concentrations at the PVN, Me/Arc and Pit in Fast and Slow rats in each of the treatment conditions. In each of these brain regions, the concentrations of ir-CRH varied as a function of the Rat line x Stressor treatment interactions. $F_{2,54} = 6.22, 6.12$ and 4.4 , p 's < 0.01 , respectively. Given the strain- and stressor-dependent changes in corticosterone and ACTH responses, post hoc comparisons at the regions comprising the HPA axis (PVN, Me/Arc, Pit) and CeA that influence HPA activities were assessed using t-tests with Bonferroni correction. In the absence of any stressor treatment the concentrations of ir-CRH were comparable in the two rat lines in each of these three regions (see Table 1). By contrast, following stressor exposure, the peptide concentrations within the PVN, Me/Arc and Pit were moderately reduced in the Fast rats, but were increased in the Slow line. In this respect, the increase of ir-CRH relative to control levels was increased by immobilization in the PVN of Slow rats and by ferret exposure in the Me/Arc of this line. As a result, the post-stressor levels of ir-CRH were significantly higher in Slow rats than in their Fast counterparts. The effects observed in other brain regions, including several hypothalamic nuclei, were less dramatic. Within the LH, the Slow rats displayed a small but statistically significant elevation of ir-CRH, $F_{1,54} = 6.89$, $p < 0.01$. However, neither the Stressor main effect nor the Rat line x Stressor interaction reached significance. Likewise, within the VMH and AH, the ir-CRH levels were not altered as a function of any of the variables. The ir-CRH

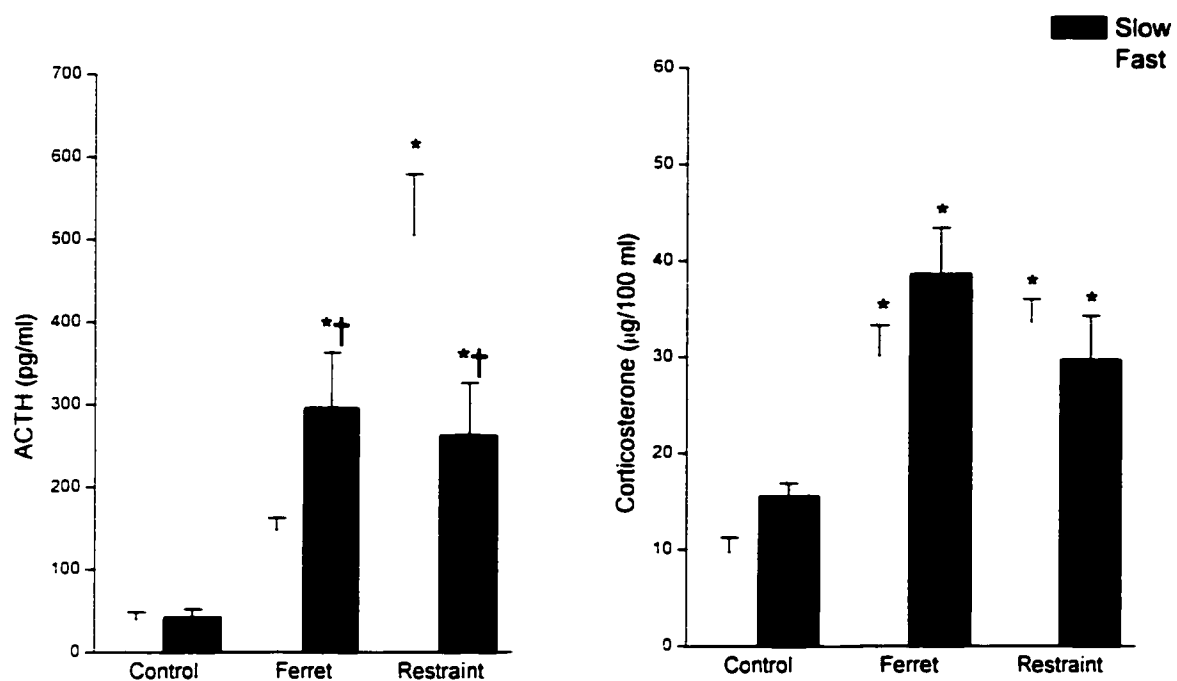


Figure 2. Effects of ferret exposure and restraint on circulating ACTH (left panel) and corticosterone (right panel) in Slow (solid bars) and Fast (shaded bars) rat lines. Values are expressed as means $\pm$ S.E.M., under control condition and following 15-min of stressor exposure. Adrenocorticotropin-releasing hormone (ACTH). * Significantly different from non-stressed (within-rat line) control values, $p<0.05$. † Significantly different from Fast (between-rat line) treatment-matched values, $p<0.05$.

concentrations at the Hipp among Slow rats exceeded that of Fast rats, $F_{1,48} = 14.08$, $p < 0.01$, but the Stressor treatment was without effect. At the CeA, the levels of ir-CRH did not vary as a function of either the Rat line or the Rat line x Stressor interaction. As the two rat lines were selected based on amygdaloid excitability, it was expected that they might be differentially affected at this site. Given this *a priori* hypothesis, Bonferroni corrected t-tests were conducted for the simple effects comprising this interaction. These comparisons confirmed that the stressors did not affect ir-CRH at the CeA of Slow rats. However, a significant rise of ir-CRH was induced by immobilization in the Fast rat line as compared to controls. The ferret exposure did not significantly increase the level from that of control rats. The concentrations of ir-CRH within the Cg Cx and S Cx were unaffected by the Stressor treatment or the line of rats. Likewise, in the M Cx, the peptide levels were unaffected by the stressor, whereas concentrations of ir-CRH in Fast rats were lower than those seen in Slow rats, $F_{1,50} = 4.25$, $p < 0.05$. Finally, striatal sites (nAcb, Cau and OT) were unaffected by the treatments and, similarly, brainstem sites, such as the LC and NTS, were comparable between the two rat lines and were unaffected by the stressor treatments.

Effects of predator exposure and immobilization on regional ir-BN levels

In contrast to the well-defined ir-CRH variations observed in response to the stressor treatments, few differences of ir-BN were observed between the Fast and Slow rats either at baseline or as a function of the stressor treatments. In neither the PVN nor the Pit were appreciable differences observed as a function of the treatments, whereas at

the Me/Arc, the levels of ir-BN of Fast rats exceeded those of the Slow line following stressor treatments although control levels were comparable between the two rat lines. $F_{1,42} = 5.59$, $p < 0.05$. Within the AH, the ir-BN levels of Fast rats appreciably exceeded that of Slow rats (see Table 2). Relative to non-stressed rats, the concentrations of the peptide in Slow rats were elevated by ferret exposure and by immobilization. $F_{1,54} = 4.40$, $p < 0.05$, but remained relatively unchanged in Fast rats. In the remaining hypothalamic nuclei (VMH, LH and DM), treatment effects were not evident. Likewise, in neither the Hipp nor the CeA were there differences as a function of the stressor treatment or the line of rats, a pattern also evident in both the M Cx and S Cx. Differences of ir-BN were not apparent as a function of the treatments within striatal regions (Cau, nAcb and OT) or within the LC. Within the NTS, however, the ir-BN levels in Fast rats exceeded that of Slow rats. $F_{1,51} = 6.56$, $p < 0.05$ (mean = 0.355 ± 0.031 and 0.274 ± 0.02 pmol/mg protein, respectively), and relative to non-stressed rats, the concentrations of the peptide were significantly reduced by ferret exposure in both Fast (mean = 0.212 ± 0.026 pmol/mg protein) and Slow rats (mean = 0.176 ± 0.021 pmol/mg protein) and by immobilization in Fast rats (mean = 0.256 ± 0.028 pmol/mg protein). $F_{2,51} = 12.36$, $p < 0.01$. Similarly, a slight but statistically non-significant decrease in post-immobilization levels of ir-BN was also observed in Slow rats (mean = 0.215 ± 0.019 pmol/mg protein).

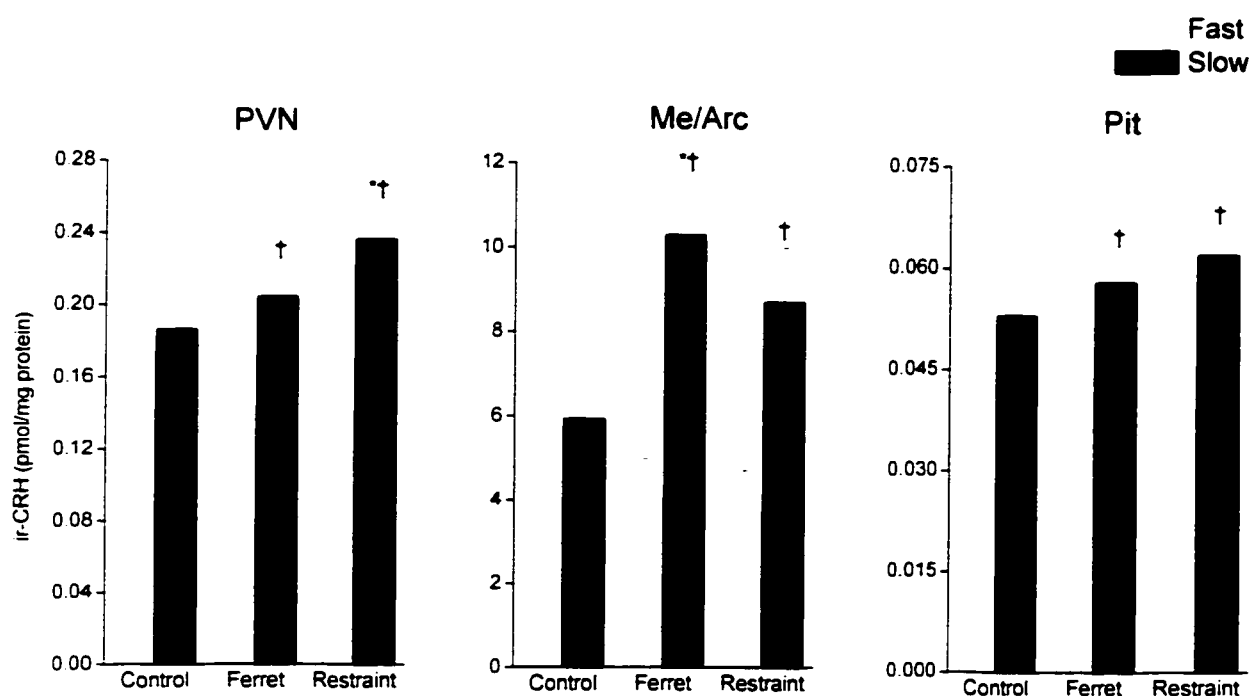


Figure 3. Effects of predator exposure and restraint on ir-CRH concentration (mean $\pm$ S.E.M.) at the paraventricular nucleus (PVN), median eminence arcuate nucleus (Me/Arc), and pituitary (Pit) in Fast (shaded bars) and Slow (dark bars) rat lines. * Significantly different from within-rat line control condition, $p < 0.05$. † Significantly different from Fast (between-rat line) treatment-matched values, $p < 0.05$.

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

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

Note. Each cell represents the mean ± S.E.M. (pmol/mg protein) under non-stressed control condition and following 15 min of ferret exposure or restraint in the following brain regions: median eminence/arcuate nucleus (Me/Arc), locus coeruleus (LC), dorsomedial (DM) and ventromedial hypothalamic (VMH) nuclei, central amygdaloid nucleus (CeA), paraventricular hypothalamic nucleus (PVN), olfactory tubercle (OT), lateral hypothalamus (LH), motor cortex (M Cx), nucleus of the solitary tract (NTS), anterior hypothalamus (AH), nucleus accumbens (nAcb), somatosensory cortex (S Cx), cingulate cortex (Cg Cx), caudate (Cau), hippocampus (Hipp) and pituitary (Pit).

* Significantly different from non-stressed (within-rat line) control values at $p < 0.05$.

† Significantly different from Fast (between-rat line) treatment-matched values at $p < 0.05$.

Experiment 2

Effects of predator exposure and immobilization on the time-course of plasma ACTH and corticosterone changes in Fast and Slow rats

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

The analysis of plasma corticosterone levels yielded significant main effects of the Rat line ($F_{1,11} = 6.98$, $p < 0.05$), indicating that corticosterone levels were higher in the Slow than in the Fast line. As well, a significant effect was found for the Time after treatment ($F_{4,44} = 13.35$, $p < 0.001$), and the t-tests indicated that corticosterone levels were elevated at each of the post-stressor periods relative to baseline values in Slow rats, but only at the 30-min post-stressor period in Fast rats. Although the interaction between the Stressor Treatment and Rat line was not significant, this experiment was specifically conducted to ascertain whether, in fact, *in vivo* corticosterone changes would mirror the

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

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

Note. Each cell represents the mean ± S.E.M. (pmol/mg protein) under non-stressed control condition and following 15 min of ferret exposure or restraint in the following brain regions: median eminence/arcuate nucleus (Me/Arc), locus coeruleus (LC), dorsomedial (DM) and ventromedial hypothalamic (VMH) nuclei, central amygdaloid nucleus (CeA), paraventricular hypothalamic nucleus (PVN), olfactory tubercle (OT), lateral hypothalamus (LH), motor cortex (M Cx), nucleus of the solitary tract (NTS), anterior hypothalamus (AH), nucleus accumbens (nAcb), somatosensory cortex (S Cx), cingulate cortex (Cg Cx), caudate (Cau), hippocampus (Hipp) and pituitary (Pit).

* Significantly different from non-stressed (within-rat line) control values at $p < 0.05$.

† Significantly different from Fast (between-rat line) treatment-matched values at $p < 0.05$.

ACTH variations induced by stressors in Experiment 1. Accordingly, Bonferroni corrected t-tests were conducted between specific means of the simple effects comprising the Rat line x Time interaction. These comparisons indicated that at baseline the rat lines displayed comparable levels of plasma corticosterone. In response to ferret exposure, a rise of plasma corticosterone levels was evident in both lines; however, among the Slow rats this increase was apparent as early as 5-min following stressor exposure, and at 15-min corticosterone levels exceeded that of the Fast rats. In both lines, the corticosterone levels continued to rise, peaking at the 30-min time point (i.e. 15-min after stressor termination), after which the levels began to decline.

Figure 5 illustrates the time-course of immobilization-induced release of plasma ACTH and corticosterone in Fast and Slow rats. Generally, in both rat lines immobilization provoked a substantially greater rise of ACTH than did ferret exposure (contrast Figure 4 and 5). For instance, while the peak ACTH level in response to the ferret was less than 180 pg/ml (in the more reactive Fast rat line), the peak ACTH level in response to immobilization exceeded 750 pg/ml in the Fast line and 350 pg/ml in the less reactive Slow line.

ANOVA revealed that plasma ACTH levels varied as a function of the Rat line x Time interaction, $F_{4,44} = 4.986$, $p < 0.01$. The t-tests of the means of the simple effects revealed that immobilization produced an increase of plasma ACTH in both rat lines, which was significantly elevated above baseline levels at the 5-, 15-, and 30-min time points for Fast rats and at the 5- and 15-min time points for Slow rats. While baseline

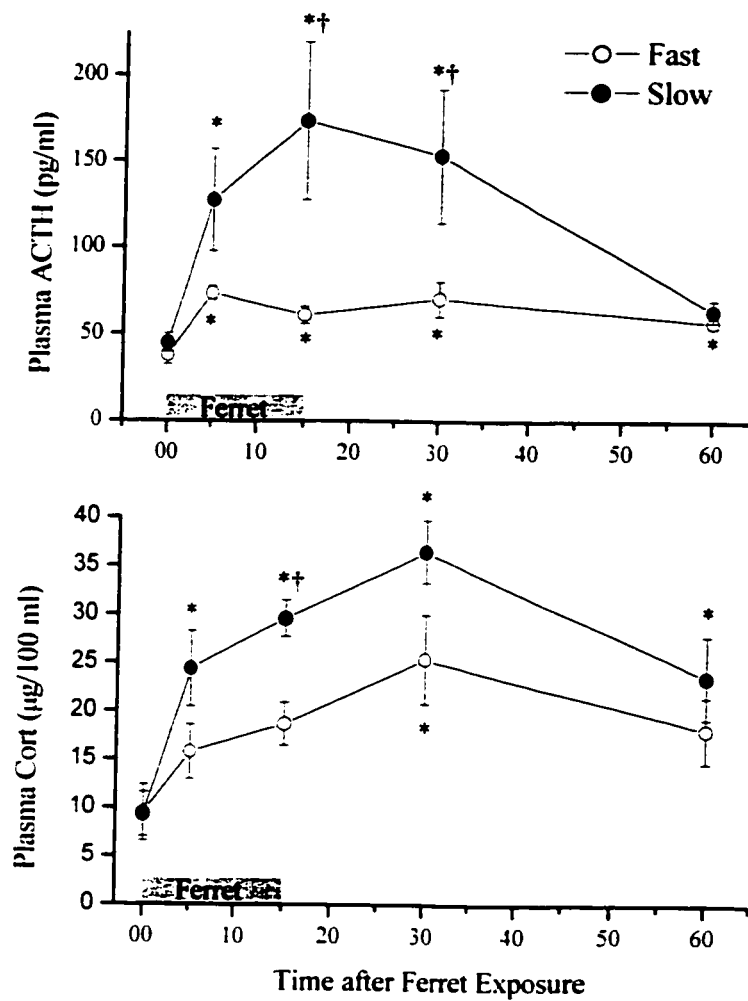


Figure 4. The *in vivo* release of plasma ACTH and corticosterone (Cort) (mean $\pm$ S.E.M.) over 60-min in Fast and Slow rats under basal (non-stressed) conditions and following 15-min of ferret exposure. *Significantly different from (within-rat line) baseline values (0 min time point) at $p < 0.05$.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

plasma ACTH levels were comparable for both rat lines. Fast rats appeared more responsive to the immobilization stressor as indicated by significantly higher plasma levels of ACTH at the 5-, 15-, and 30-min time points.

The rise of plasma corticosterone in response to immobilization was more rapid than that observed in response to the ferret. In particular, the peak corticosterone response to the ferret did not appear until the 30-min time point, whereas the peak response to immobilization was apparent at 15-min and was relatively high within 5-min of stressor onset. The ANOVA indicated that corticosterone levels varied over time. ($F_{4,44} = 13.50, p < 0.01$). In both rat lines, immobilization increased plasma corticosterone levels as early as the 5-min time point and were still markedly elevated at the 60-min time point. Unlike the rise of ACTH levels, the immobilization-induced rise of corticosterone was comparable in the two rat lines.

Experiment 3

Effects of predator exposure and immobilization on the in vivo release of ir-CRH at the CeA in Fast and Slow rats

Figure 6 shows interstitial ir-CRH levels at the CeA, as a percent of baseline, in response to ferret exposure. The ANOVA indicated that among animals with correctly positioned probes (Fast $n = 10$; Slow $n = 9$), ferret exposure influenced ir-CRH release at the CeA, $F_{6,102} = 6.84, p < 0.001$. Although the interaction between Rat line $\times$ Samples was not significant, *a priori* predictions had been made concerning the effects in the two lines, since the CeA was shown in Experiment 1 to be sensitive to stressor effects.

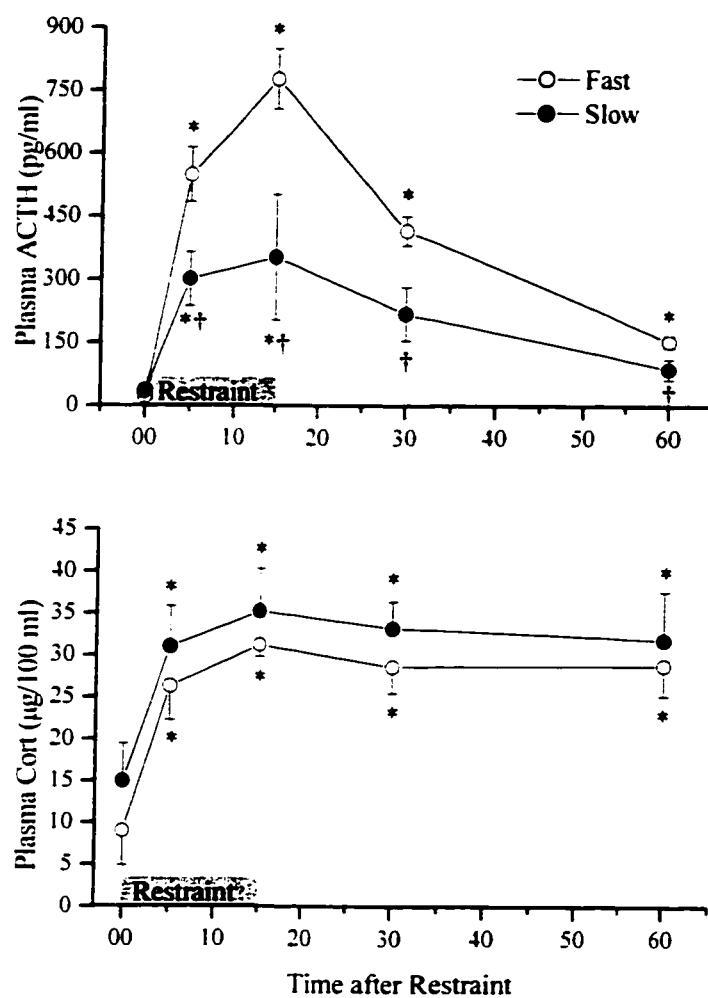


Figure 5. The *in vivo* release of plasma ACTH and corticosterone (cort) (mean $\pm$ S.E.M.) over 60-min in Fast and Slow rats under basal (non-stressed) conditions and following 15-min of restraint.
 * Significantly different from (within-rat line) baseline values (0-min time point) at $p < 0.05$.
 † Significantly different Fast (between-rat line) time-matched values at $p < 0.05$.

Accordingly, separate ANOVAs were performed for each of the strains. These analyses revealed that in both the Fast and the Slow rats CRH levels varied over the course of the test session. $F_{6, 54}$ and $F_{6, 48} = 2.97$ and 4.01 , $p's < 0.01$. The t-tests revealed that relative to baseline, predator exposure produced a pronounced, but delayed increase of ir-CRH release. In the Slow rats this increase was significant by the third post-stressor sample and remained significant until the fourth post-stress sample, whereas in the Fast rats the increase did not reach statistical significance after the Bonferroni correction. In rats with off-target CeA placements (Fast $n = 4$, Slow $n = 4$), ferret exposure had no effect on CRH levels in either the Fast or Slow rats (data not shown).

Figure 7 shows interstitial ir-CRH levels at the CeA as a function of the immobilization treatment. Among animals with correctly positioned probes (Fast $n = 8$, Slow $n = 7$), the *in vivo* ir-CRH release at the CeA varied across samples. $F_{6, 72} = 4.67$, $p < 0.001$. Once again, separate analyses were conducted for the two strains over the course of the sampling periods. These analyses indicated that in Fast rats ir-CRH varied over the course of the session. $F_{6, 36} = 3.91$, $p < 0.01$. Despite the marked struggling displayed by the Fast rats upon being immobilized, the t-tests indicated that ir-CRH elevations did not increase until the fifth post-stressor sample. In contrast to the effects seen in the Fast rats, among the Slow animals only a small rise of ir-CRH was apparent, and this effect did not reach significance. $F_{6, 36} = 1.60$, $p = 0.17$. In rats with off-target CeA placements (Fast $n = 6$, Slow $n = 7$), immobilization failed to alter ir-CRH levels in either the Fast and Slow rats (data not shown).

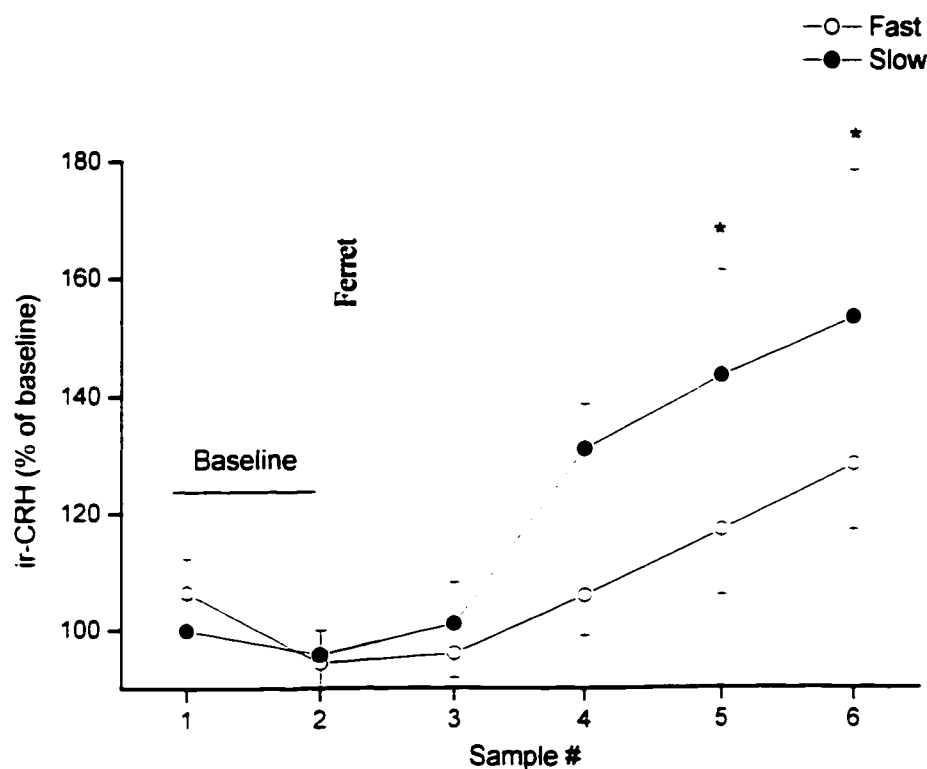


Figure 6. Levels of ir-CRH at the central nucleus of the amygdala as measured by in vivo microdialysis under baseline condition and following 15-min of non-contact ferret exposure in Fast and Slow rats. The baseline samples from each subject were averaged and defined as 100%. All values were expressed as a percentage of that baseline, with the actual values being 2.56 ± 0.57 and 4.04 ± 1.18 fmol/sample, for the Slow and Fast rats, respectively. * Significantly different from (within-rat line) baseline condition at $p < 0.05$.

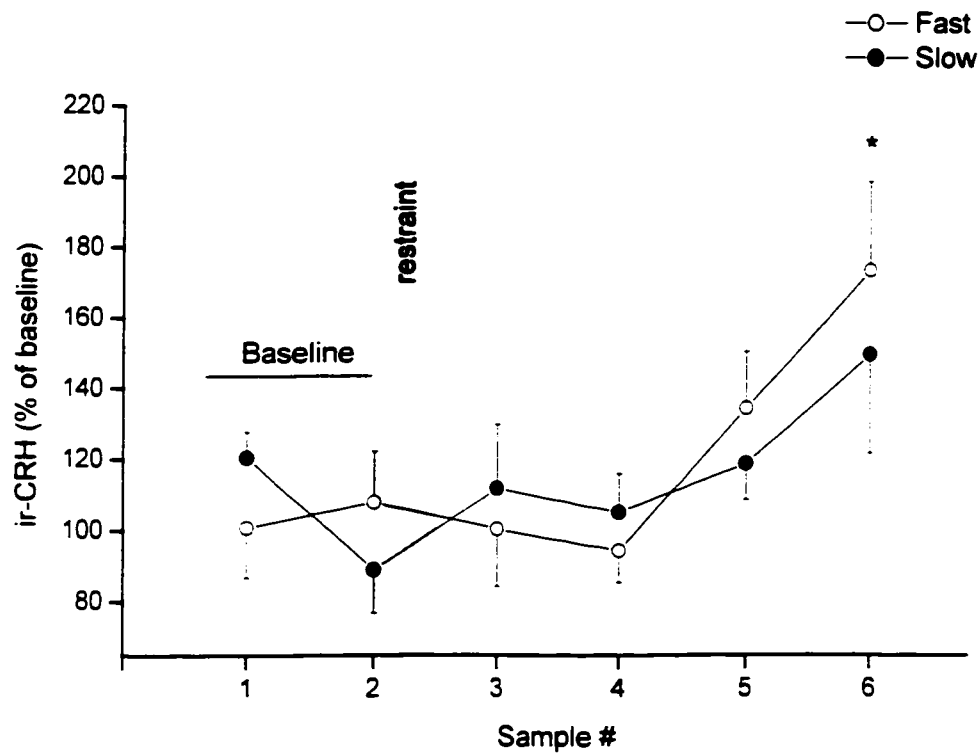


Figure 7. Levels of ir-CRH at the central nucleus of the amygdala as measured by *in vivo* microdialysis under baseline conditions and following 15-min of restraint in Fast and Slow rats. The baseline samples from each subject were averaged and defined as 100%. All values were then expressed as a percent of that baseline. Actual basal values were 2.56 ± 0.57 and 4.04 ± 1.18 fmol/sample, for the Slow and Fast rats, respectively. * Significantly different from (within-rat line) baseline condition at $p < 0.05$.

Experiment 4

Effects of non-contact exposure to estrous or non-estrous females on the release of ir-CRH at the CeA in male Fast and Slow rats

The overall ANOVAs on the Fast and Slow rats with correctly positioned probes (Fast = 8, Slow = 8) revealed that the concentration of ir-CRH levels did not differ as a function of Rat line or Female status, however they did vary over Time ($F_{1,7} = 10.833$ and 10.593 , p 's < 0.0001 , respectively). Once again, separate analyses were conducted for the two strains over the course of the sampling periods. Individual t-tests indicated that in the Fast rats the concentration of ir-CRH increased at several samples following exposure to an estrous female, while no such increase occurred in response to the non-estrous female (see Figure 8, panel A). In contrast to the Fast rats, the concentration of ir-CRH increased in a time-dependent manner in the Slow rats exposed to non-estrous females with no such change occurring in the estrous condition (see Figure 8, panel B).

Behavioral response of Fast and Slow males in the presence of estrous or non-estrous female rats

The overall ANOVA revealed that ano-genital grooming was effected by interactions between Female Status and Rat Line ($F_{1,1} = 6.763$, $p < 0.03$) and Rat line and Contact condition ($F_{1,2} = 3.388$, $p < 0.05$). The multiple t-tests indicated that ano-genital grooming increased in both rat lines when contact was permitted with an estrous female however the magnitude of this increase was greater in the Fast rats (see Figure 9, panel A). The overall ANOVA for exploratory behavior revealed a significant Rat line x Contact condition x Female status interaction ($F_{1,2} = 3.819$, $p < 0.05$). It was later

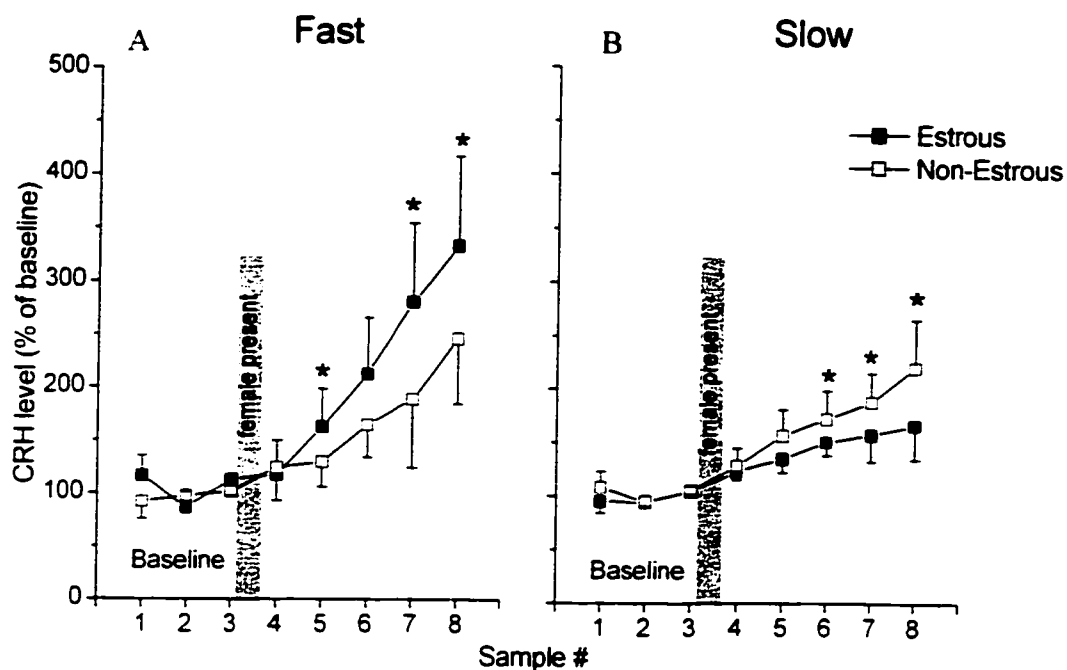


Figure 8. Levels of ir-CRH release observed at the central nucleus of the amygdala, as measured by *in vivo* microdialysis under baseline and following a 20-min non-contact exposure to either an estrous or non-estrous female among Fast (Panel. A) and Slow (Panel. B) rat lines. The baseline values from each rat were averaged and defined as 100%. All values were then expressed as a percent of that baseline (actual basal values fell between 2.5 and 4.5 fmol/sample for both rat lines). Immunoreactive-corticotropin-releasing hormone (ir-CRH). * Significantly different from average baseline, $p < 0.01$.

confirmed with multiple t-tests that this interaction was owing to a very large increase in exploratory behavior among the Fast rats during non-contact exposure with an estrous female (see Figure 9, panel B). The frequency of rearing behavior varied in a Rat line x Contact condition manner ($F_{1,2} = 12.994$, $p < 0.0001$). Both rat lines displayed similar elevations in rearing during non-contact with a non-estrous female, but the magnitude of this behavior increased to a greater extent only among the Fast rats when estrous females were in the non-contact chamber. Furthermore, while no change in this behavior occurred during contact with a non-estrous female, both rat lines displayed elevated levels of rearing in the presence of an estrous female, however the frequency was higher among the Fast rats (see Figure 9, panel C). Sniffing the non-contact chamber was monitored to indicate the level of interest the females could attract from the males. This form of sniffing varied in a Rat line x Contact condition x Female status manner ($F_{1,2} = 4.418$, $p < 0.022$). The follow-up t-tests indicated that although this behavior increased similarly in both strains during non-contact with a non-estrous female, the magnitude of this increase was much greater among the Fast rats when the non-contact chamber housed estrous females (see Figure 9, panel D). We also monitored the male's frequency of non-specific sniffing and found that this behavior also varied in a Rat line x Contact condition x Female status manner ($F_{1,2} = 4.052$, $p < 0.03$). This interaction appeared to be due to elevated non-directed sniffing among the Fast rats when an estrous female was housed in the non-contact chamber and continued when she was introduced into the testing cage. During this time (i.e. contact) the frequency of non-directed sniffing did increase among the Slow rats to a level exceeding that observed among the Fast rats (see Figure 9, panel E).

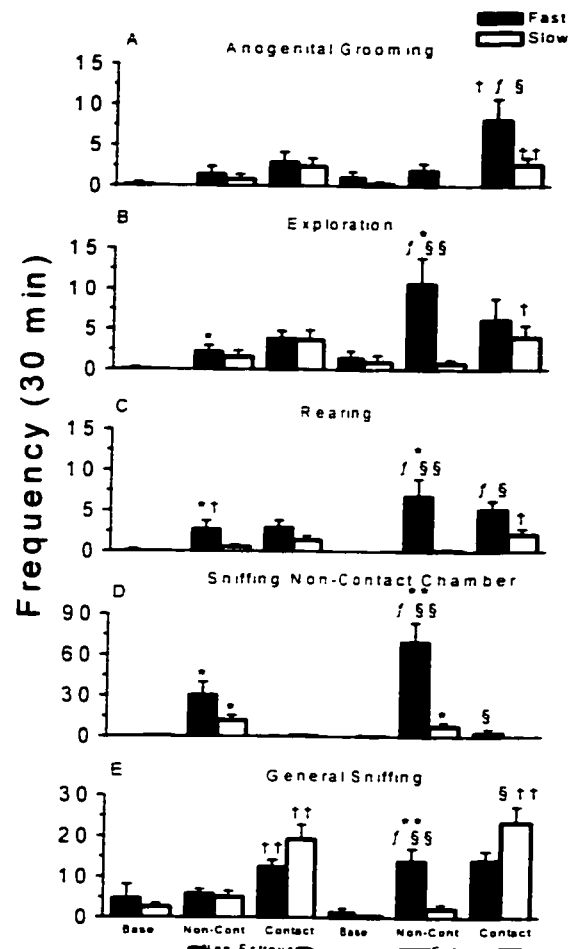


Figure 9. Several behaviors were monitored during baseline, non-contact and contract with female rats that were determined to be either non-estrous (left side) or estrous (right side). Anogenital grooming (panel A), Exploration (panel B), Rearing (panel C), Sniffing non-contact chamber (panel D) and General Sniffing (panel E). *, ** Significantly different from within rat line baseline condition at $p < 0.05$ and $p < 0.001$, respectively. †, †† Significantly different from non-contact condition at $p < 0.05$ and $p < 0.001$, respectively. f Significantly different from within rat line Non-estrous female condition at $p < 0.01$. §, §§ Significantly different from Slow (between rat line) condition for matched behavior at $p < 0.05$ and $p < 0.001$.

Some behaviors could only occur during contact with the female and so they were analyzed using a two way ANOVA with Rat line (Fast vs. Slow) and Female status (estrous vs. nonestrous) serving as the main effects. Although very few male rats (30 %) actually mounted the females during the 30-min contact session it should be noted that behavior occurred only among the Fast rats. Similarly, the frequency of mount attempts varied as a function of Rat line ($F_{1,1} = 22.067$, $p < 0.001$) and this was due to an increase in this behavior among the Fast rats, irrespective of the female's sexual status. An overall ANOVA on the frequency of female pursuit revealed a main effect of Rat line ($F_{1,1} = 20.798$, $p < 0.001$). Subsequent t-tests revealed that this behavior increased to a similar extent during contact with both females among the Fast rats, while no such change was noted in the Slow rats. When the frequency of female sniffing was analyzed it was similar between both rat lines and across both females. However, when the frequency of sniffing directed towards the female's ano-genital region was subjected to an ANOVA a main effect of Rat line was apparent ($F_{1,1} = 16.21$, $p < 0.001$). Post-hoc comparisons revealed that this behavior was highest among the Fast rats when the female was in estrous (see Figure 10).

At the end of the experiment we compared the morning levels of circulating testosterone between the Fast ($n = 10$) and Slow ($n = 10$) rats. We were surprised to find that the levels of this hormone were significantly greater in the Slow rats (mean = 1.354 ± 0.287 pg/ml) compared to the Fast rats (mean = 0.627 ± 0.097) ($p < 0.05$) (Data not shown).

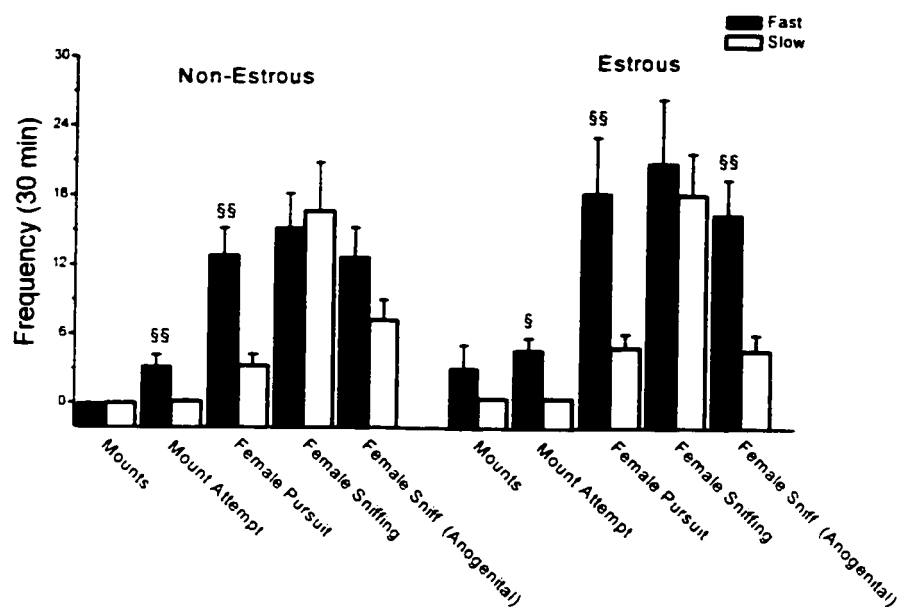


Figure 10. Several behaviors were monitored during contact with a non-estrous female (left panel) or an estrous female (right panel) in Fast and Slow rats. §§§ Significantly different from Slow (between-rat line) matched behavior at $p < 0.05$, and $p < 0.0001$, respectively.

Discussion

The major aim of the present investigation was to further characterize endocrine and neurochemical differences between Fast and Slow rats in response to ferret exposure and immobilization. These particular stressors were selected since the two rat lines were shown to display markedly different behavioral responses to these stressors. Upon immobilization, Fast rats display vigorous and sustained struggling, whereas Slow rats adopt an immobility posture. Conversely, in response to a ferret, Fast rats become immobile while Slow rats emit active avoidance responses [14:287]. As well, it has been suggested that the Slow rats may be more likely to exhibit anxiogenic responses across a variety of situations relative to the Fast rats [307].

Paralleling the behavioral effects of the two stressors, the neuroendocrine profile in response to immobilization in the rat lines was distinguishable from that associated with predator exposure. Consistent with our previous findings [14:287], ferret exposure elicited a more pronounced increase of plasma ACTH concentrations in the Slow than in the Fast rats. This effect was evident both when blood was collected immediately after stressor exposure (at a single time point) as well as when blood samples were collected *in vivo* (at several time points) through indwelling pulmonary arterial cannulation. Similarly, ferret exposure elicited a more pronounced rise of plasma corticosterone levels in the Slow rats than in the Fast rats, so that the corticosterone levels rose more quickly and were more pronounced in the Slow rats as compared to the Fast rats.

Unlike the effects of ferret exposure, immobilization elicited a more pronounced rise of plasma ACTH levels among Fast than Slow rats. This was evident both in blood taken at a single 15-min post-stressor time point, and in serially collected samples. However, in contrast to the between rat line difference in the ACTH response, the immobilization-elicited corticosterone rise was comparable in the two rat lines. Clearly, the changes of ACTH are not necessarily proportional to the variations of corticosterone. Such discrepancies between ACTH and corticosterone levels have been noted by other investigators [352:462], and may be attributable to altered adrenal sensitivity and/or the potential influence of other secretagogues on adrenal responses [11:167:190]. As well, given the very rapid rise of corticosterone levels in response to immobilization, it is possible that a ceiling effect precluded detection of differences of corticosterone between the Fast and Slow rats.

Both predator exposure and immobilization elicited brain region-specific variations of both ir-CRH and ir-BN. To a considerable degree, these effects were dependent upon the rat line being examined. Specifically, while the stressors provoked elevations of ir-CRH at the key elements of the HPA axis (PVN, Me/Arc and to a moderate extent at the Pit) among the more emotional Slow rats, the ir-CRH levels in Fast rats were moderately reduced at these sites. While the ir-CRH data cannot be directly related to HPA patterns of activity, the elevated levels of ir-CRH at the PVN, Me/Arc and Pit in Slow rats could be interpreted as either increased peptide synthesis possibly coupled with increased release, or alternatively, could reflect decreased peptide release. Given that the release of CRH from the median eminence has frequently been

shown in response to stressors [27:204:472-474], it is likely that the alterations of ir-CRH content (at least at the Me/Arc) in both Fast and Slow rats reflects enhanced peptide release, accompanied by enhanced peptide synthesis in Slow rats.

In contrast to the effects at hypothalamic-pituitary sites, at the CeA, immobilization increased ir-CRH levels among Fast rats, but had no effect in the Slow rats. There is considerable evidence for the involvement of the CeA in HPA activation [30:75:130:136:163:461], and it was previously shown that stressors give rise to ir-CRH release from the CeA [297:351:370]. Given that the rat lines were selected on the basis of amygdaloid excitability [364], it was somewhat surprising that alterations of ir-CRH content at the CeA in response to immobilization were not more pronounced in Slow rats. This is particularly the case given that the ACTH response in Slow rats was considerably more pronounced than in the Fast rats.

It is well established that analyses of neurotransmitters in post-mortem tissue collected at a single time point may not provide an adequate reflection of the dynamic changes that may occur over the course of a treatment regimen, particularly in response to stressors. This is certainly the case with respect to CRH variations, where synthesis of the peptide is particularly rapid [45] and hence changes of level may not reflect ongoing variations of activity. Post-mortem analyses are useful in that they permit the determination of proteins at a wide range of brain regions; however, the absence of an effect does not necessarily imply that variations of the neurotransmitter are not occurring as a result of the treatment [15]. In fact, using *in vivo* microdialysis, ir-CRH variations

within the CeA were detected in response to stressors. To a modest degree, these effects were dependent on the line of rat and on the specific stressor to which the animals had been exposed. Specifically, while ferret exposure elicited release of ir-CRH in both lines, the effect was apparent earlier in the Slow line. In contrast, such an effect was not apparent in response to immobilization. Indeed, the ir-CRH release elicited by immobilization was only apparent in the Fast rats, and did not reach statistical significance in the Slow line. This profile generally parallels the variations of ACTH, wherein immobilization provoked greater changes in Fast rats, whereas ferret exposure more profoundly affected ACTH in the Slow rats.

In both rat lines the stressor-elicited release of ir-CRH at the CeA was somewhat delayed, depending on the nature of the stressor (being observable between the 3rd or 5th sample post-stressor exposure). Although these findings are not concordant with our past results showing a more pronounced and rapid restraint-elicited increase in ir-CRH release at the CeA (observable by the 2nd sample post-stressor exposure) [297], there are several possible explanations for this discrepancy. In this respect, it is possible that the Fast and Slow rat lines in the present experiment differ in levels of ir-CRH and/or mRNA expression, or in the rate of synthesis, turnover and/or release of this peptide as compared to other rat strains (including Sprague Dawley rats which were employed in Chapter I). Indeed, other investigators [176] have previously observed inter-strain differences in many of these parameters [176:418]. Inter-strain differences, have also been reported in how animals respond to manipulation of CRH binding protein (CRH-BP) within the brain [353]. Since CRH-BP binds this peptide with high affinity, and the protein-bound CRH

may not cross the active membrane of the microdialysis probe, the portion of the released peptide sequestered by this binding protein may be masked. Indeed, high levels of CRH-BP are present in limbic structures such as the CeA [258], where it increases in responses to stressors and could therefore affect the dynamics of CRH release and its bioavailability [258:284:353]. At present, these explanations are speculative and efforts will be needed to determine more precisely the underlying mechanisms in these rat lines that contribute to their differential response to stressors.

It will be recalled that stressors ordinarily elicit the release of BN-LPs, as reflected by increased ir-BN [223]. In the present investigation, the stressor treatments provoked elevations of ir-BN at the AH in Slow rats, whereas a decrease of ir-BN was observed at the NTS in both rat lines. There is considerable evidence suggesting that certain brain stem nuclei, like the NTS, play a pivotal role in HPA activation [96:247:254:402]. The NTS serves as a primary relay station for information traveling *via* the glossopharyngeal and vagus nerve [366]. Moreover, this nucleus gives rise to noradrenergic projections that extend to the parvocellular division of the PVN as well as to other brain stem nuclei, which, in turn innervate the PVN [98]. BN-LPs are thought to mediate sympathetic activation via the NTS, as administration of BN into this nucleus elevates the circulating levels of epinephrine and norepinephrine [77]. Moreover, ir-BN fibers extend from the parvocellular PVN to the NTS/dorsal vagal complex, leading to the possibility that BN-LPs released at the NTS modulate HPA activity (either directly or through CRH neurons) [91:268]. The AH has been associated with fear/anxiety responses associated with stressors [115], and as such, the finding that stressor-provoked

BN-LP levels at the AH were only elevated in Slow rats is consistent with the behavioral evidence suggestive of greater fear and anxiety in the Slow line.

Aside from site-specific alterations in ir-BN and ir-CRH following stressor exposure, these two rat lines also showed brain-region specific basal differences in peptidergic concentrations. In particular, relative to Fast rats, the Slow line had higher basal levels of ir-CRH at the Hipp and LH and lower basal levels of ir-BN at the AH and NTS. These regions have all been implicated in HPA activation, leading to the possibility that pre-existing differences in the basal concentrations of stress-relevant peptides, such as CRH and/or BN-LPs, at these sites may contribute to the endocrine and behavioral differences observed between Fast and Slow rats in response to stressor exposure [96:125:333] .

In this experiment we also wanted to assess the extent to which these rat lines may differ in their neurochemical response towards a vigorous appetitive event, namely exposure to a sexually receptive female. Indeed, we have previously demonstrated that both ir-CRH and ir-BN-LPs are released at the CeA in response to food ingestion in Sprague-Dawley rats (see Chapter I). This raises the possibility that CRH and BN-LPs might likewise be responsive to other appetitive events (in this case exposure to a female cohort). We have previously noted that these rat lines elicit a robust differential behavioral activation and vocalization patterns in response to sexually receptive females. Therefore, it was of interest to assess the extent to which neuropeptides released at the CeA might underscore these rat line-dependent behaviors. In the current study the

recovery of ir-BN-LPs was below the level of detection in the RIA (i.e. <0.25 fmol/45ul sample). However, in both strains extracellular ir-CRH was increased following the placement of a female cohort into the non-contact chamber. Upon close examination of the data, this release of ir-CRH appeared to be modestly dependent upon the rat line and sexual status of the female. Specifically, extracellular ir-CRH only increased significantly among the Fast rats when the female was in estrous, while ir-CRH release only increased among the Slow rats in response to a nonestrous female. These results do sustain the contention that CRH at the CeA likely responds to the salience of a stimulus, as opposed to simply its positive or negative attributes. It should be mentioned however that because the females were inaccessible to the males there may have been an element of frustration experienced by the males that might impart changes in the release of ir-CRH at the CeA [377]. Still, both rat lines eagerly attempted to gain access to the females and this contrasts the behavioral *avoidance* that occurs in response to typical stressors (such as ferret and immobilization). While the rat line differences in ir-CRH release at the CeA were relatively modest, they elicited striking behavioral differences in response to the different females.

In general, the Fast rats reacted with heightened arousal in a non-discriminatory fashion towards the females regardless of their sexual receptiveness. In contrast, the Slow rats' behavior appeared to be dictated by the sexual status of the female (the typical response) [3]. These rat line-dependent behavioral responses are in accordance with our earlier observations that these rats elicit differential behavioral activation and vocalization patterns in response to sexually receptive female cohorts. It is of particular

interest that despite these robust behavioral differences the release of ir-CRH at the CeA was relatively similar between both rat lines. This suggests that CRH at the CeA may not be involved in the genesis of behavioral activation in general, or in response to natural appetitive stimuli, in particular.

At first glance the Fast rats appear to have a hypersexual drive, however there may be an alternative explanation for their behavior. These rats may be hyperactive/impulsive in general and not necessarily in response to sexual cues in particular. Indeed, the Fast rats do not breed as successfully as their Slow counterparts, which may be, in part, due to their lower plasma testosterone levels and/or non-discriminatory response to the different females. Successful mating requires that the male is able to focus attention towards cues that arise from the female that indicate her sexual status (e.g. ear wiggling, lordosis) [449]. It is possible that a tendency towards impulsivity among the Fast rats impairs their ability to recognize some of these cues. The observation that the Fast rats display less behavioral avoidance in response to a predator (i.e. ferret) supports the contention that they may be impaired at recognizing meaningful environmental cues. Unfortunately we were unable to acquire sufficient samples to assess the release of ir-CRH before, during and following contact with a female, so we cannot ascribe the behavioral patterns during contact to changes that might have occurred in CRH release at the CeA.

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

duration, and quality of the stressor as well as individual (or organismic) factors (e.g. species, genetics, age or gender) [210:429]. Given that a response to a stressor will vary depending on the complex interplay between these various factors underscores the need for more elaborate experiments that consider the interactions between at least some of these different variables. In the present study we demonstrated that in addition to previously reported behavioral, endocrine, and immunological differences [14:307], the Fast and Slow rat lines can also be distinguished from each other in terms of their neurochemical changes (alterations in ir-CRH and ir-BN) in response to two different stressors. Moreover, the nature of the response observed was dependent on the characteristics of the stimulus (or the behavioral changes elicited by these stressors). Furthermore, this study adds to the possibility that CRH at the CeA is involved the attribution of emotional salience to a stimulus, rather than simply responding to its positive or negative attributes. At the same time, these results are at odds with the widely held assertion that CRH at the CeA is involved in the genesis of the behavioral response associated with heightened arousal [160:255:379:403:436]. Together, the findings suggest that the Fast and Slow rat lines may provide a valuable tool to assess the mechanisms underlying the individual differences in endocrine, neurochemical and/or behavioral responses to aversive and appetitive stimuli.

Preface to Chapter III

There is sufficient evidence in both humans and experimental animal studies to sustain the notion that a response to stressors is influenced by a several factors that include the stressor regimen (acute, chronic, intermittent), stressor type (processive, systemic) as well as organismic variables, such as gender, age, and hard-wired genetic differences. As mentioned earlier, a comprehensive understanding of the stress response requires, as much as possible, a consideration of how these factors might act alone, or in combination with each other, to influence an organism's response to stressors. In the current thesis we made a concerted effort to explore the response across animals with different genetic underpinnings in response to a variety of processive stressors. It is important to stress that our intention was *not* to compare the stress response between the different rats, but rather to select out rats that display unique responses to stressors. In so doing, we then were able to assess the extent to which CRH and BN-LPs contributed to the genesis of the differential stress responses. This reasoning underscores our use of the Lewis and Fischer rats in the current study.

The Lewis and Fischer rats have a variety of well-characterized strain-dependent differences including neuroendocrine (HPA activation), immunological and behavioral responses to various stressors [120:122:169:418]. Their differences have provided the basis for many comparative studies investigating the underlying mechanisms mediating the stress response. In this respect, these strains provide a unique opportunity to further assess the contribution of CRH and BN-LPs in the mediation or modulation of behavioral

vs. neuroendocrine responses to stressors. Furthermore, they may also shed some light on mechanism(s) that contribute to the individual differences in responsivity to stressors.

In our previous experiments the application of a stressor was typically administered in the presence of the experimenter, which may have influenced the stress response. Specifically, it could be argued that the presence of the experimenter during stressor application (e.g. during administration of restraint or immobilization) may become paired with the stressor and hence every subsequent encounter with that experimenter might re-trigger a stress response. In this experiment, we decided to use noise as a stressor, as it not only represents a different stressor modality, but it can also be administered remotely (in the absence of the experimenter), providing one solution to this caveat. In the current series of experiments we assessed the effects of noise exposure on neuroendocrine and neuropeptide functioning in Fischer and Lewis rats. We examined whether their differential sensitivity to stressors would be restricted to hypothalamic-pituitary-adrenal factors or extend to certain extra-hypothalamic sites. In addition to the immediate effects, we also examined whether noise exposure would induce enduring changes on neuroendocrine and neuropeptide activity in these strains, and whether such effects would be influenced by the environmental context in which the stressor was applied.

In recognition of the inherent interpretive problems associated with the assessment of tissue peptide levels through post-mortem analyses (as discussed above), we complimented these experiments with those that more directly assessed interstitial

peptide levels using *in vivo* microdialysis and push-pull perfusion, providing more dynamic and less ambiguous mechanistic perspective(s).

CHAPTER III

Differential impact of audiogenic stressors on Lewis and Fischer rats: Behavioral, neurochemical and endocrine variations

Abstract

Exposure to intense noise can trigger a cascade of neuroendocrine events reminiscent of a stress response, including an activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. Using male Fischer and Lewis rats, which exhibit differences in their corticosterone response to stressors, this investigation assessed effects of acute noise exposure on neurochemical and neuroendocrine responses. In response to the noise exposure, Fischer rats displayed greater plasma adrenocorticotropin-releasing hormone (ACTH) and corticosterone responses than their Lewis counterparts. However, both strains responded with similar increases of plasma prolactin, suggesting that strain differences in the HPA response were not likely due to differences in noise perception. Post-mortem analyses revealed that noise exposure induced strain-dependent variations of corticotropin-releasing hormone (CRH) and gastrin-releasing peptide (GRP) across several brain regions. These effects were evident whether the rats were noise-exposed in a familiar (home-cage) or unfamiliar environment. *In vivo*, dynamic assessment of CRH at the pituitary revealed that noise exposure elicited an immediate rise in CRH among Fischer rats, relative to the delayed response in Lewis rats. Similarly, the rise in local interstitial corticosterone was more rapid and pronounced in Fischer rats. In contrast to these differences, CRH released at the central nucleus of the amygdala (CeA) was gradual and protracted following noise exposure in both strains. Behaviorally, the Fischer rats displayed an active response whereas the Lewis strain adopted a defensive coping strategy. The role of CRH in the genesis of the overall strain-dependent response to stressors is discussed.

Introduction

Despite its ubiquitous and intrusive nature, community noise has yet to be firmly established as a significant risk factor for the development of stress-related disease [39]. Nevertheless, intense audio signals provoke neuroendocrine variations in humans and experimental animals reminiscent of those associated with stressor exposure, including increased circulating levels of ACTH, glucocorticoids, and enhanced secretion of catecholamines, reflecting autonomic activation [464:486]. As hypothalamic CRH is the primary secretagogue for pituitary ACTH, which in turn evokes the release of adrenal glucocorticoids, it seems likely that CRH contributes to the neuroendocrine effects of noise. More recently, GRP has been identified as fundamental to the stress response as aversive stimuli trigger its release in several stress-relevant regions of the brain, including the amygdala [153:223:297:299]. Moreover, GRP increases the release of pituitary ACTH, an effect antagonized by the CRH antagonist, α -helical CRF, attesting to the possible interplay between GRP and CRH [299].

Several studies have demonstrated marked strain differences in response to physical and/or psychological stressors [13:121:418]. In this respect, the Lewis rat has been extensively studied as a unique strain demonstrating susceptibility to various inflammatory conditions, including streptococcal cell wall-induced arthritis [122:420] and encephalomyelitis [282]. In contrast, the histocompatible Fischer strain is relatively resistant to the deleterious effects of stressor exposure [120:308:419]. In the current study we assessed the neuroendocrine, neurochemical and behavioral responses to an acute audiogenic stressor in these two rat strains.

In addition to hypothalamic control over the activation of the HPA axis, considerable evidence sustains the notion that extra-hypothalamic structures, such as the amygdala, may also regulate the activation of the HPA axis. Indeed, lesions to the CeA block the HPA responses to acoustic stimuli [136] and restraint stressors [461]. Furthermore, the stressor-induced increase in CRH mRNA and release at the CeA [197:297:351:370] suggests that previously observed [158:418:420] HPA, autonomic and behavioral responses to stressors in the Fischer and Lewis rats might be associated with differences in the release dynamics of CRH at the CeA. In the present investigation we determined whether their differential sensitivity would be restricted to hypothalamic-pituitary-adrenal factors and/or include extra-hypothalamic sites. In addition to immediate effects, we also examined whether noise exposure would induce relatively protracted changes on neuroendocrine and neuropeptide activity in these strains, and whether such effects would be influenced by the environmental context in which the stressor was applied. Although stressor effects are usually transient [496], the duration of these effects can be influenced by contextual cues, in this instance administering the stressor in an animal's home-cage, as opposed to in a distinct environment for noise exposure.

In the present investigation we assessed CRH and GRP in differential postmortem brain punches of Lewis and Fischer rats that had been exposed to noise. Although micro-punch results provide useful information regarding which brain regions may be involved in the stress response, they preclude conclusive interpretations regarding the temporal dynamics associated with the challenge. Therefore, we complemented the postmortem

studies with experiments that assessed interstitial levels using *in vivo* microdialysis and push-pull perfusion, hence providing a more dynamic and clearer mechanistic perspective.

Materials and methods

Experiment 1a: Effect of noise exposure on brain ir-CRH, ir-GRP and plasma hormone levels

Animals and procedure

Male Lewis and Fischer rats (Charles River, Canada), weighing 300-350 g were used. The animals were housed individually in standard rodent cages (36 x 31 x 17 cm) with a 12:12-h reversed light-dark cycle (lights off 07:00-hr) and temperature maintained at 23-24°C with 60% relative humidity. Animals had *ad libitum* access to Purina™ rat chow and tap water. All experimental procedures met the guidelines on the ethical treatment of animal subjects in experimental research set forth by the University of Ottawa and Health Canada.

Noise exposure and blood collection

To minimize the effects of transporting animals from the housing room to the test area, rats were acclimated to the regimen of daily transportation for 3 weeks prior to the experiment being initiated. On test days, animals were transported in pairs (in their home cages), from the housing room to a separate room where they were immediately exposed to a 15-min burst of 90 dB continuous white noise, band-limited from 80 Hz to 20 kHz, and equalized in 1/3 octave bands. Sound pressure level varied slightly, depending on the

position in the cage and the arrangement of bedding chips. Based on measurements from 10 positions within the cage and variations in bedding distribution, overall the standard deviation for the unweighted average sound level was ± 2 dB, and in individual 1/3-octave bands it was ± 4 dB.

In the initial experiment, animals were sacrificed (by decapitation) either immediately after the stressor session (i.e., 15-min after noise/no noise onset; $n = 9$ /strain/condition) or were returned to their home cages, and then sacrificed 1-hr later ($n = 9$ /strain-noise; $n = 7$ /strain-no noise). Control rats were treated identically, except that the noise exposure was not administered. Brains and trunk blood were collected for radioimmunoassay (RIA) of peptide and hormone content [334].

Experiment 1b: The impact of environmental cues on protracted changes of brain ir-CRH, ir-GRP and plasma hormone levels 24-hr after noise exposure

Animals and procedure

Rats of each strain were brought to the test room, where half of the rats remained in their home cages ($n = 10$ /strain), while the remaining rats were placed in a similar cage that contained fresh bedding (novel cage, $n = 10$ /strain). The rats were then exposed to the noise stressor as described earlier. Animals exposed to noise in a novel cage were returned to their home cages immediately after noise exposure and all rats were returned to the vivarium. Thus, some rats were maintained in the same environment as that in which they were ordinarily housed, while the remaining rats were exposed to noise in a novel environment and then returned to their home cages before returning to the housing

room. The following day (24-hr after noise termination) rats were transported to the necropsy area where they were sacrificed immediately and brains and trunk blood taken for later neurochemical and neuroendocrine determinations.

Tissue collection

Following decapitation, brains were rapidly removed and placed into a brain matrix (McIntyre, Carleton University, ON) with coronal planes corresponding to the rat brain atlas of Paxinos and Watson (1986) [342]. Serial coronal sections (1.5-2.0 mm) were obtained and placed in ice cold 0.1M phosphate buffered saline and a total of 11 discrete brain nuclei known to be sensitive to the effects of stressors were identified and micropunched/dissected. The selected hypothalamic areas included the paraventricular nucleus of the hypothalamus (PVN), and the median eminence-arcuate complex (Me/Arc). In addition, the following extra-hypothalamic regions were taken: paraventricular thalamic nucleus (PV), prefrontal cortex (PFC), nucleus accumbens (nAcb), caudate (Cau), dorsal hippocampus (dHipp), central nucleus of the amygdala (CeA), basal lateral nucleus of the amygdala (BLA), medial nucleus of the amygdala (MeA), and locus coeruleus (LC). All nuclei were bilaterally micropunched using a customized 1 mm punch, except for the Me/Arc and PV, which were removed with a single punch, while the dHipp, and PFC were microdissected free-hand. Brain tissue was placed in clean 1.5 ml centrifuge tubes on dry ice and stored at -80°C until processing (within 4 weeks). All samples were collected between 07:00-13:00-hr.

Tissue processing

Tissue punches were sonicated (VirSonic 60, Virtus U.S.A.) in 0.5-1.0 ml of Iscove's modified Dulbecco's buffer (GIBCO) containing 0.5% fetal calf serum and a cocktail enzyme inhibitors (in mM: 100 amino-*n*-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride) for 10-sec. A portion of the homogenate (25 μ l) was then withdrawn for protein determination, and the remainder of the homogenate was centrifuged at 13000 rpm at room temperature for 9-min. Supernatant was aliquoted into 0.6 ml vials and stored at -80°C for subsequent peptide level determination. Protein assays were performed using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, ON) using the microtiter plate method.

Plasma ACTH, corticosterone and prolactin detection

Trunk blood was collected into 5.0 ml glass tubes containing EDTA (Fischer Scientific Ltd., ON), centrifuged at 1500 rpm, and the plasma aliquoted into 1.5 ml vials and stored at -80°C. Plasma ACTH, corticosterone and prolactin levels were determined, in duplicate, using commercial RIA kits (ICN Pharmaceuticals Inc., Costa Mesa, CA). These assays yielded intra- and inter-assay variations of less than 10%. The quantification of corticosterone in the perfusates from Experiment 2 was accomplished using a modification of the commercially available corticosterone RIA protocol designed to enhance the sensitivity of the assay [53].

Experiment 2a: The effect of noise exposure on the release of CRH at the CeA

Animals and procedure

Male Lewis and Fischer (n = 10/strain) rats were maintained as in Experiment 1. Rats were anesthetized (60 mg/kg i.p. pentobarbital) and stereotaxically implanted with a 20 gauge stainless steel guide cannula containing a removable 24 gauge stainless steel obturator aimed at the dorsal aspect of the CeA. The placement coordinates [342] with skull level were A/P, -2.3 mm; D/V, -7.0 mm; and L/M, $\pm$ 4.2 mm. The guide cannula protruding from a custom-manufactured Delrin[®] pedestal was secured to the skull with four stainless steel screws and dental cement. After surgical recovery (at least 7 days), animals were transferred to individual testing chambers and allowed to acclimate for 48-hr before testing. The testing chambers comprised Plexiglas cages (25 x 35 x 34 cm) with a stainless steel grid floor.

Microdialysis

Approximately 24-hr prior to testing, rats were briefly restrained (~ 3-min), and the obturator within the guide cannula was replaced with a concentric microdialysis probe, consisting of a 2.0 mm regenerated cellulose active membrane (250 μ m outer diameter; 6000 molecular weight cutoff; Spectrum Laboratories, CA). The probe was partially inserted so that the active membrane remained within the guide cannula shaft. On the following day, the anchor securing the probe was loosened and the probe was gently inserted into the CeA of the freely behaving animals. Each probe was re-secured with the anchor screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel (Instech Laboratories, PA) and a 2.5 ml gas-tight infusion syringe

attached to a pump (model 22, Harvard Apparatus, MA). Microdialysis probes were perfused at 3 $\mu\text{l}/\text{min}$ with filtered Krebs-Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K^+ , 145 Na^+ , 1.35 Ca^{2+} , 1.0 Mg^{2+} , 150 Cl^- , 0.05 ascorbate, pH 7.4 [306], and BSA (0.1%). After the probe insertion, a 60-min stabilization period was allowed (a time interval previously demonstrated to permit stabilization of peptide release) [296]. Subsequently, dialysates were collected every 20-min ($\sim 60 \mu\text{l}$) and immediately frozen on dry ice and stored at -80°C for 2 weeks until RIA analyses. The efficiency of *in vitro* peptide recovery by the microdialysis probes averaged $3.3 \pm 0.6\%$ (details previously described [297], see also Chapter 1).

Noise exposure

Following the collection of 6 baseline dialysate samples, a single 15-min burst of 90 dB continuous white noise (as described for Experiment 1) was delivered via speakers (Voxtek 85S, 8Ω 0.5W) affixed to the top of the testing cage. Following noise exposure, 12 post-noise dialysate samples were collected.

Experiment 2b: The effect of noise exposure on CRH and corticosterone availability at the anterior pituitary

Animals and procedure

Male Lewis ($n = 9$) and Fischer ($n = 12$) rats, maintained as previously described, were anesthetized with pentobarbital (60 mg/kg; i.p.) and stereotactically implanted with a sterile 20 gauge custom made stainless steel guide cannula containing a removable 24 gauge stainless steel obturator aimed at the anterior lobe of the pituitary gland. The

placement coordinates [342] with skull level were A/P, -5.3 mm; D/V, -11.0 mm; and L/M, $\pm$ 0.9 mm. The guide cannula was secured to the skull with four stainless steel screws and dental cement. After surgical recovery (at least 7 days), animals were transferred to individual testing chambers and allowed to acclimate for 24-hr before being placed individually in the noise room where they remained for another 24-hr prior to testing. Rats were tested individually in test chambers identical to those used in the microdialysis experiments.

Push-pull perfusion

Push-pull probes were custom manufactured using a 24 gauge stainless steel outer cannula as the "pull" aspect of the probe, while a concentric silica tubing (75 μ m ID x 150 μ m OD, Polymicro Technologies, AZ) protruding 0.4 mm beyond the outer cannula, constituted the "push" element of the probe. On the morning of testing the probes were calibrated for 20-min *ex vivo* to ensure the stability of the push-pull dynamics. The obturator was then carefully removed from the guide cannula of freely moving rats and replaced with the probe so that the silica tubing entered into the anterior lobe of the pituitary gland (permitting a free exchange between the KRB and interstitial fluid). Push-pull probes were secured with polyethylene tubing to a dual channel liquid swivel (Instech Laboratories, PA) and then connected to separate peristaltic pumps (Minipulse 3, Gilson, WI) that were used to perfuse the probes with KRB (as described in Experiment 2a). Typically, the 5-min sampling interval yielded 110 μ l of perfusate, which was aliquoted into two separate sample vials. These were immediately placed on dry ice and

later stored at -80°C until CRH and corticosterone assays were conducted. 2-3 weeks later.

Noise exposure

Following the insertion of the probe 12 baseline samples were collected (over a 1-hr period) followed by a 15-min burst of 90 dB continuous white noise identical to that described in the previous experiments. Three 5-min samples were collected during noise exposure followed by an additional 12 post noise samples.

Experiment 2c: Strain-dependent behavioral response to noise exposure

During the push-pull experiment, the occurrence of various behaviors was assessed using a time-sampling procedure [293]. Prior to noise onset, during noise exposure, and following noise termination the frequency of the following behaviors was noted every five seconds: *Sleeping resting*: lying still with eyes completely or partially closed; *Exploration*: actively moving about the cage; *Sniffing*: vibrissae movements accompanied by head movements directed toward the floor or walls of the chamber; *Rearing*: forelimbs off the cage floor in the absence of grooming; *Grooming*: forelimbs are wiped over the face or ventral regions of abdomen and thorax; *Freezing*: rigid posture, complete absence of visible movement, including vibrissae, with rapid respiration [160:193]. This procedure, in our laboratory, had previously been found to yield better than 90% agreement between raters [293].

Histology

At the end of the microdialysis and push-pull experiments, animals were briefly anesthetized with halothane and then sacrificed so that their brains/pituitary glands could be extracted, sectioned, and stained for histological verification of probe placement. The correct placement of the push-pull probe into the anterior lobe of the pituitary gland was readily verified under low magnification without staining. Only data from correctly placed probes were used for statistical analyses.

Radioimmunoassay (RIA)

The detection and quantification of immunoreactive (ir)-CRH was achieved using a solid-phase high-sensitivity adaptation or modification [270] of the double-antibody liquid phase RIA originally described by Vale et al. (1983) [454]. Immunoreactive-GRP was detected using a similar solid-phase RIA [356]. Briefly, protein A/G- (Calbiochem Corp., CA) coated Immulon-4 wells (Dynatec Laboratories Inc., VA) were incubated with anti-CRH serum (rC70, kindly provided by W. Vale, The Salk Institute, La Jolla, CA) or anti-BN serum (α -BN2, kindly provided by T.W. Moody, NCI, Rockville, MD) for 2-hr at 20°C. Samples, standards (diluted in tissue processing medium, ranging from 0.05 to 250 fmol/well), and blanks were incubated for 24-hr at 4°C. Next, 25 μ l of assay buffer containing 5000-6000 cpms of 125 I-[Tyr⁰]-rCRF (Amersham, ON), or 125 I-[Tyr⁴] BN (iodinated using the method of Salacinski et al. (1981) [386] was added to each well and incubated for an additional 18-hr at 4°C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted in a gamma counter (Cobra II Auto gamma, Model D5002, Packard Instrument Company, CT). A four-parameter

logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 and 2.0 fmol/well for CRH and BN, respectively.

Statistical analyses

All results are expressed as means $\pm$ S.E.M. The neuroendocrine and neuropeptide data from Experiment 1a were analyzed by a series of 2 (Strain) $\times$ 2 (Noise condition) $\times$ 2 (Time (post noise termination): 0-min vs. 1-hr) analyses of variance (ANOVA). Similarly, the data from Experiment 1b were analyzed by a 2 (Strain: Fischer vs. Lewis) $\times$ 2 (Stressor condition: home cage vs. novel environment) ANOVA. Subsequent comparisons of means of significant main effects or simple effects of significant interactions were analyzed using Bonferroni corrected t tests. At times the degrees of freedom varied because of missing samples.

For the analysis of the *in vivo* CRH and corticosterone release (i.e. Experiment 2a & b), the 3 baseline values preceding noise exposure from each animal were averaged and defined as 100%. All values were then expressed as a percentage of that baseline value. A significant main effect of Sample was followed-up with Bonferroni corrected t tests at relevant sample points. For the behavioral data obtained in Experiment 2c, separate mixed measures ANOVAs with Sample blocks (baseline, noise, and post-noise) as the within group variable, were performed for each behavior with Strain (Fischer vs. Lewis) serving as the between group factor.

Results

Experiment 1a: Effect of noise exposure on brain CRH/GRP and plasma hormone levels

Figure 1 (panel A) depicts the changes of plasma ACTH associated with noise exposure. The ANOVA revealed that ACTH levels varied as a function of the Noise x Time and the Noise x Strain interactions. $F_{1,60} = 4.37$ and 5.96 , $p's < 0.05$, respectively. The comparisons of the means comprising the simple effects of these interactions confirmed that immediately after noise exposure the ACTH concentrations were elevated, but returned to control values within 1-hr of noise termination. The comparisons of the simple effects comprising the Strain x Time interaction indicated that in the Fischer rats the levels of ACTH immediately after noise exposure were elevated relative to those seen in Lewis rats, but returned to control levels 1-hr later. The effects of the noise exposure on plasma corticosterone levels essentially paralleled the ACTH changes (see Figure 1, panel B). The ANOVA revealed a significant Strain x Noise x Time interaction. $F_{1,60} = 11.36$, $p < 0.01$. The multiple t-tests indicated that corticosterone levels were elevated in Fischer rats immediately after noise exposure, but not at the 1-hr interval. In the Lewis rats, in contrast, the noise stressor failed to significantly alter corticosterone levels.

Figure 1 (panel C) shows that basal plasma prolactin in Lewis rats were slightly, but significantly, higher than in Fischer rats. $F_{1,60} = 4.57$, $p < 0.05$. As well, prolactin levels varied as a function of the Noise x Time interaction. $F_{1,60} = 45.89$, $p < 0.01$. Unlike the ACTH and corticosterone, the level of prolactin was comparably elevated in both strains immediately after the noise exposure. In plasma taken 1-hr later prolactin had returned to basal levels.

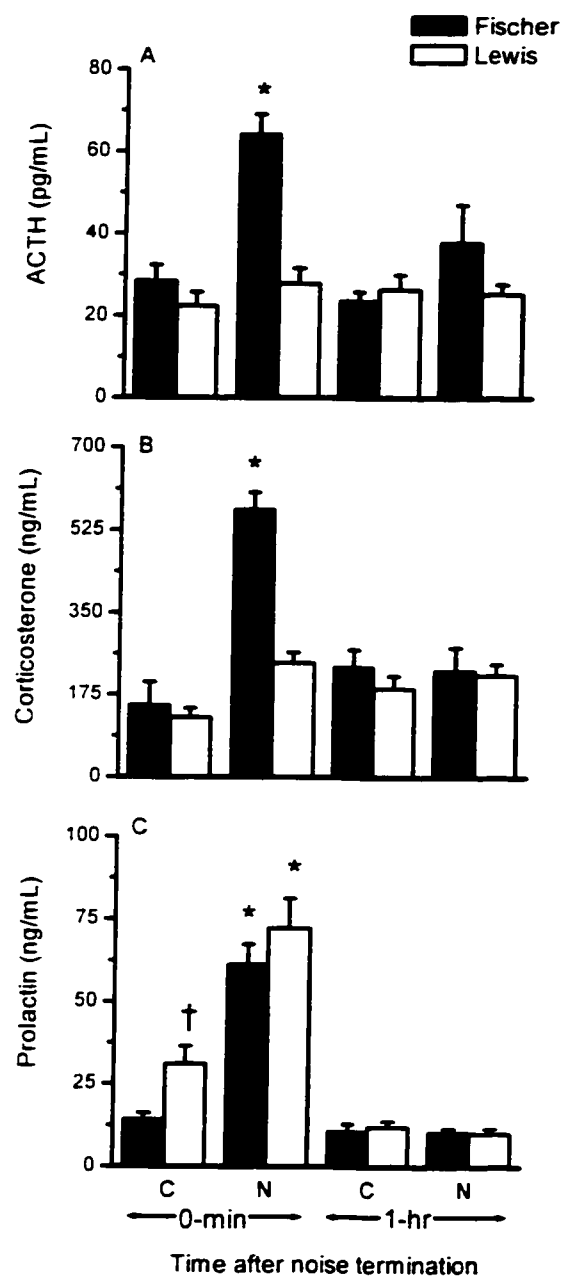


Figure 1. Variations of plasma hormone levels at 0-min and 1-hr after 15-min of noise exposure are depicted as means ($\pm$ SEM) for ACTH (panel A), corticosterone (panel B) and prolactin (panel C). Adrenocorticotropin-releasing hormone (ACTH). Control group (C), Noise exposed animals (N). * Significant within strain difference from matched time control group, $p < 0.0001$. † Significant between strain difference at matched time, $p < 0.05$.

Hypothalamic-pituitary CRH and GRP variations following noise exposure

Figure 2 shows the changes of ir-CRH (left panel) and ir-GRP (right panel) within the PVN (upper panel) and Me-Arc (lower panel). At the PVN the levels of ir-CRH varied as a function of the Noise x Time interaction, $F_{1,50} = 16.14$, $p < 0.001$. Immediately after the noise exposure, ir-CRH was slightly elevated in both strains, while in noise-exposed rats sacrificed 1-hr later, ir-CRH levels were lower than among non-stressed controls. The latter effect was due to a significant increase of ir-CRH in control animals sacrificed at the 1-hr interval, relative to control groups sacrificed at the earlier time point (see Figure 2, panel A). There was a tendency for the ir-CRH levels at the Me-Arc to be higher in the Fischer rats; however, due to the high variability, these effects failed to reach statistical significance (see Figure 2, panel B).

At the PVN the levels of ir-GRP varied over time following noise exposure, $F_{1,58} = 6.16$, $p < 0.01$. Immediately after noise exposure, the levels of ir-GRP in noise-exposed rats did not differ from control levels. However, 1-hr following noise exposure, levels were lower relative to control conditions. Similar to ir-CRH, the latter effect stemmed from a significant increase of ir-GRP in control animals sacrificed at the 1-hr interval, relative to control groups sacrificed at the earlier times (see Figure 2, panel C). The noise stressor also provoked a modest reduction of ir-GRP within the Me-Arc, $F_{1,56} = 4.33$, $p < 0.05$ (see Figure 2, panel D).

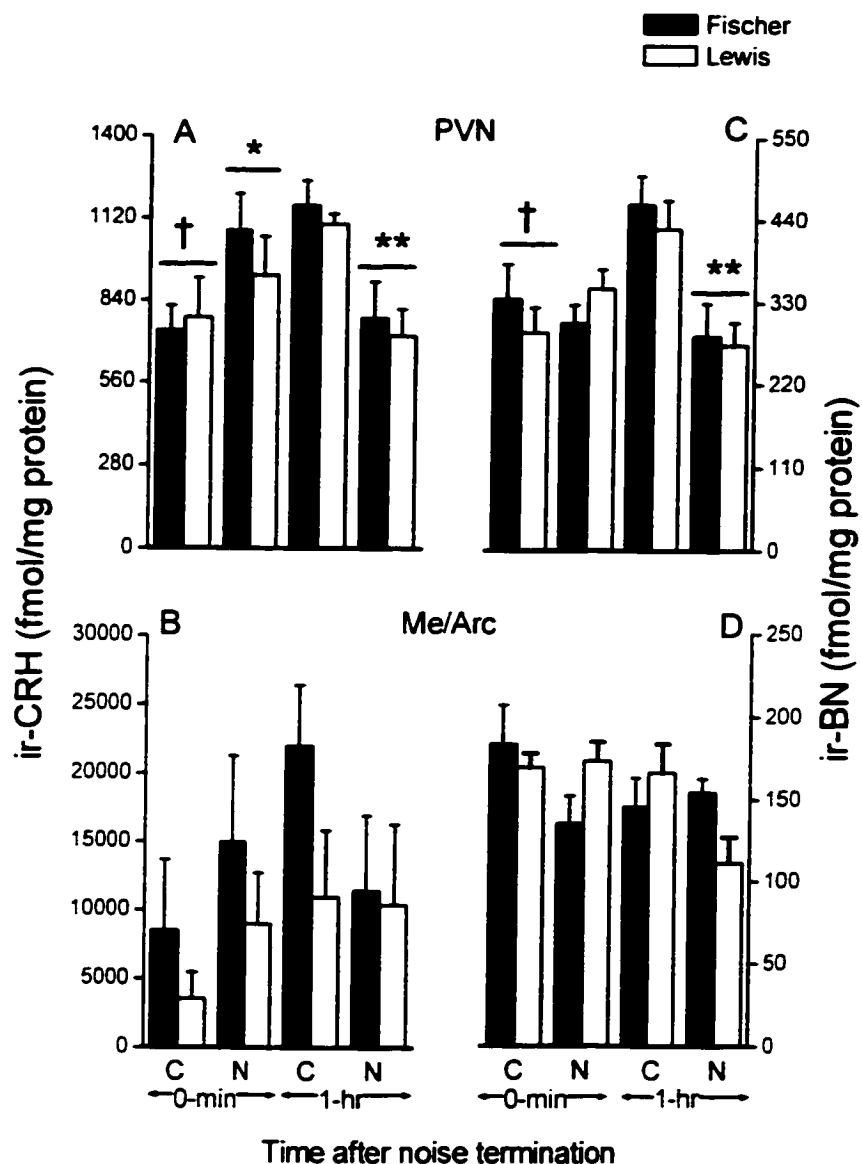


Figure 2. The two panels on the left represent the mean concentrations ($\pm$ SEM) of ir-CRH at 0-min and 1-hr after 15-min of noise exposure at the PVN (panel A), and Me/Arc (panel B). The panels on the right represent the mean concentration ($\pm$ SEM) of ir-BN at the PVN (panel C) and Me/Arc (panel D), at 0-min and 1-hr after 15-min of noise exposure. Corticotropin-releasing hormone (CRH), Bombesin (BN), Paraventricular nucleus of the hypothalamus (PVN), Median eminence/Arcuate complex (Me/Arc), Immunoreactive (ir). Control group (C). Noise exposed animals (N). *, ** Significant difference from matched time control group, $p < 0.05$ and $p < 0.01$, respectively. † Significant difference from 1-hr time point, $p < 0.05$.

Variations of CRH and GRP at amygdaloid nuclei following noise exposure

Figure 3 represents changes in the content of ir-CRH and ir-GRP at the amygdala. The ir-CRH changes at the CeA varied as a function of a significant Noise x Time interaction, $F_{1,48} = 21.18$, $p < 0.01$. In both strains, the level of ir-CRH immediately after stressor exposure did not differ from that of controls. However, 1-hr after noise exposure the levels of ir-CRH in non-stressed animals was reduced relative to that of rats that had been exposed to noise or to control conditions 1-hr earlier (see Figure 3, panel A).

At the MeA, ir-CRH varied as a function of the Strain x Noise interaction, $F_{1,58} = 3.93$, $p < 0.05$. As seen in Figure 3 (panel B) the levels of ir-CRH were comparable in the two strains in the absence of noise. The level of ir-CRH in Fischer rats was elevated both immediately and 1-hr following exposure to noise. Among the Lewis rats the increase of ir-CRH elicited by the stressor was less pronounced. Immediately after noise exposure ir-CRH was increased in the MeA of Lewis rats, but this elevation was not evident 1-hr after noise exposure. Finally, in the BLA the level of ir-CRH was greater in Lewis than in Fischer rats, $F_{1,59} = 8.06$, $p < 0.01$. However, the noise treatment had no effect on the peptide level (see Figure 3, panel C).

At the CeA ir-GRP levels were modestly reduced immediately after the noise exposure, and further reduced 1-hr thereafter, leading to a significant Noise x Time interaction, $F_{1,59} = 13.23$, $p < 0.01$ (Figure 3, panel D). In contrast, the noise did not affect the concentration of ir-GRP at the MeA or the BLA (see Figure 3, panels E-F).

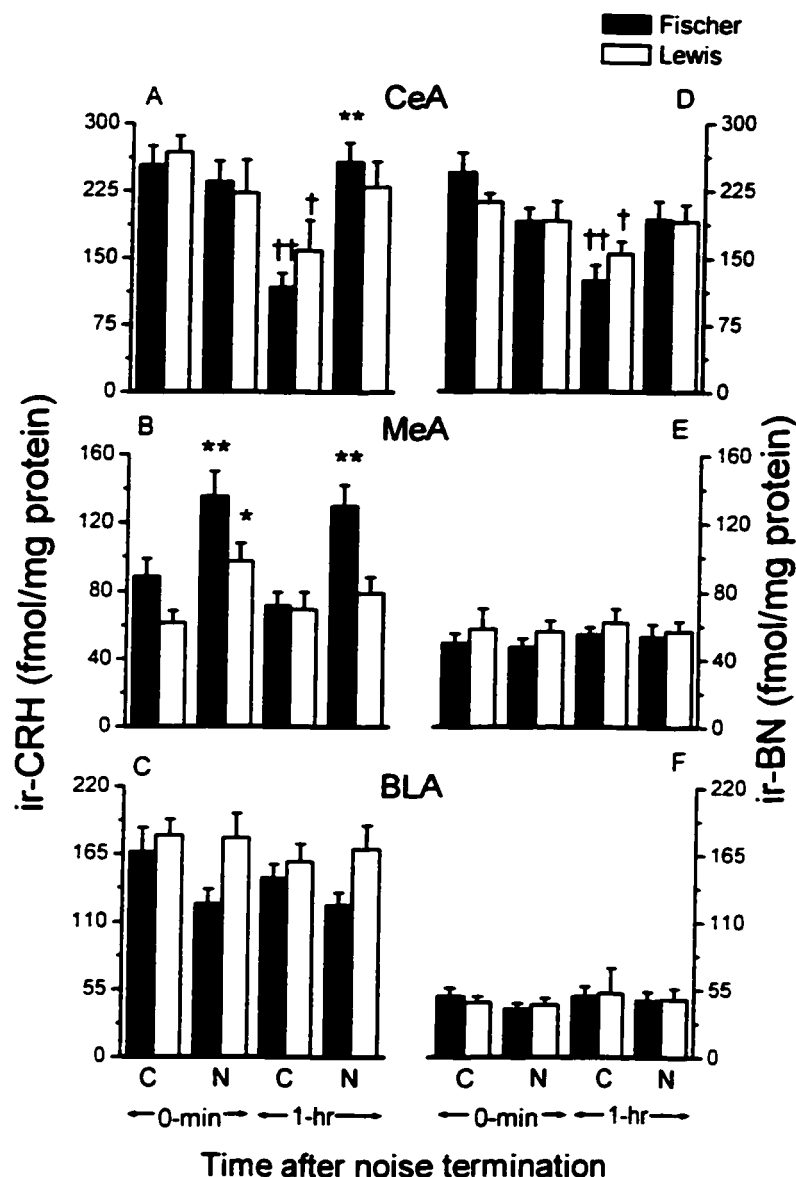


Figure 3. The three panels on the left represent the mean concentrations (± SEM) of ir-CRH at 0-min and 1-hr after 15-min of noise exposure at the CeA (panel A), MeA (panel B), and BLA (panel C). The panels on the right represent the mean concentration (± SEM) of ir-BN at the CeA (panel D), MeA (panel E), and BLA (panel F) at 0-min and 1-hr after 15-min of noise exposure. Corticotropin-releasing hormone (CRH), Bombesin (BN), Immunoreactivity (ir), Central nucleus of the amygdala (CeA), Medial nucleus of the amygdala (MeA), Basal lateral nucleus of the amygdala (BLA), Control group (C), Noise exposed animals (N). *, ** Significant within strain difference from matched time control group, $p < 0.02$ and $p < 0.01$, respectively. †, †† Significant within strain difference from 0-min time point, $p < 0.02$ and $p < 0.001$, respectively.

Variations of CRH and GRP in other stressor-sensitive brain regions following noise exposure

The levels of ir-CRH within the LC varied as a function of the Strain x Noise interaction. $F_{1,39} = 8.54$, $p < 0.01$. The t-tests confirmed that there was no change of ir-CRH in the Lewis rats. However, while the measured levels of ir-CRH increased appreciably at both time points in the Fischer rats, they only reached statistical significance 1-hr after noise exposure (Figure 4, panel A). Immunoreactive-GRP levels at the LC were reduced in rats exposed to noise. $F_{1,58} = 7.30$, $p < 0.01$. At 1-hr noise exposure was associated with significantly lower levels of ir-GRP compared to control levels (see Figure 4, panel B). Contrary to the effects at the LC, noise slightly reduced the level of ir-CRH within the dHipp. $F_{1,56} = 8.13$, $p < 0.01$, however ir-GRP levels were not effected by noise exposure at this site (data not shown).

In other brain regions, including the PFC, nAcb, Cau, and PV the noise stressor did not affect either ir-CRH or ir-GRP at the designated sampling times (see Table 1).

Experiment 1b: The impact of environmental cues on protracted changes in brain CRH and GRP and plasma hormone levels 24-hrs following noise exposure

Plasma ACTH, corticosterone, and prolactin levels were unaffected by noise exposure administered 24-hr earlier, irrespective of whether the stressor was administered to rats in their home cages or in novel (distinct) environments (data not shown). Similarly, ir-CRH levels within the PVN and Me-Arc at 24-hr were unaffected by the environment in which the noise had been applied (data not shown). Within the CeA the

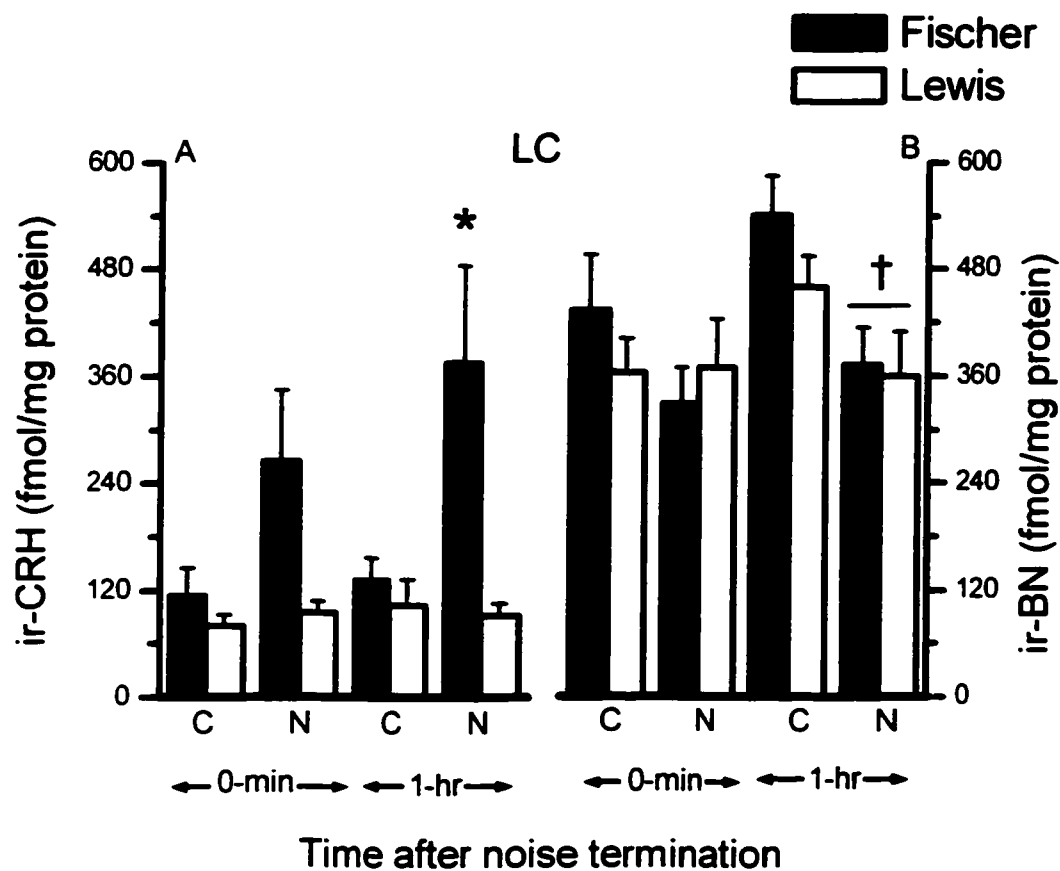


Figure 4. Variations of peptide concentration 0-min and 1-hr after 15-min of noise exposure are depicted as means ($\pm$ SEM) for ir-CRH (panel A), ir-BN (panel B). Corticotropin-releasing hormone (CRH), Bombesin (BN), Locus coeruleus (LC), Immunoreactive (ir), Control group (C), Noise exposed animals (N). * Significant within strain difference from matched time control group, $p < 0.05$. † Significant difference from matched time control condition, $p < 0.01$.

Table 1 Content of ir-CRH & ir-BN at various brain sites in Fischer and Lewis rats

Strain	Peptide	Region	Time after noise termination			
			0-min		1-hr	
			Control	Noise	Control	Noise
Fischer	ir-CRH	nAcb	51.4 ± 9.1	94.8 ± 19.2	62.9 ± 18.9	130.8 ± 19.4 *
		Cau	57.8 ± 4.5	45.9 ± 3.5	46.2 ± 2.7 †	52.3 ± 4.9
		dHipp	136.5 ± 13.9	126.0 ± 11.9	165.0 ± 12.5	138.0 ± 10
		PFC	668.3 ± 31.9	717.2 ± 84.4	420.6 ± 67.6 ††	414.4 ± 55.9 ††
		PV	173.8 ± 16.4	149.8 ± 11.1	147.7 ± 9.6	163.1 ± 12.1
	ir-BN	nAcb	40.4 ± 7.7	36.2 ± 7.8	67.5 ± 6.7 †	38.3 ± 7.7 **
		Cau	63.2 ± 9.1	68.2 ± 11.5	58.3 ± 10.8	76.5 ± 11.7
		dHipp	19.8 ± 1.8	18.5 ± 6.0	16.2 ± 2.0	14.9 ± 1.5
		PFC	61.0 ± 5.1	55.4 ± 6.0	57.5 ± 6.2	52.0 ± 5.4
		PV	162.3 ± 15.7	155.0 ± 14.8	194.8 ± 16.4	187.6 ± 24.2
Lewis	ir-CRH	nAcb	134.1 ± 28.1 §§	85.6 ± 18.3	70.9 ± 11.7	72.2 ± 10.5 §
		Cau	39.4 ± 2.9 §§	41.0 ± 5.7	52.5 ± 5.2 †	41.9 ± 4.9
		dHipp	170.5 ± 19.5	128.2 ± 7.8	145.1 ± 14.7	119.4 ± 7.8
		PFC	528.8 ± 62.9	521.5 ± 49.2	400.5 ± 25.1	515.8 ± 43.3 *
		PV	124.4 ± 11.0	164.3 ± 9.3	148.0 ± 11.0	158.8 ± 25.1
	ir-BN	nAcb	44.5 ± 6.2	60.5 ± 11.6	66.7 ± 5.6 †	64.5 ± 11.6
		Cau	61.4 ± 8.2	54.1 ± 6.2	92.5 ± 14.5	71.6 ± 10.0
		dHipp	17.3 ± 1.7	13.5 ± 1.3	11.3 ± 1.5	13.8 ± 2.5
		PFC	55.2 ± 4.7	61.5 ± 3.8	49.7 ± 3.0	59.5 ± 3.2
		PV	172.2 ± 30.8	143.0 ± 10.0	190.1 ± 33.8	128.0 ± 25.1

Table 1. Each cell represents the mean ± S.E.M. (fmol/mg protein) 0- and 1-hr following exposure to 15-min of noise or no noise (control) in the following brain regions. Caudate (Cau), paraventricular thalamic nucleus (PV), nucleus accumbens (nAcb), prefrontal cortex (PFC) and dorsal hippocampus (dHipp). *, ** Significant within strain difference from same time control condition. †, †† Significant within strain difference from 0-min after noise termination. §, §§ Significant between strain difference. All effects are, $p < 0.05$ and $p < 0.02$, respectively.

level of ir-CRH 24-hr after noise exposure varied as a function of the interaction between the Strain and the environment in which the noise was administered. $F_{1,29} = 22.51$, $p < 0.01$. Noise applied to Lewis rats while in the home cage resulted in ir-CRH levels at the CeA exceeding that of similarly treated Fischer rats (respective means: 325 ± 33 vs. 199 ± 12 ; $p < 0.05$) or Lewis rats exposed to noise in the novel environment (mean difference = 133 ± 21 ; $p < 0.05$).

In the remaining brain regions (MeA, BLA, dHipp, PFC, nAcb, Cau, and PV) the exposure environment had no effect on the levels of ir-CRH 24-hr after the noise exposure (data not shown). Similarly, the exposure environment had no effect on the levels of ir-GRP at any of the brain regions examined 24-hr after noise exposure (data not shown).

Experiment 2a: The effect of noise exposure on the release of CRH from the CeA

An initial comparison of the raw ir-CRH values from the dialysates constituting the baselines from animals with correctly positioned probes ($n = 5$ /Fischer; $n = 4$ /Lewis) revealed a significant difference between the two strains (respective means: 19.2 ± 5.2 and 217.3 ± 17.5 pg/45 μ l, $p < 0.0001$). An ANOVA of the percent change from baseline revealed that interstitial ir-CRH varied as a function of Sample, $F_{12,84} = 14.95$, $p < 0.001$. Post-hoc analyses revealed that noise exposure was associated with an immediate (during noise exposure) rise of ir-CRH concentration in the dialysates collected from the Fischer rats, which persisted until the end of the collection period (see Figure 5). In the Lewis rats ir-CRH levels increased during noise exposure but did not reach statistical

significance ($p = 0.06$) until the 3rd post-noise sample. Thereafter ir-CRH concentrations were significantly higher than baseline values until the end of the experiment (see Figure 5).

Experiment 2b: The effect of noise exposure on CRH and corticosterone availability at the anterior pituitary

Figure 6 (top panel insert) depicts the typical probe placement within the anterior pituitary. An initial comparison of the raw ir-CRH values from the perfusates constituting the baselines from animals with correctly positioned probes ($n = 11$ /Fischer; $n = 9$ /Lewis) revealed no strain differences (respective means: 29.9 ± 4.3 and 21.5 ± 2.5 pg/45 μ l, $p = 0.4$). An ANOVA of the percent change from baseline revealed a significant Sample x Strain interaction, $F_{17,323} = 2.92$, $p < 0.001$. Post-hoc analyses indicated that during the first 10-min of the 15-min noise exposure, ir-CRH concentrations were significantly elevated above baseline in the Fischer rats (p 's < 0.05) and decreased thereafter to basal levels (see Figure 6, top panel). In the Lewis rats, noise was associated with a more delayed rise of ir-CRH concentrations, beginning at 25-min following noise onset, and reaching a statistically significant increase above baseline by 30-min ($p < 0.01$). The ir-CRH remained elevated until about 45-min following stressor onset, and then declined. (see Figure 6, top panel).

An initial comparison of the means constituting the baselines for both strains revealed that corticosterone levels in the perfusates collected from the Fischer rats were significantly higher than those of the Lewis rats (respective means: 3.0 ± 0.3 and $1.4 \pm$

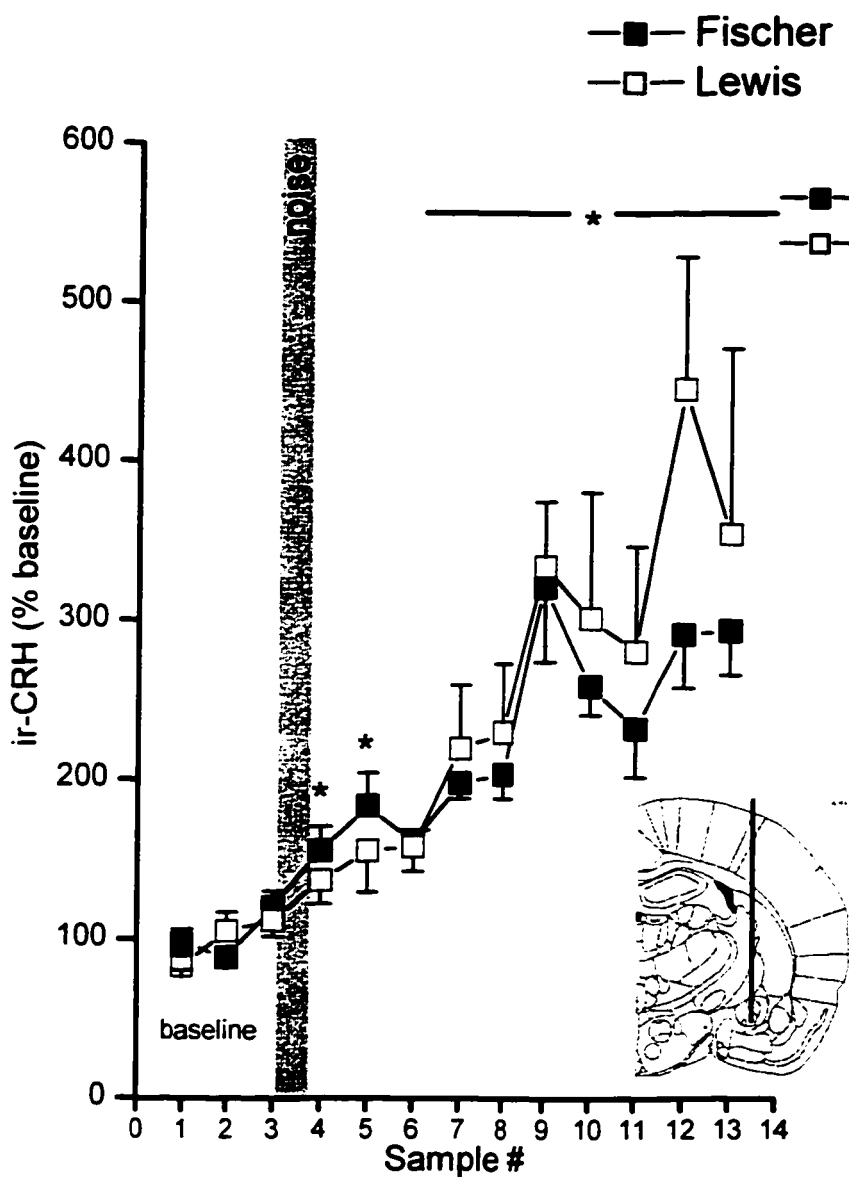


Figure 5. Levels of ir-CRH at the central nucleus of the amygdala as measured by *in vivo* microdialysis under baseline condition and following 15-min of noise exposure in Fischer (solid squares) and Lewis (open squares) rats. The baseline samples from each subject were averaged and defined as 100%. All values were then expressed as a percent of that baseline (basal values were 4.04 ± 1.1 and 45.7 ± 3.7 fmol/sample, for Fischer and Lewis strains, respectively). Insert depicts typical probe placement at the central amygdala. * Significant difference from baseline condition at $p < 0.05$.

0.2 pg/45µl, $p < 0.05$). The levels of corticosterone varied as a function of the Sample, $F_{17, 272} = 3.83$, $p < 0.0001$. During the last 5-min of the 15-min noise exposure corticosterone levels were significantly elevated in the Fischer rats and remained so for an additional 9 samples (45-min) (see Figure 6, bottom panel). In the Lewis strain noise exposure failed to significantly increase corticosterone levels in the perfusates. However, by the 8th post noise sample (40-min post onset), corticosterone levels were elevated and approached statistical significance ($p = 0.07$) (see Figure 6, bottom panel).

Experiment 2c: Strain-dependent behavioral responses to noise exposure

Individual ANOVAs of the frequency of sniffing, grooming, and exploration revealed significant Strain x Sample Blocks interactions, F 's $_{2, 38} = 11.41$, 8.95, and 9.91, p 's < 0.001 , respectively. Post-hoc analyses revealed that sniffing, grooming and exploration were significantly elevated from baseline during noise exposure in the Fischer, but not in the Lewis rats (see Figure 7, panel A-C).

Freezing was influenced by an interaction between Strain and Sample blocks, $F_{2, 38} = 14.15$, $p < 0.001$, that was due to a robust increase during noise exposure in the Lewis rats (see Figure 7, panel D). Strain x Sample blocks interactions were also evident for rearing, $F_{2, 38} = 14.28$, $p < 0.0001$ and resting, $F_{2, 38} = 3.33$, $p < 0.05$. Post-hoc analyses showed that while both strains showed almost no rearing during baseline, this behavior increased significantly with noise exposure only in the Fischer rats (see Figure 7, panel E). The Strain x Sample blocks interaction for resting stemmed from a slightly higher

amount of resting following noise exposure among the Fischer rats (see Figure 7. panel F).

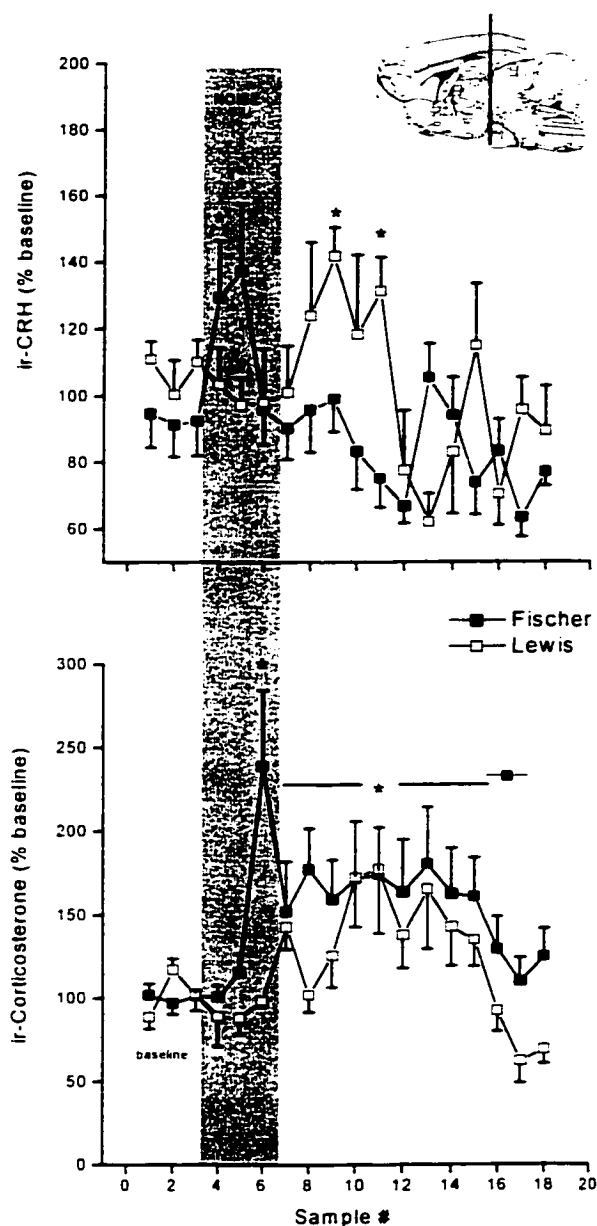


Figure 6. Levels of ir-CRH (top panel) and ir-corticosterone (bottom panel) availability at the anterior pituitary gland as measured by *in vivo* push-pull perfusion under baseline condition and following 15-min of noise exposure in Fischer (solid squares) and Lewis (open squares) rats. The baseline samples from each subject were averaged and defined as 100%. All values were then expressed as a percent of that baseline (basal ir-CRH values were 6.3 ± 0.9 and 4.5 ± 0.5 fmol/sample, for Fischer and Lewis rats, respectively and basal ir-corticosterone values were 8.7 ± 0.9 and 4.0 ± 0.6 fmol/sample, respectively). Insert depicts typical probe placement at the anterior pituitary gland * Significant difference from baseline condition at $p < 0.05$.

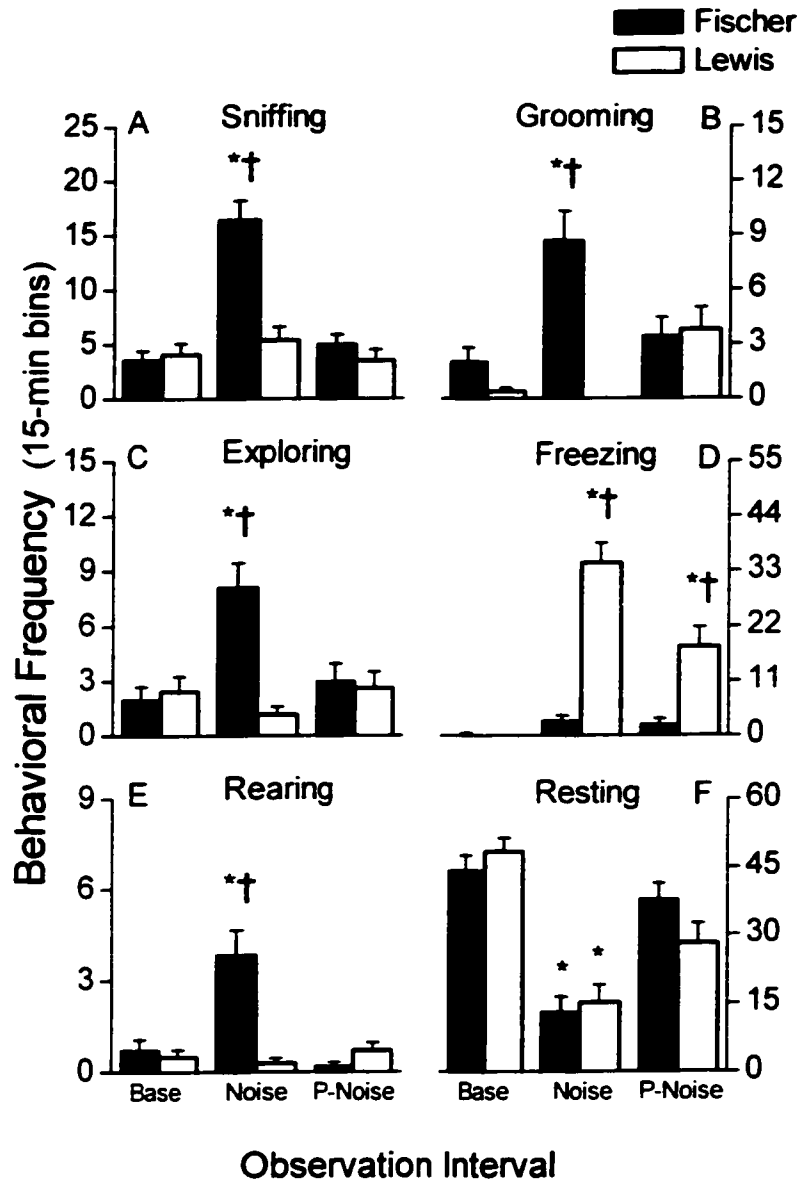


Figure 7. The frequency of sniffing (panel A), grooming (panel B), exploring (panel C), freezing (panel D), rearing (panel E) and resting (panel F) was observed before noise exposure (base) during noise exposure (noise) and following 15-min of noise exposure (P-noise) in Fischer (solid bars) and Lewis (open bars) rats. * Significant within strain difference from baseline condition, $p < 0.05$. † Significant between strain difference from matched time observation period, $p < 0.05$.

Discussion

The present study demonstrated that relative to male Lewis rats, male Fischer rats exhibited a more robust corticosterone and ACTH response to noise exposure. This is concordant with earlier reports using female rats [487] and suggests that enhanced HPA reactivity of the Fischer rat (as compared to Lewis) is gender independent. As expected, local interstitial levels of corticosterone at the pituitary paralleled the noise-induced increase in the circulating levels of this corticoid: increases being evident in the Fischer, but not in the Lewis rats. Since corticosterone at the anterior pituitary provides a feedback inhibitory signal to dampen further activation of the HPA response [269;481], it would appear that the feedback system of the Fischer rats is down regulated.

In contrast to the strain-dependent effects of the stressor on ACTH and corticosterone, noise exposure elicited a comparable increase of plasma prolactin in both strains. Most stressors inhibit the activity of tuberoinfundibular dopamine neurons in the arcuate nucleus, disinhibiting the release of prolactin from the anterior pituitary [147]. Thus, the blunted ACTH and corticosterone responses in Lewis rats are not attributable to strain-specific differences of stressor perception or sensitivity.

As seen in Experiment 1, noise exposure was also associated with site-specific alterations of tissue ir-CRH concentrations at the PVN and Me/Arc as well as in other related limbic structures, such as the amygdala. In both strains the content of ir-CRH at the PVN 15-min following noise onset was elevated compared to control conditions. By 1-hr following noise exposure ir-CRH levels were lower in both strains compared to the

control levels; however, at this time control levels of ir-CRH were greater than that seen in control animals 1-hr earlier. This pattern of change suggests that both the noise exposure and control manipulations (i.e. transportation to the test room) imparted changes in PVN ir-CRH content despite efforts to minimize these effects (i.e. exposing animals to daily transportation to and from the experimental test room, for over 3 weeks). Predictably, however, when peptidergic changes were noted they were more pronounced and enduring in rats exposed to noise.

It is quite possible for peptide release to change without altered tissue peptide levels being altered [295:370]. For instance, if increased release is accompanied by an equally enhanced rate of synthesis, then such effects would not be reflected in post-mortem analyses of tissue peptide levels. Thus, the use of *in vivo* sampling techniques has distinct advantages in detecting potential strain differences with respect to the impact of various challenges. The current study represents the only direct assessment of noise-associated alterations in the availability of ir-CRH at the anterior pituitary in the Fischer and Lewis rats. Push-pull perfusion at the anterior pituitary revealed that while noise exposure provoked a rapid rise of interstitial ir-CRH levels in Fischer rats, this change was delayed in Lewis rats.

It might have been expected that since ir-CRH availability increased at all in Lewis rats, plasma corticosterone should likewise have increased. In fact, the observed rise of corticosterone availability in the Lewis rats appeared to follow the delayed rise in ir-CRH; however, this blunted increase failed to reach statistical significance.

Accordingly, other factors that might influence the stimulatory effect of CRH on ACTH release should be considered. Lewis rats have been reported to be more sensitive to the suppressive effects of dexamethasone on CRH- or cocaine-induced ACTH secretion [408:498], suggesting that corticosterone availability at the anterior pituitary in Lewis rats need not reach the same level as in the Fischer rats, to inhibit the HPA response.

Several stressors, including noise, increase the neuronal firing rate and neurotransmitter release at the LC [409:410]. To date, the release of CRH at the LC has been alluded to, but never directly measured. Nevertheless, several indirect approaches suggest that CRH may serve as an excitatory neurotransmitter at this site [455:457-459]. In the current study we report that noise exposure elevated ir-CRH levels at the LC of the Fischer but not the Lewis strain. If lower tissue peptide levels in the Lewis rats reflect enhanced release, then it would be consistent with the greater *in vitro* firing rate of LC neurons observed by others in the Lewis as compared to the Fischer rats [169]. Ultimately, it will be necessary to assess stressor provoked *in vivo* CRH release at the LC in the two rat strains.

Considerable efforts have been made towards characterizing the role of the amygdala in the cognitive, behavioral and physiological responses to emotionally salient stimuli [136:215:432], including noise [151]. The current micropunch data (i.e. Experiment 1) suggests nucleus- and peptide-specific alterations at the amygdaloid complex. Immediately after noise exposure, a marked elevation of ir-CRH was evident at the MeA in both strains, while no differences were noted at either the CeA or the BLA.

These data appear to support the proposition that the MeA is more closely aligned with the genesis of the neuroendocrine response to emotional stressors, relative to either the CeA or the BLA [113]. The caveat, as pointed out earlier, is that the lack of change in tissue peptide levels does not necessarily indicate a lack of effect on peptide release. However, tissue ir-CRH levels at the CeA 1-hr following control manipulations (which included transport to and from the test room) was markedly reduced in comparison to the control group sacrificed at the earliest time interval. Furthermore, levels of ir-CRH at the CeA of animals sacrificed at 1-hr post stressor exposure were similar to both control and noise-exposed groups sacrificed at the 15-min sampling time. Together, these results provisionally suggest that the transportation of the rats to the test area was sufficient to increase amygdaloid ir-CRH at the earliest sampling time, and that levels of the peptide declined to pre-stress levels 1-hr later in the control groups, but remained elevated in rats exposed to the more potent noise stressor. The results from the microdialysis study support the notion that the CeA is involved in noise stressor reactivity [151], as the release of ir-CRH at the CeA occurred following noise exposure in both strains.

Although CRH, particularly at the amygdala, has been implicated in the mediation or expression of the behavioral responses to stressors, this investigation suggests that there may be a mismatch between noise-related CRH alterations and the behavioral responses mounted by the two strains. The Fischer rats displayed a more active coping style (increased exploration and grooming), whereas the Lewis strain adopted a more defensive coping strategy (freezing). However, the two strains showed a rather similar rise in the release of CRH at the CeA. Likewise, Lahmame et al. (1997) [245] concluded

that there was dissociation between CRH activity (based on tissue content) and the expression of depression-like behavior in the forced swim test. This apparent mismatch suggests that either CRH at the CeA is not a contributing factor in the behavioral response to noise stressor, or that the sensitivity to CRH varies between the two strains. The latter possibility is supported by the finding that the Fischer and Lewis rats show behavioral differences in response to centrally applied CRH [158], such that exogenously administered CRH makes the Fischer rats behaviorally similar to non-CRH exposed Lewis rats. Finally, in response to a startle stimulus, the Lewis rats appear to be more behaviorally sensitive, but it is the Fischer rats that show a more pronounced corticosterone rise [157]. Together, these observations suggest that in the Lewis rats, the brain circuits mediating the behavioral response to stressors (CRH-dependent or – independent) are up-regulated, whereas their HPA responses are either down-regulated (due to previous state of activation) or regulated by an up-regulated feedback inhibitory system. These observations are suggestive of those noted in the human condition of post-traumatic stress disorder where an individual exposed to chronic stress or a traumatic event develops, over time, exaggerated autonomic and behavioral responses to stressors. Paradoxically, the individual fails to show the predicted stressor-evoked cortisol rise, although there is a heightened response to the suppressive effects of dexamethasone [494]. Thus the Lewis rat may provide an opportunity to assess some of the neurochemical underpinnings of altered behavioral and neuroendocrine responses observed in some pathological states like post-traumatic stress disorder.

We recently reported that bombesin-related peptides (namely GRP) might contribute to some endocrine and behavioral responses to stressors, in part through the activation of CRH systems [53]. In Experiment 1, noise exposure was associated with altered ir-GRP at the PVN, Me-Arc, CeA, Cau and nAcb. The finding that both strains displayed similar changes of ir-GRP at the PVN and Me/Arc, and yet the Lewis rats still failed to show elevated ACTH and corticosterone response, suggests that these rats may have down-regulated CRH receptors. Commensurate with this suggestion, it has been shown that following stressor exposure CRH receptor binding in some brain regions is higher in Fischer compared to Lewis rats [245].

In summary, the current neurochemical, neuroendocrine and behavioral data provide evidence that the apparent hypo-adrenal response in the Lewis rats exposed to stressors (noise in this instance) is not simply a function of altered CRH release, but may have more to do with altered sensitivity (at the receptor and post receptor level). In this regard, the strain differences reported here as well as by others [158;161;487] may be reflective of strain differences in the number and/or sensitivity of pituitary corticotrophs to endogenous CRH and/or glucocorticoids, as suggested by *in vitro* findings [498]. Our results also make it tempting to suggest that noise exposure might have protracted effects on ir-CRH changes related to the exposure environment and that Lewis rats might be more sensitive to these cues compared to Fischer rats. However, it is likely that the impact of such events may be dependent on individual characteristics, including genetic disposition. And finally, we would like to suggest that the blunted corticosterone response and enhanced behavioral responses in the Lewis rats may be a reflection of

enhanced sensitivity of the negative feedback circuit (rather than diminished sensitivity of the pituitary corticotrophs) and increased sensitivity to extra-hypothalamic sites (such as the CeA), respectively: a state reminiscent to that of post-traumatic stress disorder, where behavioral hyper-reactivity to stressors is accompanied with a blunted cortisol response.

Preface to Chapter IV

The data presented thus far suggests that contrary to the current dogma, CRH (at least at the CeA) does not appear to be involved in the genesis of the behavioral response to aversive or appetitive events. More precisely, the animal's behavioral response to different types of stressors (i.e. processive vs. systemic) might differentially utilize CRH-ergic systems. Indeed, we reported that despite the robust differences in behavioral response towards a female cohort, these rats had similar release patterns of CRH at the CeA. This dissociation between behavioral output on the one hand, and release of CRH on the other, was also evident following noise exposure. In this case, the Lewis and Fischer rats had distinct behavioral reactions to noise, whereas their pattern of CRH release at the CeA was remarkably similar in both strains. To further assess the role of CRH at the CeA in context of stress and anxiety, we developed a paradigm where the release of CRH could be monitored concurrently with their behavior, as the animals transitioned from their home milieu to a more anxiogenic (novel) one.

This "novel cage paradigm" is based on the fact that a novel environment engenders anxiety-like state in rodents, because of the conflict between the need to explore the new environment for food on the one hand, and the need to be vigilant for potential threats in the unfamiliar environment. In their home cage, non-food deprived rats readily approach and consume a familiar palatable snack. However, when the same snack is presented in a novel environment, the latency to approach it gets longer and the amount consumed declines [226]. As previously observed in our laboratory, diazepam (a prototypical anxiolytic) fails to affect snack consumption in the home cage, but

completely blocks the effects of the novel environment on snack intake. This suggests that diazepam increases food intake in the novel environment by attenuating the anxiety-like effects engendered by the novel milieu. So this paradigm was selected for the next experiment. However, prior to its usage, several logistic and technical challenges had to be surmounted. Once established, this paradigm afforded us the ability to continuously assess the release of neurochemicals at the CeA (using *in vivo* microdialysis). Samples could be collected in the home cage environment and then and continued being monitored during and after their transfer into novel (presumably stressful) environment.

In the current study we assessed the impact of the novelty-stressor on the release dynamics of neuropeptides, including CRH, at the CeA. We went into the study with the hypothesis that if the novel environment was indeed a stressor, it should increase the release of ir-CRH at the CeA. Likewise, if the effects of the novel cage on behavior were mediated through CRH at the CeA, then diazepam, that prevents anxiogenic-type behavioral responses should also attenuate the release of ir-CRH at the CeA. Finally, if this stressor engendered changes in central CRH apart from that which we directly observed at the CeA, then ICV pretreatment with CRH receptor antagonists should attenuate or block the effects of the novel cage on behavior.

CHAPTER IV

Diazepam reverses the behavioral effects of novel environment stressor but fails to block novelty-elicited release of corticotropin-releasing hormone at the central nucleus of the amygdala

Abstract

The corticotropin-releasing hormone (CRH) system(s) within the brain is (are) activated by stressful stimuli and may contribute to behavioral and emotional responses. The present investigation assessed anxiety-like responses and neurochemical alterations at the central nucleus of the amygdala (CeA) evoked by exposure to an unfamiliar environment. Placement into an unfamiliar environment (new cage) markedly suppressed ingestion of a palatable snack, and this effect was antagonized in a dose-dependent manner with diazepam pretreatment. As such, this behavioral change was utilized as an index of anxiety in the rodent. This stressor also stimulated the release of immunoreactive-CRH (ir-CRH) and glutamate at the CeA. However, various CRH antagonists (e.g. α h-CRF, CP-154,526, antisauvagine-30, preproTRH₁₇₈₋₁₉₉) did not attenuate the behavioral effects elicited by the stressor. As well, central infusions of CRH (2 μ g, ICV) failed to suppress the amount of snack consumed in the home cage. Despite the potent anxiolytic effect of diazepam in this paradigm, it failed to prevent the stressor-associated release of ir-CRH and glutamate at the CeA. Thus, although exposure to an unfamiliar environment may be interpreted as being "stressful" by neural circuits involving CRH and/or glutamatergic receptors at the CeA, the data do not support the view that activation of CRH and/or glutamate receptors is necessary for the expression of anxiety-like behavioral responses. Rather than provoking anxiety, it is suggested that these systems might serve to draw attention to events or cues of biological significance, including those posing a threat to survival.

Introduction

Stressors provoke neurochemical, behavioral, and emotional alterations that may be essential for adequate defensive functioning [93:388:475]. In this respect, it is thought that the CeA contributes to emotional responses, including the acquisition and expression of fear or anxiety [107:248:475] or may influence processes by which sensory stimuli gain motivational and emotional significance [173:248:340]. Consistent with the view that CRH within the amygdala contributes to the promotion or expression of anxiety [108:248:412], stressors increased CRH mRNA expression [216] and *in vivo* ir-CRH release [351:369:370] and amygdaloid CRH manipulations influenced anxiety-related behaviors [129:437].

Like CRH, the GABA-ergic system(s) may also influence anxiety. Benzodiazepine infusion into the amygdala produces anxiolytic effects [288], and the anxiolytic action of systemically administered benzodiazepines can be blocked by intra-amygdaloid injections of GABA receptor antagonists [108]. In addition, glutamate, an excitatory transmitter at the amygdala [432], when microinfused locally, stimulates the release of ir-CRH at the median eminence [149]. Thus, glutamate at the amygdala may contribute to stress responses by affecting CRH release at the amygdala and the median eminence [149:384].

The development of "ethologically-relevant" animal models has contributed significantly to the behavioral and neurochemical characterization of anxiety, particularly as such approaches are not confounded by the effects of painful stimuli often used to

elicit anxiety. Moderately threatening situations (e.g. placement in an unfamiliar environment) induce behavioral patterns like those elicited by predator scent, including inhibition of non-defensive behaviors (e.g., reluctance to ingest food) [48], and such effects are antagonized by anxiolytic agents [51:365;428]. Although assessing food intake in an unfamiliar environment is practical, its use has been limited by methodological problems. For instance, food or water deprivation (concurrent systemic stressors) or presentation of a novel food (which may produce neophobia) can confound the anxiogenic attributes of the unfamiliar environment. As well, presentation of regular chow yields low basal feeding levels (floor effect) making further reductions of food intake less discernible. We observed that offering animals highly palatable, familiar snack in a novel environment circumvents these problems, allowing for an unadulterated appraisal of anxiety [314].

Using this procedure, the present investigation established whether 1) the anxiety-like behavioral and neurochemical responses elicited by the novel environment would be attenuated by benzodiazepine pretreatment; 2) antagonism of CRH action(s) attenuated the effects of the novel environment on snack consumption; 3) dynamic, *in vivo*, fluctuations in the release of ir-CRH, GABA and glutamate at the CeA were elicited by the novel environment, and 4) attenuation of behavioral signs of anxiety by benzodiazepine treatment would concurrently influence the stressor-elicited release of ir-CRH, GABA, and glutamate.

Materials and methods

Animals and procedures

Adult male Sprague-Dawley rats (400-550 g) obtained from Charles River Laboratories (Canada), were used in all experiments. Subjects were individually housed and maintained on a 12-hr light/dark cycle (lights on at 07:00-hr), with free access to water and standard Purina™ rat chow. The room temperature was maintained at 21-23°C with 60% relative humidity. All procedures were approved by the University of Ottawa animal care committee and met the guidelines set out by the Canadian council on animal care.

Test environment (home vs. novel cage)

The animals' home cage consisted of a standard shoebox-style clear Plexiglas container (24 x 30 x 18 cm) with the bottom lined with bedding material (beta chips about 1 cm deep). The lid of the home cage was made of a removable, stainless steel grill with a triangular indentation (about 10 cm deep) to hold rat chow pellets and the water bottle. The novel environment (test cage) consisted of a clear Plexiglas box with dimensions different from those of the home cage (i.e. 48 x 30 x 40 cm). The front of the box comprised a double-hinged door. The floor consisted of an elevated (3 cm from the Plexiglas bottom) stainless steel grid. Both the home and the test cages were located in the same room, where the testing took place. To achieve *in vivo* microdialysis in both the home cage and test cage, an adaptation of the removable stainless steel lid was made to incorporate a removable sliding microdialysis assembly consisting of a liquid swivel tethered to the animal using a flexible spring (~12 cm in length). This approach allowed

us to remove the animal from the housing cage (with swivel connected) to a novel cage that incorporated the microdialysis set up. Thus, *in vivo* sampling was possible during the home cage and upon transferring the animal to a novel cage (see Figure 1).

All surgeries were carried out in rats anaesthetised with pentobarbital (65 mg/kg: i.p.). Animals were placed in a stereotaxic instrument with level skull. For the microdialysis studies, rats were implanted with a 22 gauge guide cannula containing a removable 24 gauge obturator aimed at the dorsal aspect of the CeA using the following coordinates: A/P -2.1 mm, L/M $\pm$ 4.2 mm, and D/V -7.35 mm. For the ICV studies, guide cannulae (22 gauge) with removable 24 gauge obturator was implanted into the 3rd ventricle using the following coordinates: A/P -4.3 mm, midline, and D/V -4.3 mm. All co-ordinates were derived from the rat brain stereotaxic atlas [342]. All guide cannulae were anchored to the skull using 3 stainless steel screws and acrylic cement.

In vivo microdialysis

In all microdialysis experiments probes were implanted at least 120-min prior to baseline collection, a time frame determined by previous experience to be more than adequate to attain stable samples (see Chapter I). Rats were briefly anesthetized with halothane (~ 3-min), and the obturator within the guide cannula was replaced with a microdialysis probe, which extended 2.5 mm (includes 0.5 mm glue tip) beyond the end of the cannula. The concentric microdialysis probe had 2.0 mm of active membrane (250 μ m outer diameter) of regenerated cellulose (6000 MW cut-off: Spectrum Medical

Industries) that protruded into the CeA. Each probe was secured with a retaining screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel

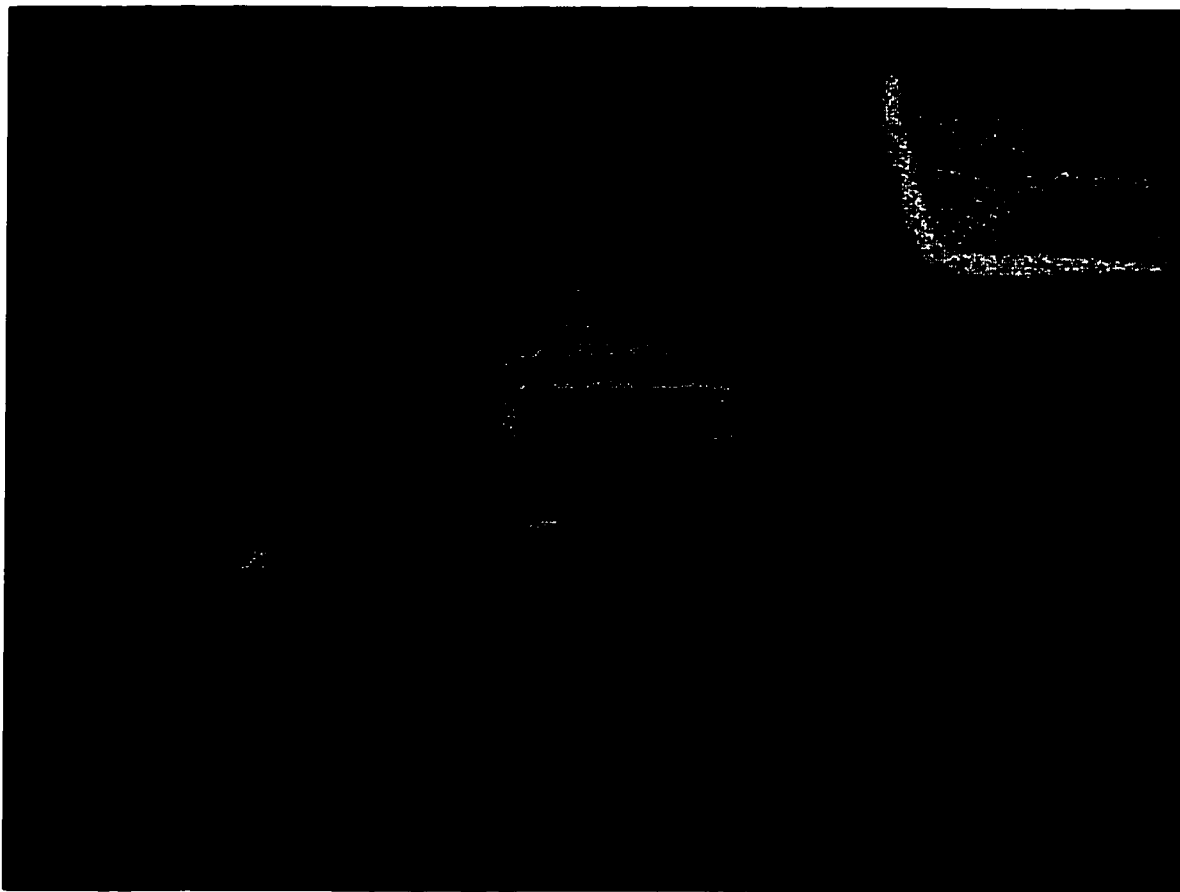


Figure 1. Photographic representation of the novel-cage paradigm. *In vivo* microdialysis is used to assess the release of neurochemicals in rats housed in typical "shoe-box" style cages. Rats are connected to a custom designed removable swivel which slides freely on two stainless steel rods fitted to the cage top. When removed, the rat can be transported with the swivel to a novel cage without any interruption in sampling.

and a 2.5 ml gas-tight infusion syringe attached to a pump (Harvard. model 22).

Microdialysis probes were perfused at 3 μ l/min with filtered Kreb's-Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K⁺, 145 Na⁺, 1.35 Ca²⁺, 1.0 Mg²⁺, 150 Cl⁻, 0.05% ascorbate, pH 7.4 [386], and BSA (0.1%). On collection, each sample (90 μ l) was immediately frozen on dry ice and stored at -80°C until radioimmunoassay (RIA) analyses. The efficiency of the microdialysis probes was assessed *in vitro* (details described in Chapter I) with the average peptide recovery for CRH being $3.3 \pm 0.6\%$.

Drugs and peptides

Diazepam (Sabex, Boucherville, QC) was dissolved in a vehicle of 50% propylene glycol, 40% distilled H₂O and 10% ethanol and injected subcutaneously (s.c., 0.1 ml/100 g body weight). PreproTRH₁₇₈₋₁₉₉, CRH, CRH-type2 receptor antagonist, antisauvagine-30 (antiSvlg-30) (Phoenix Pharmaceutical, Belmont, CA) were dissolved in saline, whereas α -helical CRF₉₋₄₁ (Phoenix Pharmaceutical, Belmont, CA) was dissolved in saline containing 0.05% acetic acid. The CRH-type1 non-peptide receptor antagonist, CP-154, 526, was kindly donated by Pfizer Inc. (Gorton, Conn., USA), and was dissolved in vehicle of saline containing 5% ethanol and 5% Tween 80.

Radioimmunoassay and Capillary Electrophoresis

The detection and quantification of ir-CRH was achieved using a highly sensitive solid-phase RIA [297]. Briefly, Protein A/G (Calbiochem, La Jolla, CA)-coated Immunilon-4 wells (Dynatech, Chantilly, VA) were incubated with anti-CRH serum (rC70 kindly provided by W.Vale, the Salk Institute, La Jolla, CA) for 2-hr at room

temperature. Samples, standards (reconstituted in the KRB solution, ranging from 0.61 to 1250 fmol/well), or blanks were incubated for 24-hr at 4°C. Next, 25 µl of assay buffer containing 4500-5000 CPM of [¹²⁵I-Tyr⁰] rCRH (Amersham, Oakville, ON) was added to each well and incubated for an additional 24-hr period at 4°C. Finally, the wells were rinsed, separated, and their residual radioactivity counted using 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 fmol/well.

The interstitial levels of glutamate, and GABA were also measured at the CeA. All *ex-vivo* analyses were performed on the Beckman P/ACE 5010 capillary electrophoresis system (Fullerton, CA), equipped with a laser-induced fluorescence (LIF) detector (488 nm Beckman P/ACE System Laser Module). Separations of analytes were achieved using fused silica capillary (Polymicro Technologies Inc., Phoenix, AZ) with 50 µm i.d. and 47 cm length. The run buffer consisted of a 100 mM boric acid adjusted with NaOH to the final pH of 9.5. Between runs, there were 30-sec and 90-sec rinses of 1 M NaOH and run buffer, respectively. The 2-sec pressure injection sample plugs were assayed at a separation voltage of 30 kV.

The amino acids GABA and glutamate were assayed in 5 µl aliquots from each sample reacted with 1.3 µl of 1.0 mM fluorescein-5-isothiocyanate (FITC) (Molecular Probes Inc., Eugene, OR) in acetone. The reaction was allowed to proceed for 16- to 18-hr before dilution in 100 µl of run buffer. Each sample was analyzed in triplicate, and peak heights were averaged and quantified from the working curves of respective amino

acid standards (Sigma, St. Louis, MO). All samples from an individual subject were assayed during the same day. Unless otherwise stated, chemicals were purchased from VWR (Ville Mont-Royal, QC).

Histology

At the end of the microdialysis and ICV experiments, animals were briefly anesthetized with halothane and then sacrificed so that their brains could be extracted, sectioned, and stained for histological verification of probe placement. The correct placement of the 3rd ventricular guide cannulae was readily observable without staining under low magnification. Only data from correctly placed probes were used for statistical analyses.

Experiment 1. Effect of an unfamiliar environment on snack intake

While in their home cages, *ad libitum* fed animals ($n = 6$) were given pre-weighed quantities (approximately 7-10 g) of graham crumbs (70.6% carbohydrate, 10.6% fat, 7.1% protein) in a round dish (10 cm diameter), once daily for a 15-min period. After 6 successive days, the snack intake reached a plateau such that the intake over the last 3 days did not vary by more than ± 0.5 g (denoted baseline). On the 7th day, rats were placed in the novel cage, and presented with the now familiar palatable graham crumbs. The intake over 15-min period was determined, after which the rats were returned to their home cages. To ascertain that the snack maintained its palatability under stress-free conditions, following 6 consecutive days of snack presentation in the novel cage, the rats

were again presented with that snack in their home cage (once daily for 15-min) for 2 days.

Experiment 2. Effect of diazepam pretreatment on novelty-induced suppression of snack intake

Subjects ($n = 6$) were injected with various doses of diazepam (0 (vehicle), 0.5, 1.0, 2.0 or 3.0 mg/kg: s.c., in a randomised order), and placed in either the home cage or the novel cage. The snack was presented 20-min later. As in Experiment 1, the food was removed after 15-min and the total amount consumed was noted. The home cage represented a familiar or non-stressful environment. If diazepam increased food intake due to non-anxiolytic or non-specific (e.g. enhanced palatability or orexigenic actions) effects, then it was expected that this would be reflected by altered snack consumption in the home cage as well as in a novel environment. It ought to be underscored that we have found that repeated tests are possible using this procedure, so long as the “unfamiliar” environment contains novel bedding or no bedding. Thus, it is possible to evaluate the same rats repeatedly for at least 6 consecutive days, using different compounds or different doses of a single compound [314].

Although both control and diazepam-pretreated rats consumed a similar amount of snack in the home cage during the 15-min test interval, the possibility that diazepam may have increased consumption further during a longer test interval was assessed in a separate group of animals. In this case, the effects of diazepam (2.0 mg/kg: s.c. a dose

deemed most effective from the earlier experiment) were reassessed in a separate group of rats ($n = 8$) given access to the snack in their home cage for 25- and 40-min.

Experiment 3a. Effect of CRH receptor antagonists on novelty-induced suppression of snack intake

In rats that had habituated to the snack in the home cage (see procedure above) the effects of pre-treatment with various CRH antagonists on snack consumption in the home and novel cage was assessed. Rats ($n = 6-8$ per group) were injected ICV with the non-selective CRH receptor antagonist, α -helical CRH (20 & 50 μg). In a separate group of animals ($n = 6-8$), CP-154, 526 (10 or 40 mg/kg, i.p.) and antiSv β -30 (2 or 10 μg , ICV) were injected in animals 20-min before being transferred to the novel cage.

Experiment 3b. Effect of CRH and BN pretreatment on snack consumption in the home cage

In this experiment we assessed the effects of CRH and BN pretreatment on snack consumption in the home cage to determine whether these compounds would elicit an anxiety-like effect similar to that observed in the novel cage. To assess whether CRH induced an anxiety-like state, the effects of this peptide (2 μg /rat; ICV) were assessed under the home cage condition at 30- or 90-min following its administration. Finally, the effects of BN (0.5 μg ; ICV) were tested under the home cage condition, 30-min following treatment.

For Experiments 3a&b, rats were removed from their home cages and peptides were injected in a volume of 3 μ l of saline (or 0.05% acetic acid-saline solution in the case of α -helical CRH), delivered over 1-min interval, using a 1.0 ml Hamilton syringe controlled an infusion pump (Harvard Apparatus, MA). The Hamilton syringe was connected via PE 20 tubing to a stainless steel injector that protruded 0.5-0.75 mm below the cannula tip, into the 3rd ventricle. Following injections, subjects were returned to their respective home cages for 20-min (and 90-min for CRH). They were then presented with the graham crumbs either in their home-cage or immediately after being transferred to the novel cage. The snack was presented for 20-min, as described in the preceding experiments.

Experiment 4. Effects of novel cage exposure on interstitial levels of CRH, GABA and glutamate at the CeA

Rats (n = 8-10/ group) equipped with permanent guide cannulae aimed at the dorsal aspect of the CeA, were briefly anaesthetised with halothane (~ 3-min) and the obturator within the guide cannula was replaced with a microdialysis probe. Rats were then placed in their home cage where sample collection began 90-min later. Following this stabilization period, 5 baseline samples (60 μ l each) were collected every 20-min. After the 4th baseline sample was collected, rats were injected with either vehicle or diazepam (2.0 mg/kg: s.c.) and one more sample was collected in the home cage. Then rats were gently transferred into the novel cage where microdialysis samples continued to be collected for 5 additional samples. Upon collection, all dialysates were frozen

immediately on dry ice and stored at -80°C immediately until neurochemical analyses (~ 2-3 weeks later).

Statistical analyses

The amount of food consumed was analyzed by either one- or two-factor within-group analyses of variance (ANOVA). In cases where each animal was repeatedly tested within the home-cage or the novel cage condition, or each animal was tested under the vehicle or drug condition, these variables (i.e. cage and drug condition) were considered to be within-group factors. When these variables were assessed in separate groups of animals they were treated as between-group factors. The concentration of the baseline levels of ir-CRH, GABA and glutamate observed were averaged for individual rats and defined as 100%. All values were then expressed as a percent of that baseline, and analyzed by a mixed measures ANOVA with Drug treatment (vehicle vs. diazepam) as a between-groups factor and Cage (Home vs. Novel), and Sample (5 home cage and 5 novel cage) serving as the within-group factors. Post-hoc analyses of significant main effects or simple effects of significant interactions were conducted using Newman-Keuls multiple comparisons, or Bonferonni adjusted t tests in the case of the *in vivo* microdialysis data.

Results

Experiment 1. The effect of a novel environment on snack intake

The amount of snack consumed in the 15-min test session varied significantly across days, $F_{13, 52} = 17.93$, $p < 0.01$. Subsequent post-hoc analyses indicated that the

consumption of the graham crumbs in the home cage was initially low but increased steadily and stabilized over days 4-6. When the rats were then presented with this now familiar palatable snack within the novel cage, consumption dropped precipitously (Day 7) relative to that seen on the final baseline day (Day 6). This suppression was evident on each of the 6 consecutive test days in the novel cage. When the rats were once again presented with the snack in their home cage, (Days 13 and 14), the intake increased sharply to levels comparable to those of the original baseline (Day 6) (see Figure 2).

Experiment 2. Effect of diazepam pretreatment on novelty-induced suppression of snack intake

The ANOVA revealed a significant main effect of Cage and Diazepam dose pretreatment on snack intake ($F_{1,4} = 22.66$; and $F_{4,16} = 5.78$, $p's < 0.01$, respectively). Likewise, snack intake was influenced in a Cage x Diazepam dose interaction, $F_{4,16} = 5.38$, $p < 0.01$. The analyses of the simple effects comprising the interaction revealed that under the novel cage condition, diazepam dose-dependently increased snack consumption, with the drug effects reaching significance at the higher doses (2 and 3 mg/kg; s.c.). In the home cage condition, only a slight increase of snack consumption was noted and in contrast to the novel cage condition, it occurred at the lower (0.5 and 1 mg/kg; s.c.) but not the higher (2 and 3 mg/kg; s.c.) doses (see Figure 3).

The modest effect of diazepam (2 mg/kg; s.c.) in the home cage was not attributable to the lack of adequate time to ingest differential amounts of the snack. Specifically, in separate groups of rats ($n = 6-8$ /group) that had been provided with the

snack in their home cage for 15-, 25- or 40-min consumption (as expected) varied with Time ($F_{2, 16} = 45.05$, $p < 0.0001$). However, no effect of Drug treatment or interaction between Drug treatment and Time was observed (data not shown).

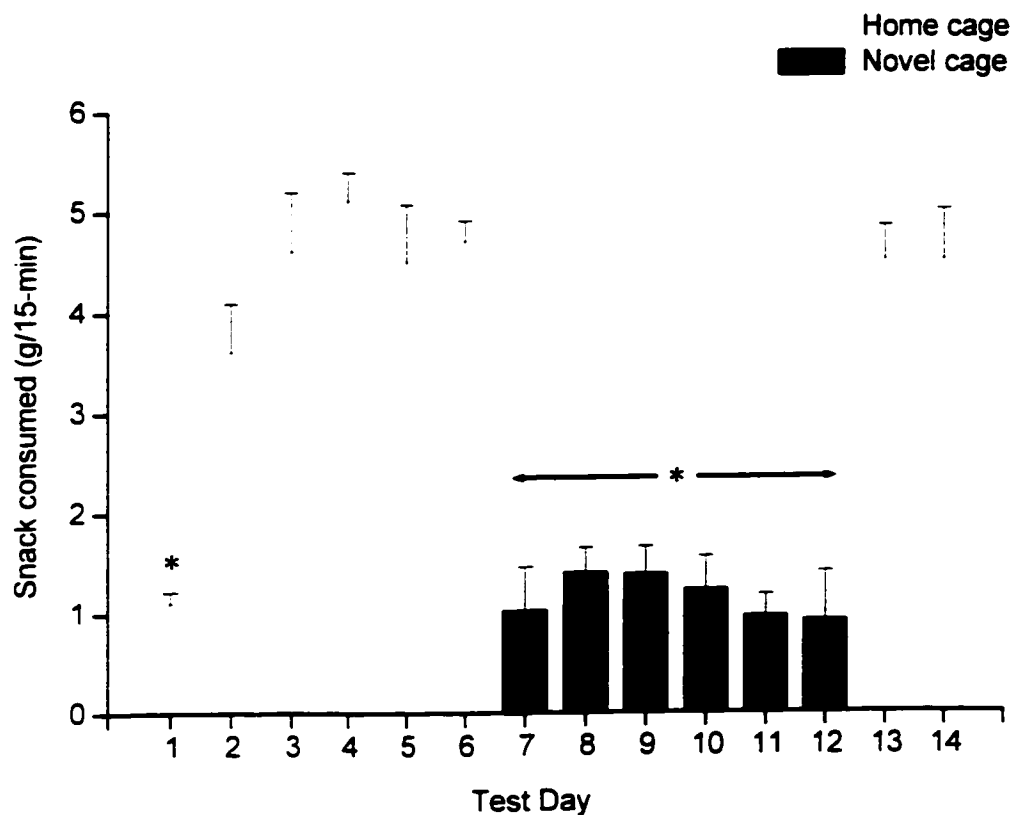


Figure 2. Rats were presented with a palatable snack (graham crumbs) over a period of 6 days in their home cage and the total amount consumed was noted. Snack intake rapidly increased with repeated exposure so that intake was stable over days 4-6. Rats were then presented with the snack for 15-min immediately after being transferred to a novel environment and the amount consumed was noted. Following 6 test sessions in the novel cage (days 7-12), rats were presented again with the snack in their home cage for 15-min on two successive days (13 & 14) and the amount consumed was noted.
 * Significantly different from the average amount consumed on Day 6 in home cage, $p < 0.0001$.

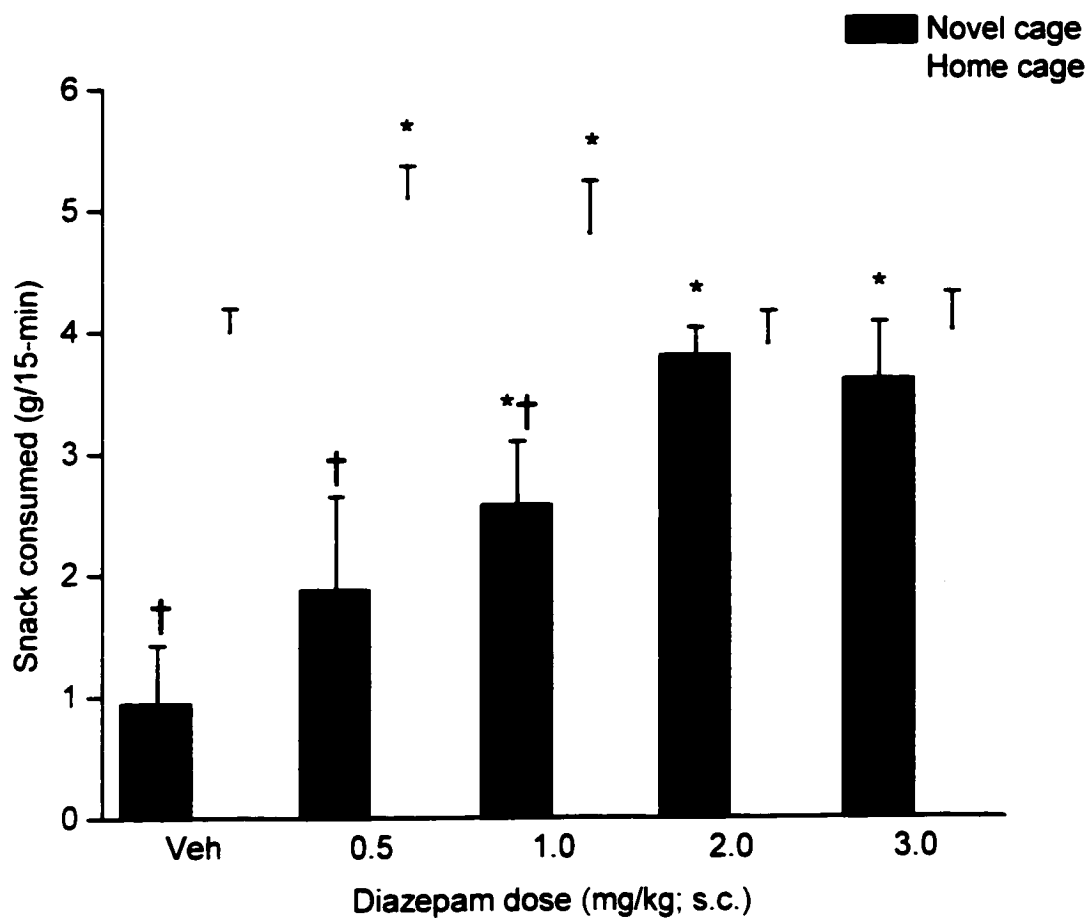


Figure 3. Effects of diazepam pretreatment on total snack (graham crumbs) intake over a 15-min test period. Rats were injected subcutaneously with various doses of diazepam 20-min prior to being presented with graham crumbs in either the home (dark bars) or novel (shaded bars) cage. * Significantly different from vehicle injection, $p < 0.01$. † Significantly different from home cage condition, $p < 0.01$.

Experiment 3a. Effect of CRH receptor antagonists on novelty-induced suppression of snack intake

The analysis of the effects of α -helical CRH at both 20 & 50 μ g (ICV) indicated that the treatment did not affect snack consumption. $F_{1,7} = 0.440$, and $F_{1,6} = 0.037$, p 's > 0.05, respectively. Likewise, snack intake observed in rats pretreated with the CRH release inhibiting factor (prepro-TRH₁₇₈₋₁₉₉ 6 μ g; ICV), was not different from control conditions in either the home or novel cage $F_{2,10} = 1.28$, $p = 0.32$. In rats pretreated with CP-154,526 (10 or 40 mg/kg; i.p.) the novel cage reduced snack intake similar to vehicle injected rats so that a main effect of Cage remained $F_{4,18} = 20.61$, $p < 0.001$, with no effect of Drug treatment (see Table 1). In rats injected with (antiSv α g-30: 2 and 10 μ g; ICV) the novel cage still suppressed the consumption of a palatable snack so that a main effect of Cage remained $F_{4,9} = 8.55$, $p < 0.001$ (see Table 2).

Experiment 3b. Effect of CRH and BN pretreatment on snack consumption in the home cage

Finally, the effects of CRH (2 μ g; ICV) and BN (0.5 μ g; ICV) were tested under the home cage condition at 30-min or 90-min following microinjection. CRH failed to alter the latency to approach the snack, or reduce the amount of snack consumed at either time point, while BN reduced the amount of snack consumed ($F_{1,7} = 15.8$, $p < 0.005$) and increased the latency to initiate consumption, $F_{1,7} = 6.98$, $p < 0.03$ (see Table 2).

Table 1. Effects of CP-154526 (CRH-type1 receptor antagonist) on novel-environment induced suppression of snack consumption.

Treatment	Test Condition	Amount consumed (g)	
		Control	Treated
Vehicle	Home cage	7.14 $\pm$ 0.66	6.53 $\pm$ 0.79
Vehicle	Novel cage	1.63 $\pm$ 0.58	2.12 $\pm$ 0.59
40 mg		3.02 $\pm$ 0.59	1.35 $\pm$ 0.28
10 mg		2.33 $\pm$ 0.48	2.43 $\pm$ 0.55

Each value represents the group mean $\pm$ SEM. CP-154626 was administered systemically (i.p.) 20-min prior to placement in the novel environment and snack presentation.

Table 2. Effects of anti Sauvagine-30 (CRH-type2 receptor antagonist) and CRH on snack consumption in a novel cage and home cage, respectively.

Treatment (ICV)	Test Condition	Amount consumed (g)	
		Baseline	Treated
AntiSav-30 (10 μ g)	Novel cage	1.30 $\pm$ 0.23	0.94 $\pm$ 0.03
AntiSav-30 (2 μ g)		1.44 $\pm$ 0.25	1.18 $\pm$ 0.24
CRH (2 μ g) 30-min	Home cage	2.38 $\pm$ 0.28	2.92 $\pm$ 0.38
CRH (2 μ g) 90-min		2.22 $\pm$ 0.20	2.62 $\pm$ 0.38

Each value represents the group mean $\pm$ S.E.M. Anti sauvagine-30 (AntiSv-30 2 or 10 μ g/rat; ICV) was administered 20-min prior placement in the novel environment and snack presentation. CRH (2 μ g/rat; ICV) was administered either 30- or 90-min before presentation of the snack in the home cage.

Experiment 4. Effects of novel cage exposure on interstitial levels of CRH, GABA and glutamate at the CeA

The ANOVA of the interstitial ir-CRH levels as a percentage of baseline revealed a significant effect of Cage x Sample interaction ($F_{4,44} = 4.45$, $p < 0.01$). Multiple comparisons of the simple effects for the interaction revealed that ir-CRH levels remained relatively stable within the home cage but increased significantly in the novel environment in both diazepam- and vehicle-injected rats. As seen in Figure 4 and confirmed by the multiple comparisons, interstitial ir-CRH levels increased progressively over the course of the collection period in both the vehicle- and diazepam-pretreated animals (see Figure 4).

The analysis of the extracellular glutamate concentrations revealed a significant effect of Sample ($F_{4,44} = 4.27$, $p < 0.01$), marked by a progressive rise of glutamate that was most notable following transfer to the novel cage. The effects of diazepam on glutamate concentrations were variable and non-significant. In fact, if anything, the levels of glutamate were slightly higher in the diazepam than vehicle treated rats. Finally, the fluctuations in observed GABA levels were similar to those of glutamate. Thus, relative to the home cage, the release of GABA increased following placement in the novel cage to a similar extent in both vehicle- and diazepam-pretreated rats, $F_{1,11} = 18.78$, $p < 0.001$ (data not shown).

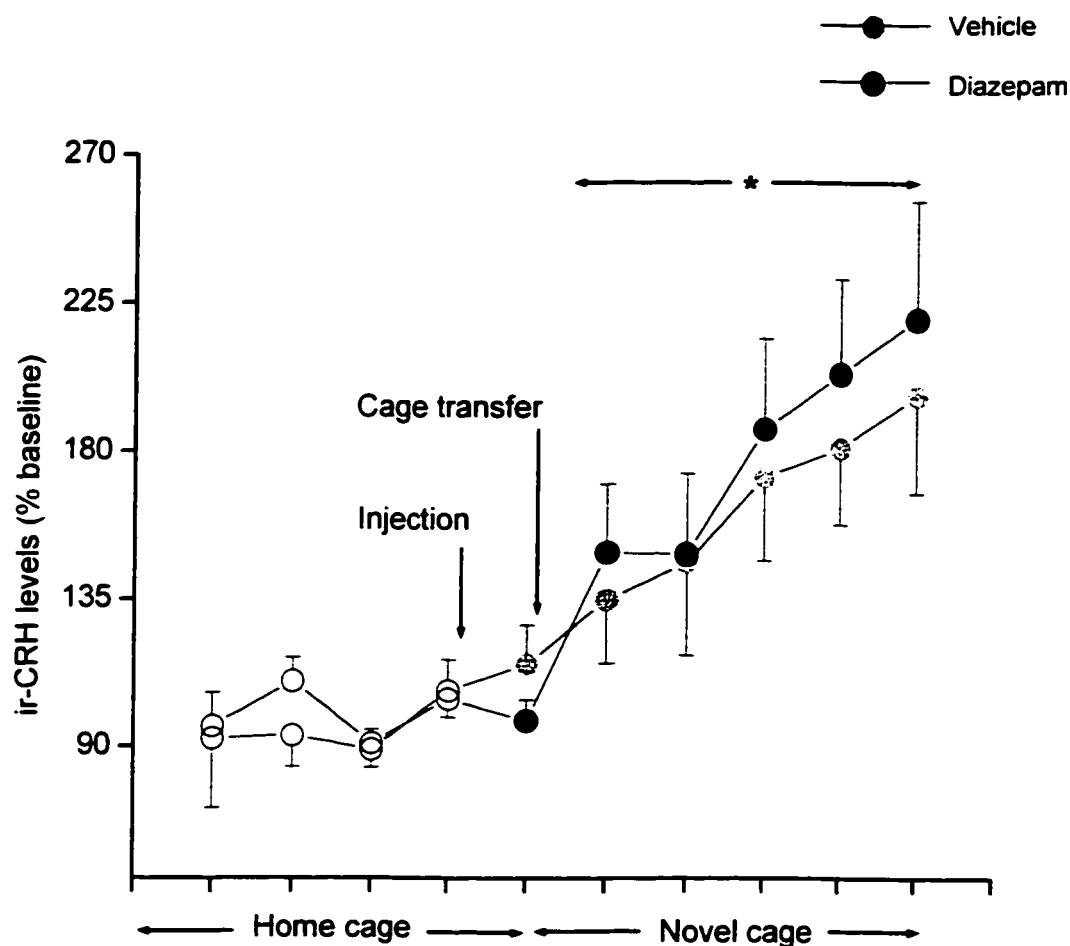


Figure 4. *Effect of novel vs home cage environment on the in vivo release of corticotropin-releasing hormone (CRH) at the CeA.* The ordinate represents CRH levels, expressed as a percentage of baseline. Dialysate samples were collected every 20-min. in the home cage and novel cage. Following collection of the fourth sample in the home cage, rats ($n = 8-10$ per group) were injected with either vehicle (hatched columns) or diazepam (2 mg/kg) (solid columns). In the home cage, interstitial CRH levels remained stable (first 5 samples). In the novel cage, levels of interstitial CRH increased progressively in both vehicle- and diazepam-treated rats over the course of 5 samples. Treatment with diazepam did not affect the novel environment-induced release of CRH. * Significantly different from home cage at $p < 0.01$.

Discussion

When placed in an unfamiliar environment, rats were reluctant to approach and consume a palatable snack, but this effect was attenuated by benzodiazepine pre-treatment. As consumption necessarily involved the initiation of an active response, this outcome could not be attributed to motor side effects or lethargy elicited by the anxiolytic. Using this paradigm, we previously observed the behavior to be modifiable by other known anxiolytic agents, whereas treatment with psychotropic agents without anxiolytic properties (e.g. acute antidepressant, neuroleptic or caffeine treatment) did not affect performance [314]. Furthermore, although benzodiazepines may have orexigenic effects, this did not account for the observed outcome, as the effects of diazepam were restricted to the novel environment. Thus, it seems that snack consumption in an unfamiliar environment is a sensitive and reliable index of anxiety, and because of its simplicity, this paradigm permits the concurrent assessment of dynamic neurotransmitter changes under conditions of high ethological validity.

The amygdaloid complex, and the CeA in particular, may be fundamental in the mediation and/or coordination of the stress response. Manipulation of this structure (i.e. lesions or stimulation) influenced the emotional and HPA response to psychological stressors in a predictable fashion [10;24;129;185;430]. Commensurate with the finding that stressors increase CRH mRNA and CRH levels [129], and the release of ir-CRH within the amygdala [351;370], the present investigation demonstrated that the anxiety associated with a novel environment was accompanied by a marked increase of ir-CRH release at the CeA.

While anxiety associated with an unfamiliar environment was attenuated by diazepam, the elevated *ir*-CRH release persisted. Furthermore, administration of CRH (2 µg; ICV) failed to suppress home cage snack consumption, and pre-treatment with various CRH antagonists failed to attenuate the anxiety associated with an unfamiliar environment. This was the case in response to α -helical CRH, prepro-TRH (inhibitor of CRH release), as well as the more selective CRH-type1 (CP-154, 526) or CRH-type2 (antiSv_g-30) receptor antagonists. Evidently, while stressful events induce amygdaloid CRH changes, CRH availability does not appear to be necessary for the expression of anxiety-like responses engendered in the unfamiliar environment. As amygdaloid *ir*-CRH release not only occurs in response to a stressor, but also in response to appetitive stimuli (see Chapter 1), CRH changes may represent a response to biologically salient stimuli, irrespective of their valence.

The effects of CRH antagonists in other anxiety models involving exploration/novelty have been inconsistent. For instance, while CRH antagonists have been reported to attenuate anxiety-like responses in the elevated plus-maze [182;415], other investigators have failed to detect such an effect [25;44;166]. In contrast, under conditions that are more threatening or painful than placement in an unfamiliar environment, including conditioned fear [189], shock-induced freezing [437], conflict-models [235], conditioned defeat, or defensive withdrawal [208], CRH antagonists were more effective. As well, in instances where CRH antagonists attenuated anxiety in exploratory situations this occurred under conditions where animals had previously been exposed to a stressor [182], or had received central administration of CRH [415]. Thus, it

may be that with increased stressor intensity, or in highly anxious organisms, CRH systems become more relevant in the behavioral manifestation of anxiety, and hence CRH antagonists are more effective.

If amygdaloid CRH release is increased in response to the naturalistic stressor of being placed in an unfamiliar environment, but does not mediate anxiety, the question emerges as to what its functional role might be? There are, of course, several possibilities in this respect. For instance, the elevated amygdala CRH release may reflect arousal, rather than fear or anxiety [181:272]. It is possible, as indicated earlier, that amygdala CRH may be released in response to salient or emotionally significant stimuli, regardless of whether they are positive or negative. In effect, amygdala neurons mediate processes by which sensory stimuli gain emotional and motivational significance [10:248]. Thus, it is possible that emotional changes (hyperactivity or vigilance), independent of anxiety *per se*, are associated with being transferred to a novel environment and these changes contribute to the *ir*-CRH release observed in this study.

A second possibility, which is not independent of an arousal hypothesis, is that CRH is fundamental in priming or preparing the organism for action. Part of the effect of increasing CRH activity, particularly at the level of the pituitary, is to increase release of adrenal glucocorticoids. A move to an unfamiliar environment may engage preparatory responses owing to the potential threat imposed. However, such a release would not necessarily occur only under stressful conditions, as positive changes to homeostasis, such as rewarding [350] or appetitive stimuli [297] also stimulate glucocorticoid secretion

[350]. Incidentally, such a multifaceted response is not unique to HPA functioning, as catecholaminergic activity at the prefrontal cortex or nucleus accumbens is also affected by both stressful and rewarding stimuli [438:477].

It is unclear to what degree the effects of CRH released in response to stressors involve CRF-type1 or CRF-type2 receptors. Both receptor subtypes are present in stress-relevant regions such as the amygdala, PVN, and ventromedial hypothalamus [133]. Inasmuch as CRF-type1 deficient mice exhibit diminished anxiety [413:446] and CRH antagonists reduce anxiety associated with stressors [119:172], particularly in highly anxious rats [221], it was suggested that CRF-type1 receptors contribute to stressor-related anxiety [417]. The role of CRF-type2 receptors is less clear, as stressor exposure or corticosterone administration may not increase CRF-type2 receptor mRNA expression [273:367]. Yet, in mice lacking the CRF-type2 receptor, the stressor-elicited HPA response terminated early, suggesting that this receptor subtype may be involved in regulation of the HPA response [90]. Furthermore, mutant mice deficient in CRF-type2 receptors are hypersensitive to stressors and display increased anxiety-like behavior [26]. This may be attributable to the reported increase of CRH mRNA at the CeA or increased mRNA for urocortin in these mice.

It has been suggested that while CRF-type1 receptors are key to mediating the anxiety introduced by stressors, CRF-type2 may be involved in other stressor-related behaviors, including the suppression of feeding elicited by CRH-like peptides [235]. Like CRH, urocortin is also released in response to stressors, has a higher affinity for the

both CRH receptors, and has potent effects on food consumption. Thus, the possibility exists that when urocortin is released preferentially to CRH, the anxiogenic actions of the stressor will be more notable with respect to behaviors such as feeding. The CRF-type1 receptors, which may contribute to processes related to executive functions (appraisal of emotions, attention, learning and memory processes related to emotion) [417], may be more apparent in response to relatively intense stressors which have more pronounced effects on CRH relative to urocortin, and may bring into play interactions between CRH and norepinephrine activity in other brain regions [235]. This notion is of course, highly speculative as data are not available regarding the differential release rates of urocortin and CRH in response to stressors of graded severity.

The role of glutamate is of particular interest as activation of glutamatergic projections to the amygdala facilitated CRH release at the median eminence and provoked the activation of the HPA axis [149]. Consistent with an NMDA receptor-driven event, glutamate-stimulated CRH release was blocked by an NMDA antagonist[92], implicating glutamatergic mechanism(s) in regulating amygdaloid CRH release. The present investigation confirmed that along with *ir*-CRH release, interstitial glutamate concentrations at the CeA were increased when rats were transferred to the unfamiliar cage. However, as with *ir*-CRH, the glutamate elevation was not attenuated by diazepam pretreatment. Thus, it seems that glutamate changes at the amygdala do not underlie the anxiogenic effects of the stressor, although may play some other role with respect to the overall stress response.

The contribution of CRH in mediating the anxiogenic effects of stressors is of considerable interest, particularly as it has figured in the development of therapeutic strategies for anxiety-related disorders, as well as those where anxiety is a poignant comorbid feature (e.g., depression). The results of the present investigation indicate that psychogenic stressors increase the *in vivo* release of ir-CRH, but the precise role of this peptide in sub-serving anxiety requires reconsideration.

Alternatively, psychogenic stressors of different categories (natural or unlearned vs. conditioned responses elicited by cues paired with neurogenic stressors) may evoke anxiety using distinct neural substrates (CRH-dependent vs. CRH-independent). Alternatively, it is possible that different stressors activate distinct neural circuits, which may differentially impact on anxiety. In particular, the presently available data are consistent with the view that while neurogenic stressors (or psychogenic stressors previously paired with neurogenic stressors) elicit a CRH mediated anxiety which is reversible by CRH antagonists. In contrast, unlearned (naturalistic) stressors activate pre-wired neural circuits, including CRH and as yet undetermined substrates. While CRH may potentially reflect the presence of anxiety, it does not mediate the response to these naturalistic stressors.

Preface to Chapter V

Although CRH has been firmly established as the primary ACTH secretagogue released in response to stressors, there is accumulating evidence supporting the notion that CRH may also act as a neurotransmitter within brain circuits [457-459]. In particular, several indirect approaches have suggested that CRH may be released onto dendrites that form synaptic connections at the locus coeruleus [457] to provoke autonomic activation during stressor exposure. However, to date, the release of CRH at the peri-locus coeruleus region has never been explicitly demonstrated. Anterograde and retrograde track tracing experiments have suggested the source (soma) of the CRH terminals at the locus coeruleus might be located at the CeA (precisely the lateral CeA) [455;458;459]. Valentino and colleagues have elegantly demonstrated that axons projecting from the lateral CeA form primarily excitatory mono-synaptic connections on dendrites emanating from locus coeruleus neurons [459;460].

The objective of the next experiment was to attempt to directly assess whether this functional connection between the amygdala and the locus coeruleus was chemically bridged by CRH. Specifically, we attempted to 1) demonstrate unequivocally that CRH is released at the locus coeruleus during stressor exposure, and 2) demonstrate that the release of CRH at the locus coeruleus is affected by direct electrical stimulation of the CeA neurons.

CHAPTER V

Stressor exposure elicits the release of corticotropin-releasing hormone at the periolocus coeruleus: Possible activation through neurons arising from the central nucleus of the amygdala

Abstract

The locus coeruleus represents the largest source of norepinephrine in the brain and plays a major role in orchestrating the sympathetic arm of the stress response. Several forebrain structures that have been studied in the context of the stress response have been shown to have both direct and indirect anatomical connections to and from the locus coeruleus. These structures include the paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BNST), medial prefrontal cortex (mPFC), hippocampus and the amygdala. It is therefore likely that these structures, together with brain stem regions like the locus coeruleus function in concert during the stress response. Of late, the neuroanatomical connections between the central nucleus of the amygdala (CeA) and the peri-locus coeruleus have been delineated using tract-tracing techniques combined with electron microscopy. These findings, together with those showing that corticotropin-releasing hormone (CRH) receptors are situated on dendrites originating from the peri-locus coeruleus and the observations that CRH manipulations at the peri-locus coeruleus influence the stress response suggest 1) that endogenous CRH may be released in the vicinity of the locus coeruleus during stressor exposure and 2) the cell bodies producing this release might reside at the CeA. Both of these contentions were assessed using *in vivo* microdialysis in freely behaving animals in the first experiment and the combination of electrical stimulation and *in vivo* push-pull perfusion in a second experiment.

The results of the first experiment demonstrate for the first time that stressor exposure elicits a rapid and sustained release of CRH at the peri-locus coeruleus in freely behaving animals. The second experiment sought to investigate if the neuroanatomical

connections between the CeA and the peri-locus coeruleus were chemically bridged by CRH. We applied a range of electrical stimulation to the lateral aspect of the CeA while collecting the concurrent release of CRH at the ipsilateral peri-locus coeruleus in sodium pentobarbital-anesthetized animals. Unexpectedly, this experiment failed to demonstrate a clear association between the release of CRH at the peri-locus coeruleus and activation of the CeA. Although we observed a clear stimulation-dependent change in the release of CRH in some animals this response was not observed consistently across all subjects. It is suggested that slight variability in stimulation and collection sites, in addition to different levels of anesthesia among the animals might have contributed to the large variability observed in this experiment. Presently, we are able to confirm that endogenous CRH is released at the peri-locus coeruleus during stressor exposure. however we are uncertain as to the source of this release.

Introduction

The prototypical stress response includes a simultaneous activation of the hypothalamic-pituitary adrenal (HPA) axis and sympathetic nervous system [321:452]. These responses serve to mobilize the energy resources and facilitate the execution of the coping responses (e.g. fight or flight response) [15]. That stressors- elicit activation of the PVN CRH neurons and the consequent release of ACTH from the anterior pituitary is firmly accepted [50:202]. In addition, stressors activate behavioral systems (including cognitive vigilance) and evoke an activation of the sympathetic nervous system [299:444]. In this regard, the locus coeruleus (which is the major source of norepinephrine in the brain) [143] is considered a primary brain site in modulating both an animal's heightened mental alertness (i.e. vigilance), and increased sympathetic outflow during stressor exposure [143:442].

In addition to the role of CRH in the endocrine arm of the stress response, several lines of evidence sustain the notion that CRH also mediates the sympathetic effector system of the stress response [457:458]. In this capacity, centrally injected CRH has been shown to elicit a dose-dependent activation of the sympathetic nervous system [63:411:483]. Of note, however, the effects of CRH are 200 times more potent when it is directly infused into the locus coeruleus [99] (as compared to ICV administration), where it activates discharge of locus coeruleus neurons and evokes the release of norepinephrine at the hippocampus and frontal cortex [99]. Several stressors, including hypotension, restraint, noise, air puff, and predator odor have been shown to increase the neuronal excitability and release of neurochemicals at the locus coeruleus [100:410]. Many of

these effects are likely mediated through an activation of CRH-type1 receptors, which have been localized at the locus coeruleus and/or on dendrites emanating from the locus coeruleus [458:459]. since CRH receptor antagonists injected into the locus coeruleus and/or peri-locus coeruleus region attenuate or block these effects [411:459]. Indeed, Van Bockstaele et al. (1998) have reported that immunoreactive (ir)-CRH neurons primarily form the excitatory synapses on dendrites arising from the rostral lateral locus coeruleus [459].

Anterograde and retrograde neuronal tract tracers injected into the CeA and the rostral lateral peri-locus coeruleus, respectively, combined with electron microscopic examination has revealed that a large proportion of the CRH axons emanating from the CeA form synaptic connections at the rostral lateral peri-locus coeruleus (where ir-CRH fibers are dense) [458-460]. These studies, taken together with those discussed above, provide a neuroanatomical and physiological basis through which CRH at the CeA might influence the brain's noradrenergic system during stressor exposure.

The objective of the current study was to use *in vivo* microdialysis to directly assess the release of ir-CRH at the peri-locus coeruleus in freely behaving animals before, during and following exposure to a processive stressor. In a subsequent experiment we attempted to determine whether electrical stimulation of the CeA resulted in the release of ir-CRH at the peri-locus coeruleus.

Materials and methods

Experiment 1: Effect of stressor exposure on the release of ir-CRH at the peri-locus coeruleus

Animals and procedure

Male Sprague-Dawley rats ($n = 16$) were individually housed in standard clear plastic cages, and were maintained on a 12-hr light-dark cycle (light phase: 06:30-18:30-hr). Animals had free access to Purina™ lab chow and tap water. The rats were anesthetized with pentobarbital (60 mg/kg; i.p.) and stereotactically implanted with a 20 gauge guide cannula containing a removable 24 gauge obturator, aimed dorsal to the peri-locus coeruleus. The coordinates, according to the atlas of Paxinos and Watson (1986) [342] were (with skull positioned 15° nose down) 3.5-3.7 mm caudal to lambda, ± 1.1 mm to the midline, and 7.0 mm ventral to the top of the skull. After a minimum of 7 days post surgical recovery, animals were transferred to individual testing chambers comprised of Plexiglas (25 x 35 x 34 cm) with a stainless steel grid floor and allowed to acclimate for at least 48-hr prior to testing.

Microdialysis

On test day (09:00-15:00-hr), the rats were restrained gently to replace the obturator with a concentric microdialysis probe, consisting of a 2.0 mm regenerated cellulose active membrane (250 μ m outer diameter; 6000 molecular weight cutoff; Spectrum Laboratories, CA). Each probe was secured to the pedestal with the anchor screw and connected via polyethylene tubing (Intermedic, Clay Adams, NJ) to a liquid swivel (Instech Laboratories, PA) and a 2.5 ml gas-tight infusion syringe attached to a

pump (model 22, Harvard Apparatus, MA). Microdialysis probes were perfused at 3 $\mu\text{l}/\text{min}$ with filtered Krebs-Ringer phosphate (KRB) solution consisting of (in mM): 2.7 K^+ , 145 Na^+ , 1.35 Ca^{2+} , 1.0 Mg^{2+} , 150 Cl^- , 0.05 ascorbate, pH 7.4 [306], and BSA (0.1%). After the probe insertion, a 60-min stabilization period was allowed (a time interval previously deemed adequate to achieve stabilized basal rate of peptide release) [296]. Subsequently, dialysates were collected every 20-min (yielding ~60 μl samples) and immediately frozen on dry ice and stored at -80°C for 2 weeks until RIA analyses. The efficiency of *in vitro* peptide recovery by the microdialysis probes averaged $3.3 \pm 0.6\%$ (details previously described [297], see also Chapter 1).

The collection of 6 baseline samples began 1-hr after the insertion of the probe. Six additional samples were collected after a 10-min restraint stressor. An assistant, not involved in the sample collection, administered the restraint stressor. This was done to minimize the potential conditioned stress effects associated with the experimenter.

Experiment 2: Effect of electrical stimulation of the lateral CeA on the release of ir-CRH at the peri-locus coeruleus

Animals and procedure

Male Sprague-Dawley rats ($n = 28$) were individually housed in standard clear plastic cages, and were maintained on a 12-hr light-dark cycle (light phase: 06:30-18:30-hr). Animals had free access to Purina™ lab chow and tap water. On test day (09:00-15:00-hr) a single rat was injected with pentobarbital (60 mg/kg; i.p.) and placed in a stereotaxic frame. An enamel-coated stimulating electrode (250 μm OD) with a 0.25 mm

enamel-free tip (angled to a 45° point) connected to an in-house designed electronic stimulator was tested *ex vivo* in albumin to verify the extent of the stimulation field. This enabled us to estimate the range of the stimulation field in the brain at the four different stimulation levels. In albumin, the stimulation field was spherical in shape, and increased slightly in accordance with the level of stimulation so that the lowest level (i.e. 125 μ A) was restricted to the tip of the stimulator, while the 1000 μ A level would likely activate the entire CeA (~ 1mm spread).

In vivo we aimed at the stimulator at the lateral CeA (a region previously determined to contain dense ir-CRH neurons that project to the peri-locus coeruleus [459]) using the coordinates of Paxinos and Watson [342] A/P – 2.8, D/V –8.0, L/M $\pm$ 4.6. Once implanted, a second custom manufactured "side-window" push-pull probe (see Figure 1) was lowered into the ipsilateral peri-locus coeruleus region to collect the release of ir-CRH. The window of the probe (i.e. collection area) was always directed away from the 4th ventricle. The animal was grounded from the tail using water-saturated cotton swabs. Samples were collected every 10-min yielding volumes of ~200 μ l. After a 90-min stabilization period, 3 baseline samples were collected followed by a single 0.1-msec bi-phasic rectangular pulse stimulation of (125, 250, 500 or 1000 μ A @ 50-Hz) delivered in a latin-square design every 30-min. Thus, on every third perfusate sample, one of the four levels of electrical stimulation was applied. The average value of ir-CRH observed for each stimulation level was compared to the sample immediately preceding it. In order to assess the effects of the anesthesia alone, a separate group of rats, (n = 10) were implanted as described above, however no stimulation was applied. To maintain a

steady state of anesthesia. rats were injected subcutaneously every hour with 10% of the original pentobarbital dose (i.e. ~ 0.6 mg/kg; s.c.).

Histology

At the end of the experiment, brains were sectioned, and stained for histological verification of the probe placements. Only data from animals with both probes correctly positioned in the lateral CeA and peri-locus coeruleus were used for the statistical analyses.

Radioimmunoassay (RIA)

The detection and quantification of ir-CRH was achieved using a solid-phase high-sensitivity adaptation or modification [270] of the double-antibody liquid phase RIA originally described by Vale et al. (1983) [454]. Briefly, protein A/G- (Calbiochem Corp., CA) coated Immulon-4 wells (Dynatec Laboratories Inc., VA) were incubated with anti-CRH serum (rC70, kindly provided by W. Vale, The Salk Institute, La Jolla, CA) for 2-hr at 20°C. Samples, standards (diluted in tissue processing medium, ranging from 0.05 to 250 fmol/well), and blanks were incubated for 24-hr at 4°C. Next, 25 µl of assay buffer containing 5000 cpms of ^{125}I -[Tyr⁰]-rCRF (Amersham, ON) was added to each well and incubated for an additional 18-hr at 4°C. Finally, the wells were rinsed and separated, and their residual radioactivity was counted in a gamma counter (Cobra II Auto gamma, Model D5002, Packard Instrument Company, CT). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay was typically ~0.1 for CRH.

Statistical analyses

The data from Experiments 1 and 2 (CRH release) was analyzed using repeated measures analysis of variance (ANOVA). All values were expressed as a percent of baseline (defined as 100%). In Experiment 2, stimulation level (125, 250, 500, and 1000 μ A) served as the within subjects factor. A significant main effect or simple effects of significant interactions were followed-up with Bonferroni corrected t tests at relevant sample points.

Results

An ANOVA on the changes observed in ir-CRH extracellular levels at the peri-locus coeruleus in rats with correctly positioned probes ($n = 9$) showed a significant effect of Sample ($F_{11, 88} = 2.323$, $p < 0.015$). The ir-CRH levels increased significantly above baseline immediately following restraint and remained elevated until the end of the experiment (see Figure 2). In Experiment 2, only animals with probes correctly positioned in both the peri-locus coeruleus and lateral CeA were selected for analyses ($n = 20$). For each of the four stimulation levels, the average concentration of ir-CRH was compared against the average baseline. Repeated measures ANOVA revealed no significant effect of amygdaloid electrical stimulation on the release of ir-CRH at the peri-locus coeruleus, $F_{3, 57} = 0.365$, $p = 0.78$ (data not shown). In the control group (sham stimulated), ir-CRH levels did not vary consistently over time, $F_{15, 120} = 1.45$, $p = 0.14$ (data not shown).

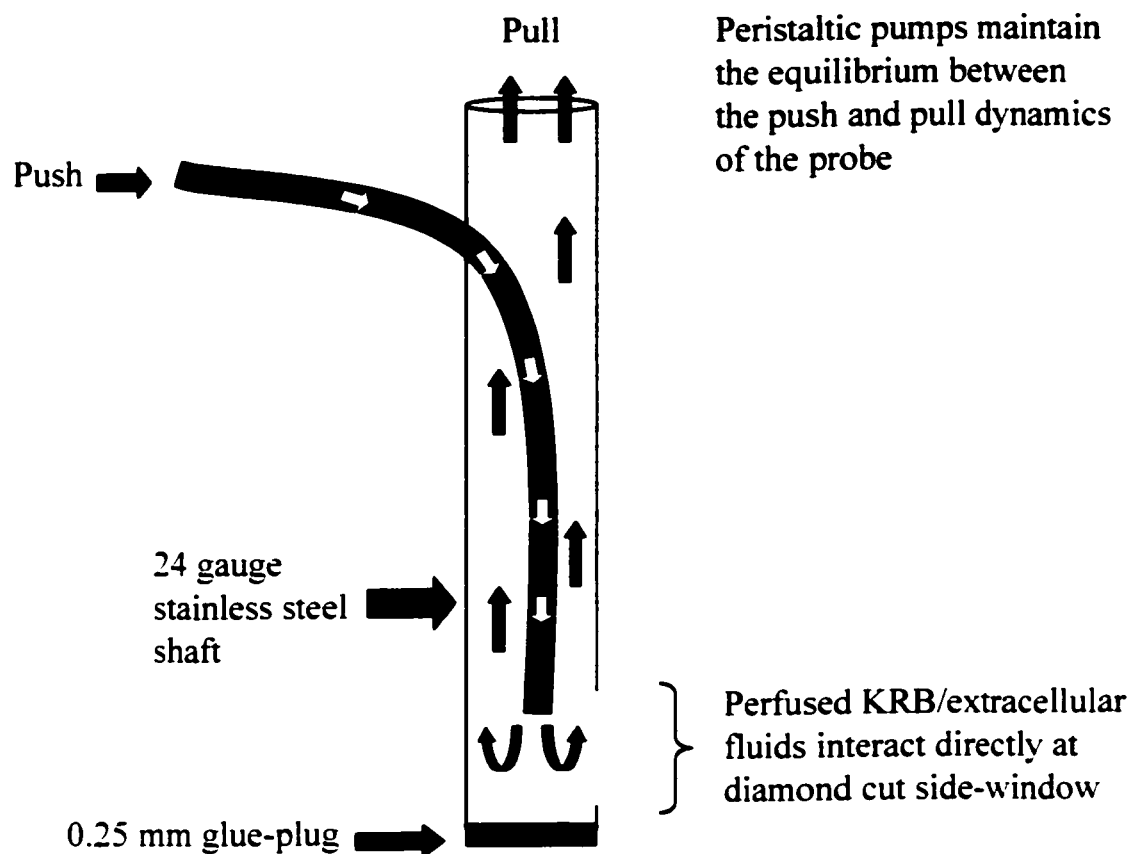


Figure 1. A schematic representation of the novel "side-window" push-pull probe used to collect the release of ir-CRH from the peri-locus coeruleus during electrical stimulation of the central nucleus of the amygdala. The diamond cut side window allowed the probe to be inserted so that the active sampling area was away from the 4th ventricle, while the glue-tip plug prevented any flow blockage during implantation.

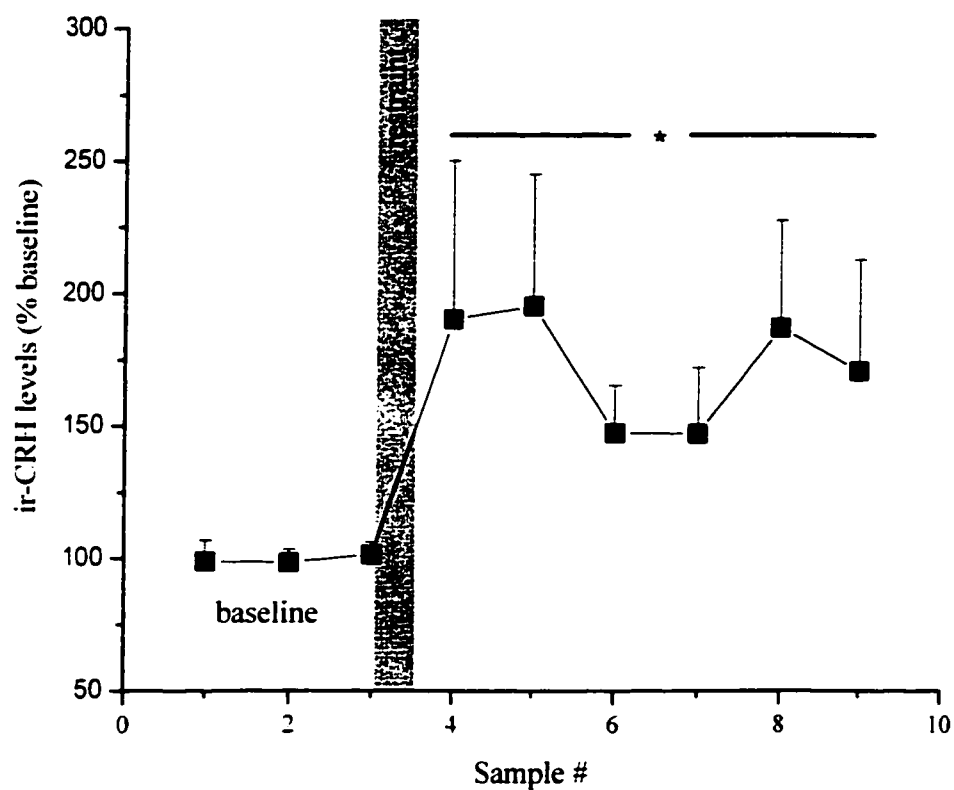


Figure 2. Levels of ir-CRH release observed at the peri-locus coeruleus, as measured by *in vivo* microdialysis under baseline and following 10-min restraint in Sprague-Dawley rats ($n = 9$). The baseline values from each rat were averaged and defined as 100%. All values were then expressed as a percent of that baseline (basal ir-CRH levels were 4.7 ± 0.7 fmol/sample). Immunoreactive-corticotropin-releasing hormone (ir-CRH). * Significantly different from average baseline, $p < 0.008$.

Discussion

The prototypical stress response includes an activation of the HPA axis as well as an increase in sympathetic outflow [321:452]. The role of CRH neurons at the PVN in the activation of the endocrine arm of the stress response is well-delineated [394:453:473]. In addition, it has been suggested that CRH might also influence sympathetic response to stressors. The precise brain locus where CRH might serve this function remains elusive, however the distribution of CRH receptors and the localization of ir-CRH at the locus coeruleus suggests that this may be one of the targets [458:459].

Neuropeptides, like classical neurotransmitters, are synthesized and stored in terminal vesicles. Once released, neuropeptides can function as neurohormones (traveling through the blood to act upon distant organs/tissues), neuromodulators (released into the extracellular fluid in the brain to influence a wide range of neurons) or as neurotransmitters (released from axon terminals to act on receptors within close vicinity) [222]. In response to stressors, the ability of CRH to act as a neurohormone is well documented. However, technical difficulties have limited our ability to directly assess CRH dynamics or function in this capacity. Indirect approaches provide strong evidence to support the idea that endogenous CRH is released in the vicinity of the locus coeruleus where it likely modulates noradrenergic transmission in the brain and sympathetic outflow during stressor exposure by influencing the excitability of locus coeruleus neurons [99].

The current study represents the first direct assessment of stressor-elicited *in vivo* release of ir-CRH at the peri-locus coeruleus in freely behaving animals. Restraint was associated with an immediate rise of extracellular ir-CRH at the peri-locus coeruleus as measured by *in vivo* microdialysis. This is consistent with the finding that pretreatment with CRH receptor antagonists in the vicinity of the locus coeruleus attenuate or block stressor-elicited changes in the firing rate of locus coeruleus neurons and the release of neurotransmitters at this region [99:331;411]. Our findings verify what several laboratories have to this point speculated.

It is of interest to note that the duration stressor-elicited rise in the interstitial levels of CRH was rather protracted—well beyond the termination of the short stressor episode. This suggests that CRH at this region may have functional implications beyond the immediate stressor responses: it may for instance be involved in maintaining hypervigilant state following stressor termination. Heightened vigilance following stressor exposure might help in the detection of subsequent threatening stimuli and prepare the organism to cope with such encounters. Increased CRH release may not only mediate such protracted effects directly, but might also serve to sensitize the locus coeruleus neurons to subsequent stressors. This is a distinct possibility because not only can exogenously administered CRH induced behavioral sensitization (e.g. to startle response) but previous stressors also sensitize the firing rate of locus coeruleus neurons to exogenously applied CRH [100;101]. Alternatively, it ought to be considered that a single stressor might have provoked long-lasting changes in an animal's perception of the environment in which the stressor was applied. Although we attempted to minimize the

after-effects of restraint (having an assistant apply the stressor). other unknown factors may have served to prolong the effects of the stressor. One such variable might be the actual testing cage. That is, restraint may have altered the animal's perception of the previously "safe" environment to one that is perceived as threatening. Indeed, such phenomenon might even explain the more transient rise of ir-CRH reported by Pich et al. (1995) [351]: these authors applied the stressor in a distinct (outside the test cage) environment. In any case, these results support the notion that CRH may act as a neurotransmitter at this brain region and play a role in activating the sympathetic arm of the stress response.

Retrograde and anterograde tract tracing experiments have revealed that a substantial number of efferent axons emanating from the CeA synapse onto dendrites of the locus coeruleus neurons [458]. Indeed, many of the behavioral and autonomic effects which result from electrical/chemical stimulation of the locus coeruleus are very similar to those observed following stimulation to the CeA [65:171:421]. Insofar as the changes evoked by amygdaloid stimulation are delayed relative to locus coeruleus stimulation, it appears as though amygdaloid excitability exerts behavioral and physiological changes via the locus coeruleus [422]. It has been suggested that the functional interplay between the amygdala and the locus coeruleus may be chemically bridged by CRH [458:459]. Similarly, noradrenergic fibers from the locus coeruleus project to the CeA where norepinephrine is thought to increase the output of the CeA that in turn feeds back to the locus coeruleus, further increasing locus coeruleus neuronal discharge [234]. In this way,

stressors that elicit changes in the neuronal output at the locus coeruleus, like restraint, are believed to provoke activation of the CeA neurons [234].

In the current experiment we attempted to test this hypothesis by directly stimulating the CeA with graded electrical stimulation, while simultaneously monitoring the release of ir-CRH at the peri-locus coeruleus. Using the current methodology we could not unequivocally demonstrate an association between electrical stimulation of the CeA and the release of ir-CRH at the peri-locus coeruleus. Several possible explanations may account for what appears to be at odds with the pharmacological and neuroanatomical expectations [422:458]. First, our tests were conducted under anesthesia and there is evidence suggesting that electrical stimulation of the amygdala under anesthesia may not evoke the type of changes observed in freely moving animals [162]. Furthermore, in this experiment we used a novel push-pull probe that had a rather small collection area that we attempted to direct towards the rostral lateral peri-locus coeruleus, the area identified by others as containing the majority of ir-CRH terminals [460]. It is possible that we failed to correctly align our probes in this area, and missed the release of CRH. This possibility is supported on two levels. One the one hand, we have previously demonstrated that restraint increased the release of ir-CRH at the CeA (an effect that presumably would follow augmented neuronal activation [422]); and secondly, restraint elicited a rapid rise in ir-CRH levels at the peri-locus coeruleus as observed using a microdialysis probe, which samples from a larger surface area. Indeed, subtle differences in probe placement had profound impact on the release of ir-CRH at the peri-locus coeruleus upon electrical stimulation of the CeA. Taken together, these possibilities

suggest that the lack of response observed following electrical stimulation of the CeA may not necessarily reflect the lack of effect, but rather we may have missed picking up such changes owing to technical considerations. Similarly, although we diligently attempted to place the stimulating electrode in the lateral aspect of the CeA, it is possible that slight errors in this regard may have accounted for the lack of effect on ir-CRH release. In the same way, our stimulation parameters might not have been optimally tuned for the activation of the CeA neurons. In sum, additional experiments are necessary before conclusions regarding the impact of electrical stimulation of the CeA upon release of ir-CRH at the peri-locus coeruleus region can be made unequivocally.

In conclusion, restraint elicited a rapid and sustained rise in ir-CRH at the peri-locus coeruleus in freely behaving animals. This finding suggests that in addition to its well-documented neuroendocrine role, endogenous CRH is released at the locus coeruleus where it likely contributes to heightened arousal, in response to stressor exposure.

General Discussion

A critical determinant of survival is often the way an organism responds to stressors (coping strategies). Thus, there has been continued interest in determining the neurochemical underpinnings of the stress response. Whereas the initial efforts were focused on the role of monoamines, more recent efforts have been directed towards the potential role of neuropeptides in the mediation or modulation of the stress response. Of all the peptides, CRH has probably received the most attention, mainly because it regulates the release of ACTH from the pituitary, and the consequently that of cortisol (or corticosterone) from the adrenal cortex, in response to stressors. However, it has become clear over the past few years that the effects of CRH may not be restricted to its endocrine action, but may also influence other stressor-related CNS responses as well. This is consistent with extensive distribution of CRH, beyond the endocrine regions, particularly within various limbic regions [362:451:463].

Since its discovery more than 20 years ago, CRH (primarily of hypothalamic origin) has gained the reputation as being the quintessential neuropeptide involved in the mediation and/or modulation of the stress response [129:321:453]. This includes the integration of the endocrine, autonomic and behavioral responses that enable an organism to cope efficiently and effectively with stressor exposure. As suggested on several occasions in this dissertation, many of the experimental approaches in this regard have relied on indirect post-mortem measurements of CRH utilization, such as *in situ* hybridization, immunohistochemistry and/or tissue content assessment obtained from micropunch [23:272:435]. Thus, central to this thesis was a direct assessment of the role

of central CRH in the coordination of the stress response, using a more dynamic assessment of peptide release before, during and following exposure to stimuli that potentially threaten the animal's internal milieu. Similarly, more recent data from our lab [223:225] and others [58:62:63:274] suggests that central BN-LPs may likewise be characterized as stressor-responsive neuropeptides. To further explore this contention we utilized a double-antibody RIA that permits the assessment of both CRH and BN-LPs within the same sample. It should be noted however that low concentrations of BN-LPs in the dialysates often precluded our ability to detect changes in this system and so our major findings are often restricted to CRH.

This general discussion begins first with a brief overview of the major goals set forth in this body of work, beginning with the technical validation of *in vivo* microdialysis as a viable tool for neuropeptide research. We then discuss and integrate some of the major findings from the course of the experiments. Outlined first, is the observation that both CRH and BN-LPs are released at the CeA, in response to stimuli of salience, be they aversive or appetitive in nature. As such, the role of the CeA in general, and the functional role of these peptides at this region in particular, are discussed. An unexpected yet recurrent finding was the observation that CRH utilization at the CeA might not be seminal to the behavioral response to stressors. On many different levels this contrasts the widely held assertion that CRH is a primary regulator of the endocrine as well as the behavioral response to stressors. Hence, a significant portion of the discussion is devoted to this finding. A second finding of some significance was the observation that not only did stressors evoke the release of CRH (and BN-LPs) at the

CeA, but so did stimuli that were appetitive in nature. Also of interest was the observation that once released, CRH (and when detectable, BN-LPs) at the CeA was quite protracted--far surpassing the duration of the stimulus. This finding might serve to provide an insight into the role of this (these) system(s) that must include processes beyond immediate stressor exposure. Thus, the possible significance of a sustained response is discussed in detail prior to concluding the general discussion with suggestions for future research.

The majority of studies to date assessing the roles of peptides have primarily relied upon indirect techniques that *infer* altered peptide utilization. More recently, some studies have attempted to address this issue more directly by employing *in vivo* microdialysis in freely behaving animals. However, very few studies have done so, and those that have, have resorted to [351:369:370] using CRH antibody within the perfusing medium to augment peptide recovery and detection: which may have confounded the results. Thus, our initial set of studies attempted to setup and validate an *in vivo* microdialysis technique to measure the release of CRH and BN-LPs in freely behaving animals, using an anti-body free perfusion medium. This objective was achieved through two sets of experiments discussed below.

In the initial set of experiments we implanted microdialysis probes at the CeA and collected dialysates every 30-min over the course of several hours in order to determine whether basal (in absence of stressor exposure) release of CRH and BN-LPs could be detected. This also afforded us the ability to address concerns that peptide release

observed in treatment conditions may be 1) an after-effect brought on by acute tissue damage following the implantation of the probe; 2) an effect associated with random behavior and not the application of the stressor *per se* or 3) an effect attributable to naturally occurring ultradian fluctuations, as has been noted with the release of other transmitters within the brain [205:206:217].

These experiments confirmed that the release of CRH and BN-LPs at the CeA was specifically triggered by the application of the treatment, and that the release of either peptide did not fluctuate significantly in undisturbed freely behaving animals. Thus, the release of CRH and BN-LPs did not appear to be a non-specific response to the implantation of the probe, nor could it be attributed to non-specific behaviors. Finally, potential ultradian rhythm related fluctuations did not appear to be a factor of concern, as basal release was fairly stable over the sampling periods. Taken together, these early experiments established that *in vivo* microdialysis, without the inclusion of antiserum in the perfusion medium, could be used as a tool to assess the release of endogenous neuropeptides in freely behaving animals. These experiments however did not permit conclusive statements regarding the authenticity of the collected peptides. Thus, the second approach to substantiating *in vivo* microdialysis as a viable tool required that we address concerns related to the authenticity of the peptides measured.

In competitive RIAs, there is a competition between the 'hot' (or labeled) the 'cold' (non-labeled) peptides for the binding sites on the antibody; thus the level of radioactivity of the bound peptide is inversely related to the concentration of the 'cold'

peptide within the sample (see Figure 3. Introduction). If the sample (or brain extract) happens to contain structurally related neurochemical(s) that share affinity for the antibody being used, the specificity of the assay becomes compromised. This potential confound is exemplified in the quantification of BN-LPs inasmuch as our BN antibody has an affinity for more than one form of the mammalian BN-LPs, the strongest of which is GRP₁₋₂₇ (110%), and GRP₁₈₋₂₇ (82%) [309]. In order to verify the authenticity of CRH and BN-LPs detected in the RIAs, pooled dialysates collected from the CeA, and synthetic standard peptides were subjected to HPLC separation and their specific elution times determined. The dialysate samples thus fractionated, are then individually subjected to RIAs. Based upon the fraction number(s) (i.e. retention time) where immunoreactivity is detected and the retention times of the known reference peptides, one can determine what specific peptides are in the unknown samples. This procedure enabled us to establish early in the course of this thesis research, that immunoreactive CRH and BN-LPs correspond respectively to authentic CRH and GRP. Thus one of the novel contributions of this work was the validation of *in vivo* microdialysis, combined with a highly sensitive RIA as a methodological approach to assess the release of CRH and BN-LPs in freely behaving animals. We were then able to exploit this methodology to explore the role of central CRH and BN-LPs in freely moving animals exposed to a variety of environmental manipulations. Moreover, this allowed dynamic assessment of specific peptides released under various behavioral states (a within subjects design), which has the added advantage of reducing the number of animals needed per experiment.

The next major objective that was set forth in this thesis included an assessment of role of central CRH in the manifestation of behavioral changes brought on by exposure to biologically salient stimuli. In this regard we focused our efforts on changes in CRH release at the CeA. Based on evidence accumulated from our lab [12:13:223] and those of others [197:378:379], there is a consensus developing that the CeA may orchestrate the behavioral, autonomic and endocrine changes that occur during stressor exposure. In terms of the behavioral responses, the CeA is thought to be critical in ascribing emotional salience to specific events [211:414:502]. In this context, it is thought to mediate some of the flight or fight as well as anxiety-like responses to specific stressors. In terms of the autonomic responses, the CeA has been shown to be the key amygdaloid structure projecting to brainstem autonomic regions where it modulates stressor-evoked increases in heart rate, blood pressure, respiration, pupillary dilation, and pallor [160:162:177]. Finally, in the context of endocrine changes, it has been suspected that the CeA may play a role in stressor-induced activation of the HPA axis, via its connection to the PVN (both direct and indirect via the BNST) [113:163]. Indeed, manipulations of this area induce autonomic, endocrine and behavioral changes similar to those seen following stressor exposure. It is thought that such changes may permit the organism to effectively cope with the challenge at hand [40:130:347:378:483]. In accordance with animal findings, neuroimaging studies reveal amygdaloid activation during fear acquisition [244], viewing films with positive and negative emotional attributes [70], subjective drug cravings [84] or recollection of unpleasant words [439]; supporting the notion that this limbic structure plays a critical role during emotional states characterized by enhanced arousal/anxiety [107-109]. More precisely, the CeA appears to play a central role in the brain's

deciphering of processive stressors: that is, stressors that require higher-order cognitive interpretation [185]. However, the neurochemical substratum at the CeA responsible for the genesis of these changes remains largely unknown.

The observation that exogenously administered CRH dose-dependently stimulates HPA activity in addition to evoking behavioral changes that resemble those noted following stressor exposure, supports the notion that central CRH might be involved in stressor-induced endocrine as well as behavioral changes [26:129:385]. The modulating role that CRH has on the release of ACTH from the anterior pituitary gland is firmly established [129:362:433]. However, it has been more difficult to define the precise role of CRH in the context of stressor-induced behavioral output. It is of interest to note that the ability of CRH antagonists to exert behavioral changes in traditional anxiety paradigms might depend on the animal's pre-test level of arousal [221], and at times the anxiolytic effects reported by diazepam pretreatment are not observed in animals pretreated with CRH antagonists [35]. Furthermore, blocking CRH-BP with CRF₆₋₃₃, an effect that would presumably augment the bioavailability of endogenous CRH, does not produce anxiogenic behavioral responses, but does increase general locomotion [181]. Likewise, centrally administered CRH potentiates the startle amplitude, however this affect only occurs at least 90-min following central injection of CRH [251].

The observation that CRH neurons and CRH receptors are heavily represented at the CeA, in part, underscores the notion that this peptide may be one of the key chemical signals influencing the output of this brain region [434:435:463], especially during

stressor exposure. However, the views regarding the role of CRH remain controversial [181:207:328]. Furthermore, there is only limited information regarding the actual utilization of CRH during exposure to environmental challenges: most studies have studied the effects of CRH only indirectly, which may account for some of the discrepant results.

To address this issue directly, we first exposed animals to stimuli that were inherently differed in their emotional qualities. Specifically, we monitored the release of CRH and BN-LPs in animals prior to, during and following exposure to a conventional stressor (i.e. restraint) or other biologically salient stimuli (e.g. meal consumption). The two stimuli can be characterized as being, aversive and appetitive, respectively, as with each evoking very different behavioral responses. Whereas exposure to an aversive event should promote natural avoidance, the meal presentation should evoke behavioral approach. Contrary to the current opinions, the results obtained from these initial experiments suggest that CRH and BN-LPs at the CeA are released both in response to aversive as well as appetitive events. As suggested in the opening chapter, the attribution of emotional significance/salience to a stimulus likely involves the CeA [414:436:502]. That both types of stimuli (aversive and appetitive) evoke a similar release in CRH and BN-LPs provides a strong basis for the contention that the neurochemical basis for this attribution of salience might involve two or more neuropeptides. These findings therefore require that we broaden our interpretation of both CRH and BN-LPs to include mechanisms beyond stressor exposure. Alternatively, it might be argued that the consumption of food constitutes a form of stressor insofar as the animal is more

vulnerable to predatory attack, or that food in itself (due to its foreign nature) evokes a stress response [175], albeit there is no doubt that the behavioral responses engendered by restraint and food consumption are very different. These data therefore challenges the notion that CRH or BN-LPs at the CeA mediate an animal's behavioral response to stressors and/or other biologically relevant stimuli (in this case food consumption).

Subsequent experiments further validated that CRH (BN-LPs were often not detectable) might not be the mediator of the immediate behavioral response to stressors or appetitive stimuli. We exposed Fast and Slow rat lines to sexually receptive/non-receptive female cohorts and noted that among the Fast rats a robust behavioral arousal was evident in response to both types of females, while CRH release was only significantly elevated in response to an estrous female. In contrast, the Slow rats' behavior was dictated more so by the sexual status of the female (more active in response to an estrous female). However, in these rats, CRH release was elevated only in response to a non-estrous female. Thus, in both rat lines, behavioral output did not correspond with changes in the release of CRH at the CeA.

Additional convincing data challenging the view that CRH at the CeA mediates the behavioral output during stressor exposure was derived from Fischer and Lewis rat strains. These rats displayed marked strain-dependent behavioral differences in response to noise exposure, which is in keeping with previous observations in other stressor paradigms including open field exposure [418:425], forced swim test [418], elevated plus maze and acoustic startle response [426]. Despite these differences in behavioral output,

both strains responded with similar increases in the release of CRH at the CeA in our experiments. Of note however, the basal release rates were much higher in the Lewis strain, which adopts a more defensive coping style in contrast to an active response displayed by their Fischer counterparts. Although our data clearly demonstrate a lack strain differences in stressor-elicited rise in CRH release (relative to their basal release rates), there were strain differences in the basal levels. Thus we cannot rule out the possibility that the elevated basal release rates of CRH at the CeA in the Lewis rats may underlie some of the strain-dependent behavioral differences observed in different paradigms.

The hypothesis put forth here that CRH might not be the mediator of the behavioral changes evoked by stressor exposure is supported by some recent observations in transgenic models. Recently, Muglia and colleagues (2001) have noted that mice completely devoid of CRH display a behavioral response to stressors that is similar to their wild-type cohorts [313]. In both the knock-out and wild-type mice, pretreatment with α -helical CRH₉₋₄₁ or CP-154, 526 (a non-peptide CRH-type1 specific receptor antagonist), blocked stressor-induced behavioral changes suggesting that an unknown CRH-type1 receptor ligand may mediate the behavioral changes observed during stressor exposure that are traditionally ascribed to CRH [313]. These findings are in line with those of Weninger et al. (1999) demonstrating that stressor-induced behavioral change required CRH receptors, but not CRH [475]. It is tempting to speculate that this "unknown" ligand might actually be urocortin, given its high affinity to both CRH-type1 and CRH-type2 receptors, and the observed up-regulation of urocortin in CRH knock out

mice [385:476]. However, the distribution of urocortin is primarily restricted to the Edinger-Westphal nucleus in the midbrain making it doubtful that this peptide drives behavioral changes during stressor exposure in mice lacking CRH or in normal controls [313]. It is possible that future research might reveal an as yet unidentified CRH-like neuropeptide that may be mediating some of these effects. These findings, taken together with our research, strongly challenge the notion that CRH *per se* is necessary for orchestrating an animal's behavioral response to stressors.

It is important to note that many animal stressor paradigms used to assess the biological and behavioral processes involved, seem to rely on the expression of punished and/or avoidant behaviors. Such is the case with the elevated plus maze, open-field exploration, light-dark box, punished drinking/feeding. In humans, stress-related disorders like anxiety, agoraphobia and panic are troublesome because they impede behaviors that the person would normally *engage* in. Animal models in this regard are scarce and when available do not readily lend themselves to ongoing neurochemical assessment using *in vivo* microdialysis.

In one of the experiments, we fine-tuned a stressor paradigm that would allow us to directly assess the release of CRH during behavioral responses to a naturalistic stressor exposure. In this particular study we exposed animals to a novel environment while monitoring the release of CRH at the CeA. The transfer of the animals from the home cage (familiar environment) to a novel cage (unfamiliar environment) was associated with a robust elevation in the release of CRH at the CeA, supporting the view that

processive stressors activate CRH at this area [272]. In addition, relative to the home cage “stress-free” condition, placing animals in a novel environment was accompanied with a significant decline in the consumption of a palatable snack as well as an increase in the latency to approach the snack. These behavioral effects were completely reversed by diazepam (a prototypical anxiolytic) [51:365]. Of particular interest however was the observation that the anxiolytic effects of diazepam pretreatment occurred *in spite of* a concurrent rise in CRH release at the CeA. This study, taken together with those already discussed, supports the position that CRH at the CeA is not involved in the genesis of the behavioral responses to stressors. Furthermore, central pretreatment with various CRH receptor antagonists failed to alter the behavioral effects of the novel cage, suggesting that amygdaloid or extra-amygdaloid CRH does not appear to be involved in the behavioral changes associated with stressor exposure.

To summarize, the results presented in this dissertation contribute novel information in this field of research. Specifically, we 1) demonstrated the viability of using *in vivo* microdialysis combined with a highly sensitive RIA, to monitor the release of CRH and BN-LPs in freely behaving rats, during exposure to various environmental challenges; 2) revealed that the concurrent detection of CRH and BN-LPs in the RIAs corresponds, respectively, to authentic CRH and GRP; 3) re-affirmed that the CeA is responsive to a range of stimuli that fall on a continuum from aversive on the one end, to appetitive on the other; 4) demonstrated that the release of CRH at the CeA, did not correspond to the behavioral responses to stressors, and 5) under conditions whereby

behavioral output is altered by the environment. CRH is not the neurochemical mediator of stressor-induced behavioral changes.

Finding meaning in the pattern of response: Possible function of a sustained release

The prototypical stress response, as discussed in detail in the Introduction, is influenced by several factors, including the history of stressor exposure [15], and the current results should be interpreted in the framework of the experimental design utilized herein. In this context, it is important to recall that contact with the stressors represented the *first* exposure to these particular stressful events. As such, one must consider the possibility that neurochemical signals mobilized at this time may serve several functions, including 1) coping with the immediate threat imposed by the stimulus; 2) processing specific cues associated with the stressor; 3) maintaining a heightened state of vigilance even in the absence of the stressor, and/or 4) initiating memory traces that will serve to associate those cues with their salience. The initial rise in CRH release at the CeA likely plays a key role in mobilizing the organism's endocrine and autonomic responses that occur within a very short time following stressor onset. Both of these functions have been ascribed to this region and CRH in the past [171;255;362]. However, the more protracted rise that was noted across all experiments suggests that elevated CRH levels constitute a neurochemical signal involved in processes that persist well after stressor termination.

It is conceivable that survival depends to a large extent on the animal's ability to predict danger and/or to generalize from one stimulus to another. Indeed, many of these

processes likely represent hard-wired neurocircuitry, especially in the case of predator recognition and avoidance. For example, predator odor alone has been reported to elicit changes in behavior and in the activation of the HPA axis, which resemble those elicited in response to the actual predator exposure [311;312]. It has been well documented that acute stressor exposure enhances memory consolidation and recall [405] and that centrally injected CRH and BN-LPs have similar effects on memory processes [95;142;250]. Thus, the protracted rise in the release of either CRH or BN-LPs at the CeA might be related to the relevant memory processes. This possibility could be addressed in future experiments that compare the release of peptides in animals with and without a history of exposure to the stressful stimulus. If the sustained release of CRH or BN-LPs is involved in processing and storing cues associated with the stimulus, one might expect the release to return to baseline more quickly in animals with prior stressor exposure.

Some researchers have recently suggested that central CRH, like stressor exposure, might sensitize brain mechanisms to subsequent stressors [101;411]. Specifically, pretreatment with CRH has been shown to augment the electrophysiological output of the locus coeruleus during stressor exposure [99;411], in effect priming the brain to respond to external stimuli. If endogenous CRH serves a similar role, the sustained release observed at the CeA might sensitize the brain to subsequent stressors. Again, this might be especially important during the animal's initial encounter with biologically salient stimuli insofar as ensuing stressors might then prompt a more biologically efficient response. On the other hand, it is reasonable to suggest that what

constitutes an adaptive coping mechanism would be the termination of an organism's response (i.e. ensuring conservation of resources) once the stressor has been terminated or removed. Indeed, many systems within the brain display habituation to sustained activation [188:290]. In this context, it is of interest that the neuronal activity of the locus coeruleus displays abrupt habituation following multiple exposures to the same type of stimulus, but increases rapidly in response to novel or heterotypic stressors [22:320].

Our attempts to directly assessing the release of CRH at the locus coeruleus in freely moving animals appear to be the only one of its kind. These experiments revealed a rapid and sustained rise in CRH levels at the locus coeruleus in response to acute restraint. The sustained release makes it tempting to suggest that elevated CRH release contributes to the habituation processes at this region. Although restraint in this case was applied as a single episode (i.e. 20-min) the length of exposure far exceeds that which an animal might encounter in the wild. This is especially true in experiments where CRH release at the CeA was monitored during predator exposure, which continued for a relatively lengthy 30-min interval. In their natural environment one might expect that survival is contingent upon limiting predator exposure to seconds! We should therefore consider the possibility that lengthy stressor exposure might evoke the activation of mechanisms involved in habituation, which might include sustained release of CRH and/or BN-LPs. It would therefore be of interest to assess the release of CRH and/or other neuropeptides in response to stressors applied for different intervals.

A final explanation that may account for the protracted rise in peptide release is based on the suggestion that endogenously released CRH and related peptides may serve a neuroprotective role at certain brain regions [146:253:343]. It has been well documented that stressor-associated cognitive disorders such as Alzheimer's, Huntington's, and Parkinson's disease are correlated with progressive decreases in CRH levels within the brain [137:263:363], changes in the morphology of CRH-expressing neurons [116] and decreased memory capability [74:302] (a possible side-effect of blunted CRH levels; see above discussion). These observations have lead investigators to assess the possibility that decreased CRH levels in the brain might underscore a susceptibility to the onset of disorders such as Alzheimer's disease. By extension, it follows that a certain optimal level of endogenous CRH might likewise prevent such disorders.

Fox et al. (1993) [146] determined that CRH delivered *in vitro* to hippocampal brain sections damaged by hypoxia resulted in a marked recovery of synaptic function. More recently, Lezoualc'h et al. (2000) [253] confirmed that CRH, acting through CRH₁ receptors, was neuroprotective against oxidative stress by way of its ability to inhibit the activity of NF- κ B, which is considered to be among the transcription factors responsible for neuronal degeneration observed in Alzheimer's patients [253]. It should be noted that others have detected neuronal *protection* in animals pretreated with α -helical CRH₉₋₄₁ [146:253:343], which is seemingly inconsistent with the notion that CRH may be neuroprotective. On the other hand, α -helical CRH₉₋₄₁ is a non-selective CRH receptor antagonist that has been demonstrated to have partial agonistic effects as well

[253:292]. Thus, more research is required before a firm position can be taken one way or the other regarding the role of CRH in processes related to cognitive disorders.

Having said this, the proposal that CRH is neuroprotective does provide an intriguing explanation as to why stressor exposure would result in a sustained rise in the release of CRH. It is also of interest that while glucocorticoids have been repeatedly demonstrated to inhibit the activation of CRH at the PVN, they appear to exert the opposite effect at the CeA [272]. Elevated CRH mRNA levels at the CeA have been observed for up to 2-hr following local glucocorticoid injections [111:272:273]. It is possible that different areas of the brain respond differently to stressor-induced increases in central glucocorticoid concentrations to ensure 1) that the activation of the HPA axis is maintained within a stable range and 2) that CRH levels continue to rise to counteract neuronal degenerative effects associated with stressors in general and glucocorticoids in particular [194:266]. This contention could be directly assessed using *in vivo* microdialysis in animal models of global or focal ischemia.

The above arguments appear to be difficult to reconcile with the finding that food consumption (an appetitive event), which the rats are accustomed to, also evokes a sustained release of CRH and BN-LPs (see Chapter 1). A sustained response in this regard might reflect post-ingestion processes related to memory formation as to the nature of the food (i.e. that it is something non-aversive and safe to consume in the future). Alternatively, these peptides might represent neurochemical signals mediating the characteristics specific to food ingestion, such as, the onset/termination of a meal, as well as inter-meal interval. Indeed, both of these peptides (but especially BN-LPs) have

received a great deal of attention as satiety signals [155:298:354:355]. These contentions could be addressed further in free-feeding animals across multiple meals.

Conclusions and future directions

It is highly probable that CRH acts as a neurotransmitter in the brain, based on the localization and distribution of this peptide (or related peptides) and its (their) receptors throughout the brain [45]. During the last decade, the development and use of *in vivo* microdialysis has substantially furthered our knowledge about the role of various classical transmitters (such as dopamine, serotonin and norepinephrine) in central processes. However, until recently, such an approach could not readily be applied to the assessment of neuropeptide function, mainly due to their larger size, microdialysis membrane permeability and relatively insensitive assays. One of the major contributions to come from the current body of work was the validation of *in vivo* microdialysis paired with a highly sensitive *ex vivo* RIA, in the assessment of neuropeptide *release* in freely behaving animals. As scientific advancements are often tightly linked to methodological or technological advancements, we hope that this research will serve to advance our knowledge of the role of neuropeptides in behavioral expression and underlying biological processes. In this vein, we have for instance, successfully used this technique to clearly demonstrated that CRH is released at the CeA and around the vicinity of the locus coeruleus, supporting the notion that this peptide mediates several aspects of both the acute and protracted phases of the stress response. This technique will therefore be an invaluable approach to elucidate the role of neuropeptides in both normal and pathophysiological conditions. Since this approach can be extended to other

neuropeptides it can be used in combination with HPLC separation to collect and identify as yet unknown compounds that may be important in the stress response. In this way we may glean a more comprehensive understanding of the neuropeptides that have been shown to respond to stressors, including orexin [200:243], NPY [135], substance P [440], and somatostatin [59:186]. Similarly, we have recently validated the use of *in vivo* microdialysis combined with ELISA to assess the release of cytokines in the brain following systemic cytokine loading.

The experiments in this dissertation confirm that a wide range of processive stressors evoke the immediate rise of CRH and in some cases, that of BN-LPs (specifically GRP). Furthermore, the changes in CRH and BN-LPs were not restricted to conventional stressors (as previously thought) but were evident in response to biologically salient stimuli including those of appetitive nature. This observation not only extends the role of these peptides beyond stressor responsivity but also challenges the contention that CRH released at the CeA mediates the behavioral aspect of the stress response. This concept was investigated using a range of stressors in animals with different genetic and behavioral backdrops. Although we consistently observed increased utilization of CRH (and BN-LPs) across a variety of stressful conditions, we were unable to ascribe any clear relationship between the utilization of either CRH or BN-LPs at the CeA and the behavioral output during exposure to aversive or appetitive events. Thus, although the neuropeptides at the CeA respond to biologically meaningful stimuli, our data do not support the position that either of these peptides orchestrates the behavioral elements of this response.

With respect to the specific pattern of change, we noted that across all experiments the response was protracted suggesting that CRH and/or BN-LPs at the CeA may also sub-serve functions that go beyond the immediate stress response. These could possibly be related to 1) processing and storing relevant information; 2) enhancing cognitive vigilance; 3) sensitization and/or habituation of the brain's neurocircuitry and 4) protecting against stressor-induced neuronal degeneration. Our initial findings provide the impetus to investigate such possibilities.

This research also makes a significant contribution to our understanding of the dynamic relationship between CRH and BN-LPs (likely GRP). In all cases where both peptides could be detected, differential release patterns were readily observed in comparison to one another and in response to a range of stressors. In general, the response of BN-LPs tended to follow changes in CRH release, which on the one hand supports the notion that these peptides might interact at some level, but questions the purported notion that BN-LPs act through the activation of the CRH system(s) [225:355]. One interesting concept might be that elevated BN-LPs might play a role in maintaining the functions of CRH, either by directly sustaining the release of CRH, or by indirectly influencing other systems that in turn influence CRHergic processes. These hypotheses remain speculative, and future research in this area will profit from assessing the specific relationship between not only CRH and BN-LPs, but also that of other peptides/transmitters.

Although it was clearly not our intention to make direct comparisons between the different strains used in these studies, it is worth noting that certain patterns were apparent, that might warrant future research in this area. Of particular note was the observation that although restraint evoked the release of CRH at the CeA in Sprague-Dawley, Fast as well as the Slow rats, the profiles of peptidergic change were different across these strains/lines. The onset of release was most robust in Sprague-Dawley rats and least responsive in Slow rats with the Fast rat line falling in between. Therefore, it would be of interest to study these strains within the same experiment so that one may glean a better understanding of the genetic contributions to the strain/line differences and possibly to individual differences.

Future directions

Most of the studies reported here have focused on the release of CRH and BN-LPs at the CeA and we feel they have provided a strong basis for future research that might include the following:

1. Determine the source of CRH and/or BN-LPs collected at the CeA. It is apparent that the release of peptides at the CeA may reflect local changes and/or more distal changes in the cell bodies that produce these peptides whose efferents synapse on neurons at the CeA. Therefore, additional studies are needed to focus on brain regions that are anatomically positioned to influence peptide release at the CeA with therapeutic targets directed towards not only these particular peptidergic systems but also those co-expressed with them. We have recently

devised a multi-probe pedestal that permits the simultaneous assessment of neurochemical changes at two different brain regions. Since compounds can also be delivered through the microdialysis membrane (reverse microdialysis), future studies may profit by administering antagonists/agonists at one region while collecting the release at another.

2. As mentioned earlier, the duration of stressor exposure was relatively lengthy, compared to that which might be expected under 'normal' situations. Exposure times are to a large extent based on the time required to collect a sufficient amount of the peptide so that it is detectable in the RIA. Future research in this area will undoubtedly benefit from the improved detection methods that are currently being developed that include HPLC combined with mass spectrometry. More recently, Dr. Cook (2001) and colleagues have been developing an immunosensor based electrochemical methodology that might permit close to *real time* assessment of CRH release in freely behaving animals [88]. Of note, in Cook's trial experiments he exposed animals to restraint and observed changes in the release of CRH at the CeA consistent with our results. Specifically, stressor exposure provoked a transient rise in the release of CRH that increased again well after stressor termination [88].
3. Of course, it would be very useful to extend these findings to other stress-relevant brain regions (prefrontal cortex, hippocampus, BNST, locus coeruleus, as well as other CRH-expressing brain stem nuclei, like Barrington's nucleus). Such

experiments would help characterize the neural circuits involved in stress responses.

As future research builds upon the work presented here, we can only hope that this research will represent pieces of the puzzle depicting a more comprehensive understanding of the neuronal systems involved in the mediation of various aspects of the stress response, including endocrine and behavioral changes. In this way, the identity of the neurochemical mediators, as well as their loci of action, will facilitate the development of effective treatments for various conditions impacting on human health and well-being.

Bibliography

1. Abercrombie, E.D. and Jacobs, B., Single unit response of noradrenergic neurons in locus coeruleus of freely moving cats. II. Adaptation to chronically presented stressful stimuli. (1987) *J. Neurosci.*, 7, 2844-2848.
2. Adell, A., Casanovas, J.M., and Artigas, F., Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. (1997) *Neuropharmacology*, 36, 735-741.
3. Agmo, A., Male rat sexual behavior. (1997) *Brain Res. Prot.*, 1, 203-209.
4. Al-Damluji, S., Iverson, T., Thomas, J.M., Pendlebury, D.J., Rees, L.H., and Besser, G.M., Food-induced cortisol secretion is mediated by central alpha-1 adrenoceptor modulation of pituitary ACTH secretion. (1987) *Clin. Endocrinol.*, 26, 629-636.
5. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1998) *Molecular biology of the cell*, Garland Publishing Inc., New York.
6. Alonso, G. and Assenmacher, I., Radioautographic studies on the neurohypophysial projections of the supraoptic and paraventricular nuclei in the rat. (1981) *Cell Tissue Res.*, 219, 525-534.
7. Alves, S.E., Akbari, H.M., Anderson, G.M., Azmitia, E.C., McEwen, B.C., and Strand, F.L., Neonatal ACTH administration elicits long-term changes in forebrain monoamine innervation. Subsequent disruptions in hypothalamic-pituitary-adrenal and gonadal function. (1997) *Ann. NY Acad. Sci.*, 814, 226-251.
8. Amorapanth, P., Nader, K., and Ledoux, J.E., Lesions of periaqueductal gray dissociate-conditioned freezing from conditioned suppression behavior in rats. (1999) *Learn. Mem.*, 6, 491-499.
9. Anastasi, A., Erspamer, V., and Bucci, M., Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians Bombina and Alytes. (1971) *Experientia*, 27, 166-167.

10. Anderson, A.K. and Phelps, E.A., Lesions of the human amygdala impair enhanced perception of emotionally salient events. (2001) *Nature*. 411. 305-309.
11. Andreis, P.G., Belloni, A.S., Cavallini, L., Mazzocchi, G., and Nussdorfer, G.G., Evidence that long-term administration of a methionine-enkephalin analogue stimulates the growth and steroidogenic capacity of rat inner adrenocortical cells. (1988) *Neuropeptides*. 12. 165-170.
12. Anisman, H., Kelly, O., Hayley, S., Borowski, T., Merali, Z., and McIntyre, D.C., Acoustic startle and fear-potentiated startle in rats selectively bred for fast and slow kindling rates: relation to monoamine activity. (2001) *Eur. J. Neurosci.* 12. 4405-4416.
13. Anisman, H., Lacosta, S., Kent, P., McIntyre, D.C., and Merali, Z., Stressor-induced corticotropin-releasing hormone, bombesin, ACTH and corticosterone variations in strains of mice differentially responsive to stressors. (1998) *Stress*. 2. 209-220.
14. Anisman, H., Lu, Z.W., Song, C., Kent, P., McIntyre, D.C., and Merali, Influence of psychogenic and neurogenic stressors on endocrine and immune activity: differential effects in fast and slow seizing rat strains. (1997) *Brain. Beh. Immun.* 11. 63-74.
15. Anisman, H. and Merali, Z., Understanding stress: characteristics and caveats. (1999) *Alcoh. Res. Hlth.* 23. 241-249.
16. Anisman, H. and Zacharko, R.M., Depression: the predisposing influence of stress. (1982) *Beh. Brain Sci.* 5. 89-137.
17. Antoni, F.A., Novel ligand specificity of pituitary vasopressin receptors in the rat. (1984) *Neuroendocrinology*. 39. 186-188.
18. Antoni, F.A., Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. (1993) *Front. Neuroendocrinol.* 14. 76-122.
19. Armario, A., Gavalda, A., and Marti, J., Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats. (1995) *Psychoneuroendocrinology*. 20. 879-890.

20. Aston-Jones. G. and Bloom. F.E.. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. (1981) *J. Neurosci.* 1. 876-886.
21. Aston-Jones. G.. Rajkowski. J.. and Cohen. J.. Role of locus coeruleus in attention and behavioral flexibility. (1999) *Biol. Psychia.* 46. 1309-1320.
22. Aston-Jones. G.. Rajkowski. J.. Kubiak. P.. and Alexinsky. T.. Locus coeruleus neurons in monkey are selectively activated by attended cues in a vigilance task. (1994) *J. Neurosci.* 14. 4467-4480.
23. Aubry. J.M.. Bartanusz. V.. Jezova. D.. Belin. D.. and Kiss. J.Z.. Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity. (1999) *J. Neuroendocrinol.* 11. 377-384.
24. Baklavadzhyan. O.G.. Pogosyan. N.L.. Arshakyan. A.V.. Darbinyan. A.G.. Khachatryan. A.V.. and Kikogosyan. T.G.. Studies of the role of the central nucleus of the amygdala in controlling cardiovascular functions. (2000) *Neurosci. Behav. Physiol.* 30. 231-236.
25. Baldwin. H.A.. Hitchcott. P.K.. and File. S.E.. Evidence that the increased anxiety detected in the elevated plus-maze during chlordiazepine withdrawal is not due to enhanced noradrenergic activity. (1989) *Pharmacol. Biochem. Behav.* 34. 931-933.
26. Bale. T.L.. Contarino. A.B.. Smith. G.W.. Chan. R.. Gold. L.H.. Sawchenko. P.E.. Koob. G.F.. Vale. W.W.. and Lee. K.F.. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. (2000) *Nat. Gen.* 24. 410-414.
27. Barbanel. G.. Ixart. G.. Malaval. F.. Mas. N.. Didier. M.. and Assenmacher. I.. Adrenergic activation of the liberation of corticotropin-releasing hormone (CRH41) in the median eminence of unanesthetized rats. (1987) *Comptes Rendus de l'Academie des Sciences - Serie III, Sciences de la Vie.* 305. 703-708.
28. Barbanel. G.. Ixart. G.. Szafarczyk. A.. Malaval. F.. and Assenmacher. I.. Intrahypothalamic infusion of interleukin-1 beta increases the release of corticotropin-releasing hormone (CRH 41) and adrenocorticotrophic hormone (ACTH) in free-moving rats bearing a push-pull cannula in the median eminence. (1990) *Brain Res.* 516. 31-36.

29. Bartanusz, V., Jezova, D., Bertini, L.T., Tilders, F.J., Aubry, J.M., and Kiss, J.Z., Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. (1993) *Endocrinology*, 132, 895-902.
30. Beaulieu, S., Di Paolo, T., and Barden, N., Control of ACTH secretion by the central nucleus of the amygdala: implication of the serotonergic system and its relevance to the glucocorticoid delayed negative feedback mechanism. (1986) *Neuroendocrinology*, 44, 247-254.
31. Beaulieu, S., Di Paolo, T., Cote, J., and Barden, N., Participation of the central amygdaloid nucleus in the response of adrenocorticotropin secretion to immobilization stress: opposing roles of the noradrenergic and dopaminergic systems. (1987) *Neuroendocrinology*, 45, 37-46.
32. Beaulieu, S., Di Paolo, T., Cote, J., and Barden, N., Participation of the central amygdaloid nucleus in the response of adrenocorticotropin secretion to immobilization stress: opposing roles of the noradrenergic and dopaminergic systems. (1987) *Neuroendocrinology*, 45, 37-46.
33. Beaulieu, S., Pelletier, G., Vaudry, H., and Barden, N., Influence of the central nucleus of the amygdala on the content of corticotropin-releasing factor in the median eminence. (1989) *Neuroendocrinology*, 49, 255-261.
34. Beckett, S.R., Duxon, M.S., Aspley, S., and Marsden, C.A., Central c-fos expression following 20kHz/ultrasound induced defence behaviour in the rat. (1997) *Brain Res. Bull.*, 42, 421-426.
35. Belzung, C., Rodent models of anxiety-like behaviors: are they predictive for compounds acting via non-benzodiazepine mechanisms?. (2001) *Curr. Opin. Invest. Drugs*, 2, 1108-1111.
36. Ben Jonathan, N., Dopamine: a prolactin-inhibiting hormone. (1985) *Endocrine Rev.*, 6, 564-589.
37. Benker, G., Jaspers, C., Hausler, G., and Reinwein, D., Control of prolactin secretion. (1990) *Klinische Wochenschrift*, 68, 1157-1167.

38. Berecek, K.H., Olpe, H.R., Jones, R.S., and Hofbauer, K.G., Microinjection of vasopressin into the locus coeruleus of conscious rats. (1984) *Am. J. Physiol.*, 247, H675-H681.
39. Berglund, B., Lindvall, T., and Schwela, D.H., Guidelines for community noise. (1999) *Report by World Health Organization*.
40. Berman, R.F. and Kesner, R.P., Posttrial hippocampal, amygdaloid, and lateral hypothalamic electrical stimulation: effects on short- and long-term memory of an appetitive experience. (1976) *J. Comp. Physiol. Psychol.*, 90, 260-267.
41. Berridge, C.W. and Dunn, A.J., Restraint-stress-induced changes in exploratory behavior appear to be mediated by norepinephrine-stimulated release of CRF. (1989) *J. Neurosci.*, 9, 3513-3521.
42. Berridge, K.C., Food reward: brain substrates of wanting and liking. (1996) *Neurosci. Biobehav. Rev.*, 20, 1-25.
43. Berridge, K.C. and Robinson, T.E., What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience. (1998) *Brain Res. - Brain Res. Rev.*, 28, 309-369.
44. Biro, E., Sarnyai, Z., Penke, B., Szabo, G., and Telegdy, G., Role of endogenous corticotropin-releasing factor in mediation of neuroendocrine and behavioral responses to cholecystokinin octapeptide sulfate ester in rats. (1993) *Neuroendocrinology*, 57, 340-345.
45. Bissette, G. (1991) Neuropeptides involved in stress and their distribution in the mammalian central nervous system. In McCubbin, J.A., Kauffman, G., Nemeroff, C.B. (Eds.) *Stress, Neuropeptides, and Systemic Disease*, Academic Press, San Diego, CA, pp.55-72.
46. Bittencourt, J.C. and Sawchenko, P.E., Do centrally administered neuropeptides access cognate receptors?: An analysis in the central corticotropin-releasing factor system. (2000) *J. Neurosci.*, 20, 1142-1156.
47. Blackburn, J.R., Pfaus, J.G., and Phillips, A.G., Dopamine functions in appetitive and defensive behaviours. (1992) *Prog. Neurobiol.*, 39, 247-279.

48. Blanchard, R.J., Griebel, G., Henrie, J.A., and Blanchard, D.C., Differentiation of anxiolytic and panicolytic drugs by effects on rat and mouse defense test batteries. (1997) *Neurosci. Biobehav. Rev.*, 21, 783-789.
49. Block, C.H., Hoffman, G., and Kapp, B.S., Peptide-containing pathways from the parabrachial complex to the central nucleus of the amygdala. (1989) *Peptides*, 10, 465-471.
50. Bloom, F.E., Battenberg, E.L., Rivier, J., and Vale, W., Corticotropin-releasing factor (CRF): Immunoreactive neurons and fibers in rat hypothalamus. (1982) *Regul. Pep.*, 4, 43-48.
51. Bodnoff, S.R., Suranyi-Cadotte, B.E., Quirion, R., and Meaney, M.J., A comparison of the effects of diazepam versus several typical and atypical antidepressant drugs in an animal model of anxiety. (1989) *Psychopharmacology*, 97, 277-279.
52. Bohus, B. and Koolhaas, J.M. (1993) Stress and the cardiovascular system: Central and peripheral physiological mechanisms. In Stanford, S.C., Salmon, P. (Eds.) *Stress: From Synapse to Syndrome*. Academic Press, London, pp.75-117.
53. Bonsall, R.W., Weiss, F., and Weiss, F., Corticosterone levels in rat brain microdialysates following exposure to noise stress or to lipopolysaccharide. (1996) *Soc. Neurosci. Abs.*, 22, 453.
54. Bragin, E.O., Moody, T., Pert, K.B., Pert, A., and Durinian, R.A., Changes of opiate-, bombesin-, and substance P-like substances in stress-induced analgesia in rats. (1982) *Voprosy Meditsinskoi Khimii*, 28, 44-48.
55. Britton, D.R., Varela, M., Garcia, A., and Rosenthal, M., Dexamethasone suppresses pituitary-adrenal but not behavioral effects of centrally administered CRF. (1986) *Life Sci.*, 38, 211-216.
56. Britton, K.T., Lee, G., Vale, W., Rivier, J., and Koob, G.F., Corticotropin releasing factor (CRF) receptor antagonist blocks activating and anxiogenic actions of CRF in the rat. (1986) *Brain Res.*, 369, 303-306.
57. Brown, G.W., Life events and affective disorder: Replications and limitations. (1993) *Psychosom. Med.*, 55, 248-259.

58. Brown, M.. Corticotropin releasing factor: central nervous system sites of action. (1986) *Brain Res.*, 399, 10-14.
59. Brown, M., Rivier, J., and Vale, W.. Somatostatin: central nervous system actions on glucoregulation. (1979) *Endocrinology*, 104, 1709-1715.
60. Brown, M., Tache, Y., and Fisher, D.. Central nervous system action of bombesin: mechanism to induce hyperglycemia. (1979) *Endocrinology*, 105 (3), 660-665.
61. Brown, M.R.. Central nervous system sites of action of bombesin and somatostatin to influence plasma epinephrine levels. (1983) *Brain Res.*, 276, 253-257.
62. Brown, M.R., Carver, K., and Fisher, L.A.. Bombesin: central nervous system actions to affect the autonomic nervous system. (1988) *Ann.NY Acad. Sci.*, 547, 174-182.
63. Brown, M.R. and Fisher, D.A.. Glucoregulation and the sympathetic nervous system: CNS control by brain peptides. (1980) *Soc. Gen.Physiol. Series*, 35, 81-97.
64. Brown, M.R. and Fisher, L.A.. Corticotropin releasing factor: effects on the autonomic nervous system and visceral systems, (1985) *Fed. Proc.*, 44, 243-248.
65. Brown, M.R. and Gray, T.S.. Peptide injections into the amygdala of conscious rats: effects on blood pressure, heart rate and plasma catecholamines. (1988) *Regula. Pep.*, 21, 95-106.
66. Brown, M.R., Rivier, J., and Vale, W.W.. Bombesin affects the central nervous system to produce hyperglycemia in rats, (1977) *Life Sci.*, 21, 1729-1734.
67. Buckingham, J.C., Cowell, A.-M., Gillies, G.E., Herbison, A.E., and Steel, J.H. (1997) Neuroendocrine responses to stress. In Buckingham, J.C., Gillies, G.E., Cowell, A.-M. (Eds.) *Stress, stress hormones and the immune system*, John Wiley & Sons, New York, pp.9-47.
68. Burrows, H.L., Nakajima, M., Lesh, J.S., Goosens, K.A., Samuelson, L.C., Inui, A., Camper, S.A., and Seasholtz, A.F.. Excess corticotropin releasing hormone-

- binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice. (1998) *J. Clin. Invest.*, 101, 1439-1447.
69. Buwalda, B., Nyakas, C., Koolhaas, J.M., and Bohus, B., Neuroendocrine and behavioral effects of vasopressin in resting and mild stress conditions. (1993) *Physio. Beh.*, 54, 947-953.
 70. Cahill, L., Haier, R.J., Fallon, J., Alkire, M.T., Tang, C., Keator, D., Wu, J., and McGaugh, J.L., Amygdala activity at encoding correlated with long-term, free recall of emotional information. (1996) *Proc. Nat. Acad. Sci. U.S.A.*, 93, 8016-8021.
 71. Cahill, L., Weinberger, N.M., Roozendaal, B., and McGaugh, J.L., Is the amygdala a locus of "conditioned fear"? Some questions and caveats. (1999) *Neuron*, 23, 227-228.
 72. Campeau, S., Akil, H., and Watson, S.J., Lesions of the medial geniculate nuclei specifically block corticosterone release and induction of c-fos mRNA in the forebrain associated with audiogenic stress in rats. (1997) *J. Neurosci.*, 17, 5979-5992.
 73. Campeau, S. and Watson, S.J., Connections of some auditory-responsive posterior thalamic nuclei putatively involved in activation of the hypothalamo-pituitary-adrenocortical axis in response to audiogenic stress in rats: An anterograde and retrograde tract tracing study combined with Fos expression. (2000) *J. Comp. Neurol.*, 423, 474-491.
 74. Camus, J.F., Nicolas, S., Wenisch, E., Morrone, I., Blanchard, F., and Bakchine, S., Implicit memory for words presented in short texts is preserved in Alzheimer's Disease. (2001) *Revue Neurologique*, 157 Suppl 10, 52.
 75. Canteras, N.S. and Swanson, L.W., Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat. (1992) *J. Comp. Neurol.*, 324, 180-194.
 76. Carlson, N.R. (2001) *Physiology and Behavior*, Allyn and Bacon, Boston.
 77. Carver-Moore, K., Gray, T.S., and Brown, M.R., Central nervous system site of action of bombesin to elevate plasma concentrations of catecholamines. (1991) *Brain Res.*, 541, 225-231.

78. Chakraborti, S., Chakraborti, T., Mandal, M., Das, S., and Batabyal, S.K.. Hypothalamic-pituitary-thyroid axis status of humans during development of ageing process. (1999) *Clinica Chimica Acta*. 288, 137-145.
79. Chalmers, D.T., Lovenberg, T.W., and De Souza, E.B.. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: Comparison with CRF1 receptor mRNA expression. (1995) *J. Neurosci.* 15, 6340-6350.
80. Chaouloff, F.. Physiopharmacological interactions between stress hormones and central serotonergic systems. (1993) *Brain Res. Rev.* 18, 1-32.
81. Chaouloff, F.. Serotonin, stress and corticoids. (2000) *J. Psychopharmacol.* 14, 139-151.
82. Chaouloff, F., Baudrie, V., and Coupry, I.. Behavioural and biochemical evidence that glucocorticoids are not involved in DOI-elicited 5-HT₂ receptor down-regulation. (1993) *Eur. J. Pharmacol.* 249, 117-120.
83. Chappell, P.B., Smith, M.A., Kilts, C.D., Bissette, G., Ritchie, J., Anderson, C., and Nemeroff, C.B.. Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. (1986) *J. Neurosci.* 6, 2908-2914.
84. Childress, A.R., Mozley, P.D., McElgin, W., Fitzgerald, J., Reivich, M., and O'Brien, C.P.. Limbic activation during cue-induced cocaine craving. (1999) *Am. J. Psychia.* 156, 11-18.
85. Childs, G.V., Westlund, K.N., and Unabia, G.. Characterization of anterior pituitary target cells for arginine vasopressin: including cells that store adrenocorticotropin, thyrotropin-beta, and both hormones. (1989) *Endocrinology*. 125, 554-559.
86. Chisari, A., Carino, M., Perone, M., Gaillard, R.C., and Spinedi, E.. Sex and strain variability in the rat hypothalamo-pituitary-adrenal (HPA) axis function. (1995) *J. Endocrinol. Invest.* 18, 25-33.
87. Chodobski, A., Loh, Y.P., Corsetti, S., Szmydynger-Chodobska, J., Johanson, C.E., Lim, Y.P., and Monfils, P.R.. The presence of arginine vasopressin and its

- mRNA in rat choroid plexus epithelium. (1997) *Brain Res., Mol. Brain Res.*, 48, 67-72.
88. Cook, C.J.. Measuring of extracellular cortisol and corticotropin-releasing hormone in the amygdala using immunosensor coupled microdialysis. (2001) *J. Neurosci. Met.*, 110, 95-101.
 89. Cortright, D.N., Nicoletti, A., and Seasholtz, A.F.. Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. (1995) *Mol. Cell. Endocrinol.*, 111, 147-157.
 90. Coste, S.C., Kesterson, R.A., Heldwein, K.A., Stevens, S.L., Heard, A.D., Hollis, J.H., Murray, S.E., Hill, J.K., Pantely, G.A., Hohimer, A.R., Hatton, D.C., Phillips, T.J., Finn, D.A., Low, M.J., Rittenberg, M.B., Stenzel, P., and Stenzel-Poore, M.P.. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. (2000) *Nat. Genet.*, 24, 403-409.
 91. Costello, J.F., Brown, M.R., and Gray, T.S.. Bombesin immunoreactive neurons in the hypothalamic paraventricular nucleus innervate the dorsal vagal complex in the rat. (1991) *Brain Res.*, 542, 77-82.
 92. Cratty, M.S. and Birkle, D.L.. N-methyl-D-aspartate (NMDA)-mediated corticotropin-releasing factor (CRF) release in cultured rat amygdala neurons. (1999) *Peptides*, 20, 93-100.
 93. Crestani, F., Lorez, M., Baer, K., Essrich, C., Benke, D., Laurent, J.P., Belzung, C., Fritschy, J.M., Luscher, B., and Mohler, H.. Decreased GABA(A)-receptor clustering results in enhanced anxiety and a bias for threat cues. (1999) *Nat. Neurosci.*, 2, 833-839.
 94. Crofford, L.J., Jacobson, J., and Young, E.. Modeling the involvement of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes in autoimmune and stress-related rheumatic syndromes in women. (1999) *J. Womens Hlth.*, 8, 203-215.
 95. Croiset, G., Nijsen, M.J., and Kamphuis, P.J.. Role of corticotropin-releasing factor, vasopressin and the autonomic nervous system in learning and memory. (2000) *Eur. J. Pharmacol.*, 405, 225-234.

96. Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H., and Watson, S.J.. Pattern and time course of immediate early gene expression in rat brain following acute stress. (1995) *Neuroscience*, 64, 477-505.
97. Cunningham, E.T.Jr., Bohn, M.C., and Sawchenko, P.E.. Organization of adrenergic inputs to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. (1990) *J. Comp. Neurol.*, 292, 651-667.
98. Cunningham, E.T.Jr., and Sawchenko, P.E.. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. (1988) *J. Comp. Neurol.*, 274, 60-76.
99. Curtis, A.L., Lechner, S.M., Pavcovich, L.A., and Valentino, R.J.. Activation of the locus coeruleus noradrenergic system by intracoeular microinfusion of corticotropin-releasing factor: effects on discharge rate, cortical norepinephrine levels and cortical electroencephalographic activity, (1997) *J. Pharmacol. Exp. Therapeut.*, 281, 163-172.
100. Curtis, A.L., Pavcovich, L.A., Grigoriadis, D.E., and Valentino, R.J.. Previous stress alters corticotropin-releasing factor neurotransmission in the locus coeruleus. (1995) *Neuroscience*, 65, 541-550.
101. Curtis, A.L., Pavcovich, L.A., and Valentino, R.J.. Long-term regulation of locus ceruleus sensitivity to corticotropin-releasing factor by swim stress. (1999) *J. Pharmacol. Exp. Therapeut.*, 289, 1211-1219.
102. Daftary, S.S., Boudaba, C., Szabó, K., and Tasker, J.G.. Noradrenergic excitation of magnocellular neurons in the rat hypothalamic paraventricular nucleus via intranuclear glutamatergic circuits. (1998) *J. Neurosci.*, 18, 10619-10628.
103. Daftary, S.S., Boudaba, C., and Tasker, J.G.. Noradrenergic regulation of parvocellular neurons in the rat hypothalamic paraventricular nucleus. (2000) *Neuroscience*, 96, 743-751.
104. Dahlstrom, A. and Fuxe, K.. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. (1964) *Acta Physiologica Scandinavica*, 62, 5-55.
105. Dallman, M.F., Akana, S.F., Strack, A.M., Hanson, S., and Sebastian, R.J.. The neural network that regulates energy balance is responsive to glucocorticoids and

- insulin and also regulates HPA axis responsivity at sites proximal to CRF neurons. (1995) *Ann. NY Acad. Sci.* 771. 730-742.
106. Danielsen. E.H., Magnuson. D.J., and Gray. T.S., The central amygdaloid nucleus innervation of the dorsal vagal complex in rat: a Phaseolus vulgaris leucoagglutinin lectin anterograde tracing study. (1989) *Brain Res. Bull.* 22. 705-715.
 107. Davis. M., The role of the amygdala in fear and anxiety. (1992) *Ann. Rev. Neurosci.* 15. 353-375.
 108. Davis. M., Rainnie. D., and Cassell. M., Neurotransmission in the rat amygdala related to fear and anxiety. (1994) *Trends Neurosci.* 17. 208-214.
 109. Davis. M. and Whalen. P.J., The amygdala: vigilance and emotion. (2001) *Mol. Psychia.* 6. 13-34.
 110. Day. H.E., Badiani. A., Uslaner. J.M., Oates. M.M., Vittoz. N.M., Robinson. T.E., Watson. S.J., Jr., and Akil. H., Environmental novelty differentially affects c-fos mRNA expression induced by amphetamine or cocaine in subregions of the bed nucleus of the stria terminalis and amygdala. (2001) *J. Neurosci.* 21. 732-740.
 111. Day. H.E., Campeau. S., Watson. S.J.J., and Akil. H., Expression of alpha(1b) adrenoceptor mRNA in corticotropin-releasing hormone-containing cells of the rat hypothalamus and its regulation by corticosterone. (1999) *J. Neurosci.* 19. 10098-10106.
 112. Dayas. C.V., Buller. K.M., Crane. J.W., Xu. Y., and Day. T.A., Stressor categorization: Acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. (2001) *Eur. J. Neurosci.* 14. 1143-1152.
 113. Dayas. C.V., Buller. K.M., and Day. T.A., Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. (1999) *Eur. J. Neurosci.* 11. 2312-2322.
 114. de Jonge. F.H., Tonnaer. J.A., van Leeuwe. H., Tielemans. A.J., Louwerse. A.L., and van de Poll. N.E., Cerebral glucose utilization during conditioned sexual arousal. (1992) *Physiol. Behav.* 52. 1009-1013.

115. De Oliveira, L., Hoffmann, A., and Menescal-de-Oliveira, L., Participation of the medial and anterior hypothalamus in the modulation of tonic immobility in guinea pigs. (1997) *Physiol. Behav.*, 62, 1171-1178.
116. De Souza, E.B., Whitehouse, P.J., Price, D.L., and Vale, W.W., Abnormalities in corticotropin-releasing hormone (CRH) in Alzheimer's disease and other human disorders. (1987) *Ann. NY Acad. Sci.*, 512, 237-247.
117. De Wied, D., Long-term effect of vasopressin on the maintenance of a conditioned avoidance response in rats. (1971) *Nature*, 232, 58-60.
118. De Wied, D., Diamant, M., and Fodor, M., Central nervous system effects of the neurohypophyseal hormones and related peptides. (1993) *Front. Neuroendocrinol.*, 14, 251-302.
119. Deak, T., Nguyen, K.T., Ehrlich, A.L., Watkins, L.R., Spencer, R.L., Maier, S.F., Licinio, J., Wong, M.L., Chrousos, G.P., Webster, E., and Gold, P.W., The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. (1999) *Endocrinology*, 140, 79-86.
120. Dhabhar, F.S., McEwen, B.S., and Spencer, R.L., Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels--a comparison between Sprague-Dawley, Fischer 344 and Lewis rats. (1993) *Brain Res.*, 616, 89-98.
121. Dhabhar, F.S., McEwen, B.S., and Spencer, R.L., Adaptation to prolonged or repeated stress--comparison between rat strains showing intrinsic differences in reactivity to acute stress. (1997) *Neuroendocrinology*, 65, 360-368.
122. Dhabhar, F.S., Miller, A.H., McEwen, B.S., and Spencer, R.L., Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats. (1995) *J. Neuroimmunol.*, 56, 77-90.
123. Di Michele, S., Sillen, U., Engel, J.A., Hjalmas, K., Rubenson, A., and Soderpalm, B., Desmopressin and vasopressin increase locomotor activity in the rat via a central mechanism: implications for nocturnal enuresis. (1996) *J. Urol.*, 156, 1164-1168.
124. Dietl, H., Temporal relationship between noradrenaline release in the central amygdala and plasma noradrenaline secretion in rats and tree shrews, (1985) *Neurosci. Lett.*, 55, 41-46.

125. Diorio, D., Viau, V., and Meaney, M.J.. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. (1993) *J. Neurosci.*, 13, 3839-3847.
126. Doherty, M.D. and Gratton, A.. NMDA receptors in nucleus accumbens modulate stress-induced dopamine release in nucleus accumbens and ventral tegmental area. (1997) *Synapse*, 26, 225-234.
127. Dominguez, J.M.H.. Stimulation of the medial amygdala enhances medial preoptic dopamine release: implications for male rat sexual behavior. (2001) *Brain Res.*, 917, 225-229.
128. Dufresne, C., Kelly, M.E., and McIntyre, D.. New fast and slow kindling rat strains. (1989) *Epilepsia*, 30, 652.
129. Dunn, A.J. and Berridge, C.W.. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses?. (1990) *Brain Res. Rev.*, 15, 71-100.
130. Dunn, J.D. and Whitener, J.. Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. (1986) *Neuroendocrinology*, 42, 211-217.
131. Duval, F., Mokrani, M.C., Bailey, P., Correa, H., Diep, T.S., Crocq, M.A., and Macher, J.P.. Thyroid axis activity and serotonin function in major depressive episode. (1999) *Psychoneuroendocrinology*, 24, 695-712.
132. Eaves, M., Thatcher, B.K., Rivier, J., Vale, W., and Koob, G.F.. Effects of corticotropin releasing factor on locomotor activity in hypophysectomized rats. (1985) *Peptides*, 6, 923-926.
133. Eghbal-Ahmadi, M., Avishai-Eliner, S., Hatalski, C.G., and Baram, T.Z.. Differential regulation of the expression of corticotropin-releasing factor receptor type 2 (CRF2) in hypothalamus and amygdala of the immature rat by sensory input and food intake. (1999) *J. Neurosci.*, 19, 3982-3991.
134. Esposito, S., Prange, A.J., and Golden, R.N.. The thyroid axis and mood disorders-overview and future prospects. (1997) *Psychopharmacol. Bull.*, 33, 205-217.

135. Fekete, C., Kelly, J., Mihaly, E., Sarkar, S., Rand, W.M., Legradi, G., Emerson, C.H., and Lechan, R.M., Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis. (2001) *Endocrinology*, 142, 2606-2613.
136. Feldman, S., Conforti, N., Itzik, A., and Weidenfeld, J., Differential effect of amygdaloid lesions on CRF-41, ACTH, and corticosterone responses following neural stimuli. (1994) *Brain Res.*, 658, 21-26.
137. Ferrier, I.N. and Leake, A., Peptides in the neocortex in Alzheimer's disease and ageing. (1990) *Psychoneuroendocrinology*, 15, 89-95.
138. Finlay, J.M. and Zigmond, M.J., The effects of stress on central dopaminergic neurons: possible clinical implications. (1997) *Neurochem. Res.*, 22, 1387-1394.
139. Fisher, L.A., Cave, C.R., and Brown, M.R., Central nervous system cardiovascular effects of bombesin in conscious rats. (1985) *Am. J. Physiol.*, 248, H425-H431.
140. Fitch, D., Lesage, A., Seguin, M., Trousignant, M., Bankelfat, C., Rouleau, G.A., and Turecki, G., Suicide and the serotonin transporter gene. (2001) *Mol. Psychia.*, 6, 127-128.
141. Fliers, E., Alkemade, A., and Wiersinga, W.M., The hypothalamic-pituitary-thyroid axis in critical illness. (2001) *Best Prac. Res. Clin. Endocrinol. Metabol.*, 15, 453-464.
142. Flood, J.F. and Morley, J.E., Effects of bombesin and gastrin-releasing peptide on memory processing. (1988) *Brain Res.*, 460, 314-322.
143. Florin-Lechner, S.M., Druhan, J.P., Aston-Jones, G., and Valentino, R.J., Enhanced norepinephrine release in prefrontal cortex with burst stimulation of the locus coeruleus. (1996) *Brain Res.*, 742, 89-97.
144. Folkow, B., Hallback-Nordlander, M., Martner, J., and Nordborg, C., Influence of amygdala lesions on cardiovascular responses to alerting stimuli, on behaviour and on blood pressure development in spontaneously hypertensive rats. (1982) *Acta Physiologica Scandinavica*, 116, 133-139.

145. Follenius, M., Brandenberger, G., and Hietter, B., Diurnal cortisol peaks and their relationships to meals. (1982) *J. Clin. Endocrinol. Metab.*, 55, 757-761.
146. Fox, M.W., Anderson, R.E., and Meyer, F.B., Neuroprotection by corticotropin releasing factor during hypoxia in rat brain. (1993) *Stroke*, 24, 1072-1076.
147. Freeman, M.E., Kanyicska, S., Lerant, A., and Nagy, G., Prolactin: structure, function, and regulation of secretion. (2000) *Physiol. Rev.*, 80, 1523-1631.
148. Fukuda, M. and Ono, T., Amygdala-hypothalamic control of feeding behavior in monkey: Single cell responses before and after reversible blockade of temporal cortex or amygdala projections. (1993) *Behav. Brain Res.*, 55, 233-241.
149. Gabr, R.W., Birkle, D.L., and Azzaro, A.J., Stimulation of the amygdala by glutamate facilitates corticotropin-releasing factor release from the median eminence and activation of the hypothalamic-pituitary-adrenal axis in stressed rats. (1995) *Neuroendocrinology*, 62, 333-339.
150. Gaffan, E.A., Gaffan, D., and Harrison, S., Disconnection of the amygdala from visual association cortex impairs visual reward-association learning in monkeys. (1988) *J. Neurosci.*, 8, 3144-3150.
151. Galeno, T.M., Van Hoesen, G.W., and Brody, M.J., Central amygdaloid nucleus lesion attenuates exaggerated hemodynamic responses to noise stress in the spontaneously hypertensive rat. (1984) *Brain Res.*, 291, 249-259.
152. Garcia, R., Stress, hippocampal plasticity, and spatial learning. (2001) *Synapse*, 40, 180-183.
153. Garrido, M.M., Martin, S., Ambrosio, E., Fuentes, J.A., and Manzanares, J., Role of corticotropin-releasing hormone in gastrin-releasing peptide-mediated regulation of corticotropin and corticosterone secretion in male rats. (1998) *Neuroendocrinology*, 68, 116-122.
154. Gibbs, J., Fauser, J.D., Rowe, E.A., Rolls, B.J., Rolls, E.T., and Maddison, S.P., Bombesin suppresses feeding in rats. (1979) *Nature*, 282, 208-210.
155. Gibbs, J. and Smith, G.P., The actions of bombesin-like peptides on food intake. (1988) *Annals of the New York Academy of Sciences*, 547, 210-216.

156. Gillies, G.E., Linton, E.A., and Lowry, P.J., Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin, (1982) *Nature*, 299, 355-357.
157. Glowa, J.R., Geyer, M.A., Gold, P.W., and Sternberg, E.M., Differential startle amplitude and corticosterone response in rats, (1992) *Neuroendocrinology*, 56, 719-723.
158. Glowa, J.R., Sternberg, E.M., and Gold, P.W., Differential behavioral response in LEW/N and F344/N rats: Effects of corticotropin releasing hormone. (1991) *Prog. Neuro-Psychopharm. Biol. Psychia.*, 18, 549-560.
159. Goldberg, S. (1997) *Lipidland. Clinical biochemistry made ridiculously simple*. Memaster Inc., Miami.
160. Goldstein, L.E., Rasmusson, A.M., Bunney, B.S., and Roth, R.H., Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress, (1996) *J. Neurosci.*, 16, 4787-4798.
161. Gomez, F., De Kloet, E.R., and Armario, A., Glucocorticoid negative feedback on the HPA axis in five inbred rat strains, (1998) *Am. J. Physiol.*, 274, R420-R427.
162. Gray, T.S. (1991) Amygdala: role in autonomic and neuroendocrine responses to stress. In McCubbin, J.A., Kauffman, G., Nemeroff, C.B. (Eds.) *Stress, neuropeptides and systemic disease*. Academic, New York, pp.37-53.
163. Gray, T.S., Carney, M.E., and Magnuson, D.J., Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release, (1989) *Neuroendocrinology*, 50, 433-446.
164. Gray, T.S. and Magnuson, D.J., Neuropeptide neuronal efferents from the bed nucleus of the stria terminalis and central amygdaloid nucleus to the dorsal vagal complex in the rat. (1987) *J. Comp. Neurol.*, 262, 365-374.
165. Gresch, P.J., Sved, A.F., Zigmond, M.J., and Finlay, J.M., Stress-induced sensitization of dopamine and norepinephrine efflux in medial prefrontal cortex of the rat. (1994) *J. Neurochem.*, 63, 575-583.

166. Griebel, G., Perrault, G., and Sanger, D.J., Characterization of the behavioral profile of the non-peptide CRF receptor antagonist CP-154,526 in anxiety models in rodents. Comparison with diazepam and buspirone. (1998) *Psychopharmacology*, 138, 55-66.
167. Guaza, C., Zubiaur, M., and Borrell, J., Corticosteroidogenesis modulation by beta-endorphin and dynorphin 1-17 in isolated rat adrenocortical cells. (1986) *Peptides*, 7, 237-240.
168. Guillemin, R. and Rosenberg, B., Humoral hypothalamic control of anterior pituitary: a study with combined tissue cultures. (1955) *Endocrinology*, 57, 599-607.
169. Guitart, X., Kogan, J.H., Berhow, M., Terwilliger, R.Z., Aghajanian, G.K., and Nestler, E.J., Lewis and Fischer rat strains display differences in biochemical, electrophysiological and behavioral parameters: Studies in the nucleus accumbens and locus coeruleus of drug naive and morphine-treated animals. (1993) *Brain Res.*, 611, 7-17.
170. Gunion, M.W., Tache, Y., Rosenthal, M.J., Miller, S., Butler, B., and Zib, B., Bombesin microinfusion into the rat hypothalamic paraventricular nucleus increases blood glucose, free fatty acids and corticosterone. (1989) *Brain Res.*, 478, 47-58.
171. Guo, J., Yin, W.P., Huang, W.Q., Xu, B., and Qian, Z.M., Effect of microinjection of CRH into rat central nucleus of amygdala on blood pressure and its central mechanism. (1997) *Sheng Li Hsueh Pao - Acta Physiologica Sinica*, 49, 110-114.
172. Habib, K.E., Weld, K.P., Rice, K.C., Pushkas, J., Champoux, M., Listwak, S., Webster, E.L., Atkinson, A.J., Schulkin, J., Contoreggi, C., Chrousos, G.P., McCann, S.M., Suomi, S.J., Higley, J.D., and Gold, P.W., Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates (2000) *Proc. Nat. Acad. Sci. U.S.A.*, 97, 8745.
173. Hall, J., Parkinson, J.A., Connor, T.M., Dickinson, A., and Everitt, B.J., Involvement of the central nucleus of the amygdala and nucleus accumbens core in mediating Pavlovian influences on instrumental behaviour. (2001) *Eur. J. Neurosci.*, 13, 1984-1992.

174. Hallbeck, M., Hermanson, O., and Blomqvist, A., Distribution of preprovasopressin mRNA in the rat central nervous system. (1999) *J. Comp. Neurol.*, 411, 181-200.
175. Hansen, K., Sickelmann, F., Pietrowsky, R., Fehm, H.L., and Born, J., Systemic immune changes following meal intake in humans. (1997) *Am. J. Physiol. Reg. Integ. Comp. Physiol.*, 273, R548-R553.
176. Harbuz, M.S., Jessop, D.S., Lightman, S.L., and Chowdrey, H.S., The effects of restraint or hypertonic saline stress on corticotropin-releasing factor, arginine vasopressin, and proenkephalin A mRNAs in the CFY, Sprague-Dawley and Wistar strains of rat. (1994) *Brain Res.*, 667, 6-12.
177. Harper, R.M., Frysinger, R.C., Trelease, R.B., and Marks, J.D., State dependent alteration of respiratory cycle timing by stimulation of the central nucleus of amygdala. (1984) *Brain Res.*, 306, 1-8.
178. Hassanyeh, F. and Marshall, E.F., Measures of serotonin metabolism in anorexia nervosa. (1991) *Acta Psychiatr Scand.* 84, 561-563.
179. Health Canada and Statistics Canada, Statistical report on the health of Canadians. (1999) *Report by Government of Canada.* 49-52.
180. Heilig, M., Koob, G.F., Ekman, R., and Britton, K.T., Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. (1994) *Trends Neurosci.*, 17, 80-85.
181. Heinrichs, S.C. and Joppa, M., Dissociation of arousal-like from anxiogenic-like actions of brain corticotropin-releasing factor receptor ligands in rats. (2001) *Beh. Brain Res.*, 122, 43-50.
182. Heinrichs, S.C., Menzaghi, F., Pich, E.M., Baldwin, H.A., Rassnick, S., Britton, K.T., and Koob, G.F., Anti-stress action of a corticotropin-releasing factor antagonist on behavioral reactivity to stressors of varying type and intensity. (1994) *Neuropsychopharmacology*, 11, 179-186.
183. Herbert, T.B. and Cohen, S., Stress and immunity in humans. (1993) *Psychosom. Med.*, 55, 364-379.

184. Herman, J.P., In situ hybridization analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids. (1995) *J. Comp. Neurol.* 363, 15-27.
185. Herman, J.P. and Cullinan, W.E., Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. (1997) *Trends Neurosci.* 20, 78-84.
186. Hernandez, D.E., Nemeroff, C.B., Orlando, R.C., and Prange, A.J., Jr., The effect of centrally administered neuropeptides on the development of stress-induced gastric ulcers in rats. (1983) *J. Neurosci. Res.* 9, 145-157.
187. Hernandez, L. and Hoebel, B.G., Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens. (1988) *Physiol. Behav.* 44, 599-606.
188. Herve-Minvielle, A. and Sara, S.J., Rapid habituation of auditory responses of locus coeruleus cells in anaesthetized and awake rats. (1995) *Neuroreport* 6, 1363-1368.
189. Hikichi, T., Akiyoshi, J., Yamamoto, Y., Tsutsumi, T., Isogawa, K., and Nagayama, H., Suppression of conditioned fear by administration of CRF receptor antagonist CP-154,526. (2000) *Pharmacopsychiatry* 33, 189-193.
190. Hinson, J.P., Paracrine control of adrenocortical function: a new role for the medulla?. (1990) *J. Endocr.* 124, 7-9.
191. Hisano, S., Fukui, Y., Chikamori-Aoyama, M., Aizawa, T., and Shibasaki, T., Reciprocal synaptic relations between CRF-immunoreactive and TRH-immunoreactive neurons in the paraventricular nucleus of the hypothalamus. (1993) *Brain Res.* 620, 343-346.
192. Honkaniemi, J., Kainu, T., Ceccatelli, S., Rechardt, L., Hokfelt, T., and Peltto-Huikko, M., Fos and jun in rat central amygdaloid nucleus and paraventricular nucleus after stress. (1992) *Neuroreport* 3, 849-852.
193. Horvitz, J.C., Williams, G., and Joy, R., Time-dependent actions of D₂ family agonist quinpirole on spontaneous behavior in the rat: Dissociation between sniffing and locomotion. (2001) *Psychopharmacology* 154, 350-355.

194. Hoschl, C. and Hajek, T., Hippocampal damage mediated by corticosteroids--a neuropsychiatric research challenge. (2001) *Eur. Arch. Psychia. Clin. Neurosci.*, 251 Suppl 2, II81-II88.
195. Hotta, M., Shibasaki, T., Arai, K., and Demura, H., Corticotropin-releasing factor receptor type 1 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats. (1999) *Brain Res.*, 823, 221-225.
196. Houben, H., Vandenbroucke, A.T., Verheyden, A.M., and Deneef, C., Expression of the genes encoding bombesin-related peptides and their receptors in anterior pituitary tissue. (1993) *Mol. Cell. Endocrinol.*, 97, 159-164.
197. Hsu, D.T., Chen, F.-L., Takahashi, L.K., and Kalin, N.H., Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: An in situ hybridization study. (1998) *Brain Res.*, 788, 305-310.
198. Hulbert, A.J., Thyroid hormones and their effects: a new perspective. (2000) *Biol. Rev.*, 75, 519-631.
199. Hull, E.M., Du, J., Lorrain, D.S., and Matuszewich, L., Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. (1995) *J. Neurosci.*, 15, 7465-7471.
200. Ida, T., Nakahara, K., Murakami, T., Hanada, R., Nakazato, M., and Murakami, N., Possible involvement of orexin in the stress reaction in rats. (2000) *Biochem. Biophys. Res. Commun.*, 270, 318-323.
201. Imaki, T., Nahan, J.L., Rivier, C., Sawchenko, P.E., and Vale, W., Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. (1991) *J. Neurosci.*, 11, 585-599.
202. Imaki, T., Shibasaki, T., Hotta, M., and Demura, H., Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress, (1993) *Brain Res.*, 616, 114-125.
203. Itoh, S., Takashima, A., Itoh, T., and Morimoto, T., Open-field behavior of rats following intracerebroventricular administration of neuromedin B, neuromedin C, and related amphibian peptides. (1994) *JPN. J. Physiol.*, 44, 271-281.

204. Ixart, G., Barbanel, G., Conte-Devolx, B., Grino, M., Oliver, C., and Assenmacher, I., Evidence for basal and stress-induced release of corticotropin releasing factor in the push-pull cannulated median eminence of conscious free-moving rats, (1987) *Neurosci. Lett.*, 74, 85-89.
205. Ixart, G., Barbanel, G., Nouguiere-Soule, J., and Assenmacher, I., A quantitative study of the pulsatile parameters of CRH-41 secretion in unanesthetized free-moving rats, (1991) *Exp. Brain Res.*, 87, 153-158.
206. Ixart, G., Siaud, P., Barbanel, G., Mekaouche, M., Givalois, L., and Assenmacher, I., Circadian variations in the amplitude of corticotropin-releasing hormone 41 (CRH41) episodic release measured in vivo in male rats: correlations with diurnal fluctuations in hypothalamic and median eminence CRH41 contents, (1993) *J. Biol. Rhythms*, 8, 297-309.
207. Jacobson, L., Muglia, L.J., Weninger, S.C., Pacak, K., and Majzoub, J.A., CRH deficiency impairs but does not block pituitary-adrenal responses to diverse stressors, (2000) *Neuroendocrinology*, 71, 79-87.
208. Jasnow, A.M., Banks, M.C., Owens, E.C., and Huhman, K.L., Differential effects of two corticotropin-releasing factor antagonists on conditioned defeat in male Syrian hamsters (*Mesocricetus auratus*), (1999) *Brain Res.*, 846, 122-128.
209. Jedema, H.P., Sved, A.F., Zigmond, M.J., and Finlay, J.M., Sensitization of norepinephrine release in medial prefrontal cortex: Effect of different chronic stress protocols, (1999) *Brain Res.*, 830, 211-217.
210. Johnson, E.O., Kamilaris, T.C., Chrousos, G.P., and Gold, P.W., Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis, (1992) *Neurosci. Biobehav. Rev.*, 16, 115-130.
211. Jones, B. and Mishkin, M., Limbic lesions and the problem of stimulus-reinforcement associations, (1972) *Exp. Neurol.*, 36, 362-377.
212. Kaehler, S.T., Singewald, N., and Philippu, A., Release of serotonin in the locus coeruleus of normotensive and spontaneously hypertensive rats (SHR), (1999) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 359, 460-465.

213. Kakucska, I., Qi, Y., and Lechan, R.M., Changes in adrenal status affect hypothalamic thyrotropin-releasing hormone gene expression in parallel with corticotropin-releasing hormone. (1995) *Endocrinology*, 136, 2795-2802.
214. Kakucska, I., Rand, W., and Lechan, R.M., Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependent upon feedback regulation by both triiodothyronine and thyroxine. (1992) *Endocrinology*, 130, 2845-2850.
215. Kalin, N.H., Shelton, S.E., Davidson, R.J., and Kelley, A.E., The primate amygdala mediates acute fear but not the behavioral and physiological components of anxious temperament. (2001) *J. Neurosci.*, 21, 2067-2074.
216. Kalin, N.H., Takahashi, L.K., and Chen, F.-L., Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. (1994) *Brain Res.*, 656, 182-186.
217. Kalra, S.P., Circadian rhythm in luteinizing hormone-releasing hormone (LH-RH) content of preoptic area during the rat estrous cycle. (1976) *Brain Res.*, 104, 354-358.
218. Karbonits, M., Trainer, P.J., Nelson, M.L., Howse, I., Kopelman, P.G., Besser, G.M., Grossman, A.B., and Svec, F., Differential stimulation of cortisol and dehydroepiandrosterone levels by food in obese and normal subjects: relation to body fat distribution. (1996) *Clin. Endocrinol.*, 45, 699-706.
219. Kasting, N.W. and Martin, J.B., Altered release of growth hormone and thyrotropin induced by endotoxin in the rat. (1982) *Am. J. Physiol.*, 243, 332-337.
220. Kawahara, H., Yoshida, M., Yokoo, H., Nishi, M., and Tanaka, M., Psychological stress increases serotonin release in the rat amygdala and prefrontal cortex assessed by in vivo microdialysis. (1993) *Neurosci. Lett.*, 162, 81-84.
221. Keck, M.E., Welt, T., Wigger, A., Renner, U., Engelmann, M., Holsboer, F., and Landgraf, R., The anxiolytic effect of the CRH1 receptor antagonist R121919 depends on innate emotionality in rats. (2001) *Eur. J. Neurosci.*, 13, 373-380.
222. Kent, P., Peptidergic mediation of response to stress: Focus on bombesin-like peptides. (2000). *Doctoral Dissertation*.

223. Kent, P., Anisman, H., and Merali, Z., Are bombesin-like peptides involved in the mediation of stress response. (1997) *Life Sci.*, 62, 103-114.
224. Kent, P., Anisman, H., and Merali, Z., Central bombesin activates the hypothalamic-pituitary-adrenal axis. Effects on regional levels and release of corticotropin-releasing hormone and arginine-vasopressin. (2001) *Neuroendocrinology*, 73, 203-214.
225. Kent, P., Bedard, T., Khan, S., Anisman, H., and Merali, Z., Bombesin-induced HPA and sympathetic activation requires CRH receptors. (2001) *Peptides*, 22, 57-65.
226. Khan, S., Michaud, D., Anisman, H., and Merali, Z., Diazepam reverses novel environment-induced feeding suppression without blocking amygdaloid CRH release. (1999) *Soc. Neurosci. Abs.*, 25, 1126.
227. King, D., Zigmond, M.J., and Finlay, J.M., Effects of dopamine depletion in the medial prefrontal cortex on the stress-induced increase in extracellular dopamine in the nucleus accumbens core and shell. (1997) *Brain Res.*, 77, 141-153.
228. Kirby, L.G., Chou-Green, J.M., Davis, K., and Lucki, I., The effects of different stressors on extracellular 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. (1997) *Brain Res.*, 760, 218-230.
229. Kirby, L.G. and Lucki, I., The effect of repeated exposure to forced swimming on extracellular levels of 5-hydroxytryptamine in the rat. (1998) *Stress*, 2, 251-263.
230. Kirby, L.G., Rice, K.C., and Valentino, R.J., Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. (2000) *Neuropsychopharmacology*, 22, 148-162.
231. Kiss, A., Palkovits, M., and Aguilera, G., Neural regulation of corticotropin releasing hormone (CRH) and CRH receptor mRNA in the hypothalamic paraventricular nucleus in the rat. (1996) *J. Neuroendocrinol.*, 8, 103-112.
232. Kitchigina, V., Vankov, A., Harley, C., and Sara, S.J., Novelty-elicited, noradrenaline-dependent enhancement of excitability in the dentate gyrus. (1997) *Eur. J. Neurosci.*, 9, 41-47.

233. Koller, K.J., Wolff, R.S., Warden, M.K., and Zoeller, R.T., Thyroid hormones regulate levels of thyrotropin-releasing-hormone mRNA in the paraventricular nucleus. (1987) *Proc. Nat. Acad. Sci. U.S.A.*, 84, 7329-7333.
234. Koob, G.F., Corticotropin-releasing factor, norepinephrine, and stress. (199) *Biol. Psychia.*, 46, 1167-1180.
235. Koob, G.F. and Heinrichs, S.C., A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. (1999) *Brain Res.*, 848, 141-152.
236. Kostich, W.A., Chen, A., Sperle, K., and Largent, B.L., Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor. (1998) *Mol. Endocrinol.*, 12, 1077-1085.
237. Kovacs, K., c-Fos as a transcription factor: a stressful (re)view from a functional map. (1998) *Neurochem. Inter.*, 33, 287-297.
238. Kovács, K.J. and Sawchenko, P.E., Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. (1996) *J. Neurosci.*, 16, 262-273.
239. Krishnan, K.R.R., Doraiswamy, P.M., Venkataraman, S., Reed, D.A., and Richie, J.C. (1991) Current concepts in hypothalamo-pituitary-adrenal axis regulation. In McCubbin, J.A., Kaufmann, P.G., Nemeroff, C.B. (Eds.) *Stress, Neuropeptides and Systemic Disease*, Academic Press, San Diego, pp.19-35.
240. Kulkosky, P.J., Gibbs, J., and Smith, G.P., Behavioral effects of bombesin administration in rats. (1982) *Physiol. Beh.*, 28, 505-512.
241. Kuroda, Y., Mikuni, M., Nomura, N., and Takahashi, K., Differential effect of subchronic dexamethasone treatment on serotonin-2 and beta-adrenergic receptors in the rat cerebral cortex and hippocampus. (1993) *Neurosci. Lett.*, 155, 195-198.
242. Kuroda, Y., Mikuni, M., Ogawa, T., and Takahashi, K., Effect of ACTH, adrenalectomy and the combination treatment on the density of 5-HT₂ receptor binding sites in neocortex of rat forebrain and 5-HT₂ receptor-mediated wet-dog shake behaviors. (1992) *Psychopharmacology*, 108, 27-32.

243. Kuru, M., Ueta, Y., Serino, R., Nakazato, M., Yamamoto, Y., Shibuya, I., and Yamashita, H., Centrally administered orexin/hypocretin activates HPA axis in rats. (2000) *Neuroreport*, 11, 1977-1980.
244. LaBar, K.S., Gatenby, J.C., Gore, J.C., Ledoux, J.E., and Phelps, E.A., Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. (1998) *Neuron*, 20, 937-945.
245. Lahmame, A., Grigoriadis, D.E., De Souza, E.B., and Armario, A., Brain corticotropin-releasing factor immunoreactivity and receptors in five inbred rat strains: relationship to forced swimming behaviour. (1997) *Brain Res.*, 750, 285-292.
246. Landgraf, R., Gerstberger, R., Montkowski, A., Probst, J.C., Wotjak, C.T., Holsboer, F., and Engelmann, M., V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. (1995) *J. Neurosci.*, 15, 4250-4258.
247. Laucher, J., Gaillet, S., Barbagli, B., Buda, M., and Tappaz, M., Differential early time course activation of the brainstem catecholaminergic groups in response to various stresses. (1991) *Neuroendocrinology*, 53, 589-596.
248. Ledoux, J.E., Emotion circuits in the brain. (2000) *Ann. Rev. Neurosci.*, 23, 155-184.
249. Ledoux, J.E., Iwata, J., Cicchetti, P., and Reis, D.J., Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. (1988) *J. Neurosci.*, 8, 2517-2529.
250. Lee, E.H. and Sung, Y.J., Differential influences of corticotropin-releasing factor on memory retention of aversive learning and appetitive learning in rats. (1989) *Beh. Neural Biol.*, 52, 285-294.
251. Lee, Y. and Davis, M., Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle response. (1997) *J. Neurosci.*, 17, 6434-6446.

252. Lemaire, V., Taylor, G.T., and Mormede, P., Adrenal axis activation by chronic social stress fails to inhibit gonadal function in male rats. (1997) *Psychoneuroendocrinology*, 22, 563-573.
253. Lezoualc'h, F., Engert, S., Berning, B., and Behl, C., Corticotropin-releasing hormone-mediated neuroprotection against oxidative stress is associated with the increased release of non-amyloidogenic amyloid beta precursor protein and with the suppression of nuclear factor-kappaB. (2000) *Mol. Endocrinol.*, 14, 147-159.
254. Li, H.-Y., Ericsson, A., and Sawchenko, P.E., Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. (1996) *Proc. Natl. Acad. Sci.*, 93, 2359-2364.
255. Liebsch, G., Landgraf, R., Gerstberger, R., Probst, J.C., Wotjak, C.T., Engelmann, M., Holsboer, F., and Montkowski, A., Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats. (1995) *Reg. Pept.*, 59, 229-239.
256. Liebsch, G., Wotjak, C.T., Landgraf, R., and Engelmann, M., Septal vasopressin modulates anxiety-related behaviour in rats. (1996) *Neurosci. Lett.*, 217, 101-104.
257. Lolait, S.J., O'Carroll, A.M., Mahan, L.C., Felder, C.C., Button, D.C., Young, W.S., III, Mezey, E., and Brownstein, M.J., Extrapituitary expression of the rat V1b vasopressin receptor gene. (1995) *Proc. Nat. Acad. Sci. U.S.A.*, 92, 6783-6787.
258. Lombardo, K.A., Herringa, R.J., Balachandran, J.S., Hsu, D.T., Bakshi, V.P., Roseboom, P.H., and Kalin, N.H., Effects of acute and repeated restraint stress on corticotropin-releasing hormone binding protein mRNA in rat amygdala and dorsal hippocampus. (2001) *Neurosci. Lett.*, 302, 81-84.
259. Lorrain, D.S., Matuszewich, L., Friedman, R.D., and Hull, E.M., Extracellular serotonin in the lateral hypothalamic area is increased during the post ejaculatory interval and impairs copulation in male rats. (1997) *J. Neurosci.*, 17, 9361-9366.
260. Lovallo, W.R. (1997) *Stress and Health*. Sage Publications. London.

261. Lovenberg, T.W., Chalmers, D.T., Liu, C., and De Souza, E.B., CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. (1995) *Endocrinology*, 136, 4139-4142.
262. Lovenberg, T.W., Liaw, C.W., Grigoriadis, D.E., Clevenger, W., Chalmers, D.T., De Souza, E.B., and Oltersdorf, T., Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. (1995) *Proc. Nat. Acad. Sci. U.S.A.*, 92, 836-840.
263. Lucassen, P.J., Tilders, F.J., Salehi, A., and Swaab, D.F., Neuropeptides vasopressin (AVP), oxytocin (OXT) and corticotropin-releasing hormone (CRH) in the human hypothalamus: activity changes in aging, Alzheimer's disease and depression. (1997) *Aging*, 9, 48-50.
264. Lucki, I., The spectrum of behaviors influenced by serotonin. (1998) *Biol. Psychia.*, 44, 151-162.
265. Lumpkin, M.D., Samson, W.K., and McCann, S.M., Arginine vasopressin as a thyrotropin-releasing hormone. (1987) *Science*, 235, 1070-1073.
266. Lupien, S., Lecours, A.R., Lussier, I., Schwartz, G., Nair, N.P.V., and Meaney, M.J., Basal cortisol levels and cognitive deficits in human aging. (1994) *J. Neurosci.*, 14, 2893-2903.
267. Lupien, S., Lecours, A.R., Schwartz, G., Sharma, S., Hauger, R.L., Meaney, M.J., and Nair, N.P.V., Longitudinal study of basal cortisol levels in healthy elderly subjects- Evidence for subgroups. (1996) *Neurobiol. Aging*, 17, 95-105.
268. Lynn, R.B., Hyde, T.M., Cooperman, R.R., and Miselis, R.R., Distribution of bombesin-like immunoreactivity in the nucleus of the solitary tract and dorsal motor nucleus of the rat and human: Colocalization with tyrosine hydroxylase. (1996) *J. Comp. Neurol.*, 369, 552-570.
269. Mahmoud, S.N., Scaccianoce, S., Scraggs, P.R., Nicholson, S.A., Gillham, B., and Jones, M.T., Characteristics of corticosteroid inhibition of adrenocorticotrophin release from the anterior pituitary gland of the rat. (1984) *J. Endocrinol.*, 102, 33-42.
270. Maidment, N.T. and Evans, C.T. (1991) Measurement of extracellular neuropeptides in the brain: microdialysis linked to sub-femtomole limits of

- detection. In Robinson.T.E., Justice.J.B. (Eds.) *Microdialysis in the neurosciences*. Elsevier. Amsterdam. pp.275-303.
271. Majzoub. J.A., Carrazana. E.J., Shulman. J.S., Baker. K.J., and Emanuel. R.L., Defective regulation of vasopressin gene expression in Brattleboro rats. (1987) *Am. J. Physiol.*, 252, E637-E642.
 272. Makino. S., Shibasaki. T., Yamauchi. N., Nishioka. T., Mimoto.T., Wakabayashi. I., Gold. P.W., and Hashimoto. K., Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. (1999) *Brain Res.*, 850, 136-143.
 273. Makino. S., Takemura. T., Asaba. K., Nishiyama. M., Takao. T., and Hashimoto. K., Differential regulation of type-1 and type-2alpha corticotropin- releasing hormone receptor mRNA in the hypothalamic paraventricular nucleus of the rat. (1997) *Brain Res. Mol. Brain Res.*, 47, 170-176.
 274. Malendowicz. L.K. and Nussdorfer. G.G., Investigations on the acute effects of neuropeptides on the pituitary-adrenocortical function in normal and cold-stressed rats. I. Bombesin and neuromedin B. (1995) *Exp. Toxicol. Pathol.*, 47, 31-34.
 275. Malpas. S.C. and Coote. J.H., Role of vasopressin in sympathetic response to paraventricular nucleus stimulation in anesthetized rats. (1994) *Am. J. Physiol.*, 266, R228-R236.
 276. Mamalaki. E., Kvetnansky. R., Brady. L.S., Gold. P.W., and Herkenham. M., Repeated immobilization stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor messenger ribonucleic acid levels in rat brain. (1992) *J. Neuroendocrinol.*, 4, 689-699.
 277. Mantey. S.A., Weber. H.C., Sainz. E., Akesson. M., Ryan. R.R., Pradhan. T.K., Searles. R.P., Spindel. E.R., Battey. J.F., Coy. D.H., and Jensen. R.T., Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates that it has a unique pharmacology compared with other mammalian bombesin receptors. (1997) *J. Biol. Chem.*, 272, 26062-26071.
 278. Martin. D.S. and Haywood. J.R., Sympathetic nervous system activation by glutamate injections into the paraventricular nucleus. (1992) *Brain Res.*, 577, 261-267.

279. Martinez, M., Phillips, P.J., and Herbert, J., Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. (1998) *Eur. J. Neurosci.*, 10, 20-33.
280. Martini, L. and Morpurgo, C., Neurohormonal control of release of adrenocorticotrophic hormone. (1955) *Nature*, 175, 1127-1128.
281. Masand, P.S. and Gupta, S., Selective serotonin-reuptake inhibitors: an update. (1999) *Harvard Rev. Psychia.*, 7, 69-84.
282. Mason, D., MacPhee, I., and Antoni, F., The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. (1990) *Immunology*, 70, 1-5.
283. McCann, S.M. and Brobeck, J.R., Evidence for a role of the supraopticohypophyseal system in the regulation of adrenocorticotrophin secretion. (1954) *Proc. Soc. Exp. Biol. Med.*, 87, 318-324.
284. McClennen, S.J., Cortright, D.N., and Seasholtz, A.F., Regulation of pituitary corticotropin-releasing hormone-binding protein messenger ribonucleic acid levels by restraint stress and adrenalectomy, (1998) *Endocrinology*, 139, 4435-4441.
285. McEwen, B.S. and Stellar, E., Stress and the individual. Mechanisms leading to disease. (1993) *Arch. Int. Med.*, 153, 2093-2101.
286. McIntyre, D.C., Kelly, M.E., and Dufresne, C., FAST and SLOW amygdala kindling rat strains: comparison of amygdala, hippocampal, piriform and perirhinal cortex kindling, (1999) *Epil. Res.*, 35, 197-209.
287. McIntyre, D.C., Kent, P., Hayley, S., Merali, Z., and Anisman, H., Influence of psychogenic and neurogenic stressors on neuroendocrine and central monoamine activity in fast and slow kindling rats. (1999) *Brain Res.*, 840, 65-74.
288. McKernan, R.M., Wafford, K., Quirk, K., Hadingham, K.L., Harley, E.A., Ragan, C.I., and Whiting, P.J., The pharmacology of the benzodiazepine site of the GABA-A receptor is dependent on the type of gamma-subunit present, (1995) *J. Recept. Signal. Transduct. Res.*, 15, 173-183.

- 289. McLaughlin, C.L. and Baile, C.A., Obese mice and the satiety effects of cholecystokinin, bombesin and pancreatic polypeptide. (1981) *Physiol. Beh.* 26, 433-437.
- 290. Melia, K.R., Ryabinin, A.E., Schroeder, R., Bloom, F.E., and Wilson, M.C., Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. (1994) *J. Neurosci.* 14, 5929-5938.
- 291. Menzaghi, F., Heinrichs, S.C., Merlo-Pich, E., Tilders, F.J., and Koob, G.F., Involvement of hypothalamic corticotropin-releasing factor neurons in behavioral responses to novelty in rats. (1994) *Neurosci. Lett.* 168, 139-142.
- 292. Menzaghi, F., Howard, R.L., Heinrichs, S.C., Vale, W., Rivier, J., and Koob, G.F., Characterization of a novel and potent corticotropin-releasing factor antagonist in rats. (1994) *J. Pharmacol. Exp. Therapeu.* 269, 564-572.
- 293. Merali, Z. and Banks, K., Does the histaminergic system mediate bombesin/GRP-induced suppression of food intake. (1994) *Am. J. Physiol. Regul. Integra. Comp. Physiol.* 267, R1589-R1595.
- 294. Merali, Z. and Kateb, C.C., Rapid alterations of hypothalamic and hippocampal bombesin-like peptide levels with feeding status. (1993) *Am. J. Physiol.* 265, R420-R425.
- 295. Merali, Z., Kent, P., and Anisman, H., Role of bombesin-related peptides in the mediation or integration of the stress response. (2001) *Mol. Chem. Neuropathol.* In press.
- 296. Merali, Z., Kent, P., Michaud, D.S., McIntyre, D.C., and Anisman, H., Differential impact of predator or immobilization stressors on central corticotropin-releasing hormone and bombesin-like peptides in Fast and Slow seizing rats. (2001) *Brain Res.* 906, 60-73.
- 297. Merali, Z., McIntosh, J., Kent, P., Michaud, D., and Anisman, H., Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. (1998) *J. Neurosci.* 18, 4758-4766.

298. Merali, Z., Moody, T.W., and Coy, D., Blockade of brain bombesin/GRP receptors increases food intake in satiated rats. (1993) *Am. J. Physiol.*, 264, R1031-R1034.
299. Merali, Z., Plamondon, H., Kent, P., and Banks, K., Corticotropin-releasing factor (CRF) antagonist blocks the autonomic, endocrine, ingestive and behavioral effects of central bombesin (BN).. (1994) *Soc. Neurosci. Abs.*, 528, 1.
300. Merchenthaler, I., Hynes, M.A., Vigh, S., Petrusz, P., and Schally, A.V., Immunocytochemical localization of corticotropin releasing factor (CRH) in the rat spinal cord. (1983) *Brain Res.*, 275, 373-377.
301. Merlo-Pich, E., Heinrichs, S.C., Rivier, C., Miczek, K.A., Fisher, D.A., and Koob, G.F., Blockade of pituitary-adrenal axis activation induced by peripheral immunoneutralization of corticotropin-releasing factor does not affect the behavioral response to social stress. (1993) *Psychoneuroendocrinology*, 18, 495-507.
302. Messer JR, W.S., Cholinergic agonists and the treatment of Alzheimer s disease. (2002) *Curr. Topics Med. Chem.*, 2, 353-358.
303. Million, M., Taché, Y., and Anton, P., Susceptibility of Lewis and Fischer rats to stress-induced worsening of TNB-colitis: protective role of brain CRF. (1999) *Am. J. Physiol...: Gastrointest. Liver Physiol.*, 276, G1027-G1036.
304. Mitchell, J.F., The spontaneous and evoked release of acetylcholine from the cerebral cortex. (1963) *J. Physiol.*, 165, 98-116.
305. Miyata, I., Shiota, C., Ikeda, Y., Oshida, Y., Chaki, S., Okuyama, S., and Inagami, T., Cloning and characterization of a short variant of the corticotropin-releasing factor receptor subtype from rat amygdala. (1999) *Biochem. Biophys. Res. Comm.*, 256, 692-696.
306. Moghaddam, B. and Bunney, B.S., Ionic composition of microdialysis perfusing solution alters the pharmacological responsiveness and basal outflow of striatal dopamine. (1989) *J. Neurochem.*, 53, 652-654.
307. Mohapel, P. and McIntyre, D.C., Amygdala kindling-resistant (Slow) or -prone (Fast) rat strains show differential fear responses. (1998) *Beh. Neurosci.*, 112, 1402-1413.

308. Moncek, F., Kvetnansky, R., and Jezova, D., Differential responses to stress stimuli of Lewis and Fischer rats at the pituitary and adrenocortical level. (2001) *Endocr. Res.*, 35, 35-41.
309. Moody, T.W., O'Donohue, T.L., and Jacobowitz, D.M., Biochemical localization and characterization of bombesin-like peptides in discrete regions of rat brain. (1981) *Peptides*, 2, 75-80.
310. Moore, H., Rose, H.J., and Grace, A.A., Chronic cold stress reduces the spontaneous activity of ventral tegmental dopamine neurons. (2001) *Neuropsychopharmacology*, 24, 410-419.
311. Morrow, B.A., Redmond, A.J., Roth, R.H., and Elsworth, J.D., The predator odor, TMT, displays a unique, stress-like pattern of dopaminergic and endocrinological activation in the rat. (2000) *Brain Res.*, 864, 146-151.
312. Morrow, B.A., Roth, R.H., and Elsworth, J.D., TMT, a predator odor, elevates mesoprefrontal dopamine metabolic activity and disrupts short-term working memory in the rat. (2000) *Brain Res. Bull.*, 52, 519-523.
313. Muglia, L.J., Jacobson, L., Weninger, S.C., Karalis, K., Jeong, K.-H., and Majzoub, J.A., The physiology of corticotropin-releasing hormone deficiency in mice. (2001) *Peptides*, 22, 725-731.
314. Muller-Gass, A., Porath, A., Anisman, H., and Merali, Z., Novelty-induced delay in snack approach: a robust and rapid naturalistic model for anxiety assessment. (2000) *Soc. Neurosci. Abs.*, 30, 659.2.
315. Murphy, B.L., Arnsten, A.F., Goldman-Rakic, P.S., and Roth, R.H., Increased dopamine turnover in the prefrontal cortex impairs spatial working memory performance in rats and monkeys. (1996) *Proc. Nat. Acad. Sci. U.S.A.*, 93, 1325-1329.
316. Muurahainen, N.E., Kissileff, H.R., and Pi-Sunyer, F.X., Intravenous infusion of bombesin reduces food intake in humans. (1993) *Am. J. Physiol.-Regul. Integra. Comp. Physiol.*, 264, R350-R354.
317. Myers, R.D., Adell, A., and Lankford, M.F., Simultaneous Comparison of Cerebral Dialysis and Push-Pull Perfusion in the Brain of Rats: A Critical Review. (1998) *Neurosci. Biobehav. Rev.*, 22, 371-387.

318. Nader, K., Majidishad, P., Amorapanth, P., and Ledoux, J.E., Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning, (2001) *Learn. Mem.*, 8, 156-163.
319. Neri, G., Malendowicz, L.K., Andreis, P., and Nussdorfer, G.G., Thyrotropin-releasing hormone inhibits glucocorticoid secretion of rat adrenal cortex- in vivo and in vitro studies. (1993) *Endocrinology*, 133, 511-514.
320. Nestler, E.J., Alreja, M., and Aghajanian, G.K., Molecular control of locus coeruleus neurotransmission. (1999) *Biol. Psychia.*, 46, 1131-1139.
321. Nijssen, M.J., Neuropeptides and the autonomic stress response. (1999). *Doctoral Dissertation*
322. Olsen, L., Knigge, U., and Warberg, J., Gastrin-releasing peptide stimulation of corticotropin secretion in male rats. (1992) *Endocrinology*, 130, 2710-2716.
323. Ono, N., Lumpkin, M.D., Samson, W.K., McDonald, J.K., and McCann, S.M., Intrahypothalamic action of corticotropin-releasing factor (CRF) to inhibit growth hormone and LH release in the rat. (1985) *Proc. Nat. Acad. Sci. U.S.A.*, 82, 7787-7790.
324. Ostrowski, N.L., Lolait, S.J., and Young, W.S., Cellular localization of vasopressin V1A receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. (1994) *Endocrinology*, 135, 1511-1528.
325. Otagiri, A., Wakabayashi, I., and Shibasaki, T., Selective corticotropin-releasing factor type 1 receptor antagonist blocks conditioned fear-induced release of noradrenaline in the hypothalamic paraventricular nucleus of rats. (2000) *J. Neuroendocrinol.*, 12, 1022-1026.
326. Owens, M.J. and Nemeroff, C.B., Physiology and pharmacology of corticotropin-releasing factor. (1991) *Pharmacol. Rev.*, 43, 425-473.
327. Pacak, K., Stressor-specific activation of the hypothalamic-pituitary-adrenocortical axis. (2000) *Physiol. Res.*, 49 Suppl 1, S11-S17.
328. Pacak, K., Stressor-specific activation of the hypothalamic-pituitary-adrenocortical axis. (2000) *Physiol. Res.*, 49 (Suppl 1):S11-S17, 2000.

329. Pacak, K. and Palkovits, M., Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. (2001) *Endocr. Rev.*, 22, 502-548.
330. Pacak, K., Palkovits, M., Makino, S., Kopin, I.J., and Goldstein, D.S., Brainstem hemisection decreases corticotropin-releasing hormone mRNA in the paraventricular nucleus but not in the central amygdaloid nucleus. (1996) *J. Neuroendocrinol.*, 8, 543-551.
331. Page, M.E. and Abercrombie, E.D., Discrete local application of corticotropin-releasing factor increases locus coeruleus discharge and extracellular norepinephrine in rat hippocampus. (1999) *Synapse*, 33, 304-313.
332. Palkovits, M., Stress-induced expression of co-localized neuropeptides in hypothalamic and amygdaloid neurons. (2000) *Eur. J. Pharmacol.*, 405, 161-166.
333. Palkovits, M., Baffi, J.S., and Dvori, S., Neuronal organization of stress response: pain-induced c-fos expression in brainstem catecholaminergic cell groups. (1995) *Ann. NY Acad. Sci.*, 771, 313-326.
334. Palkovits, M. and Brownstein, M.J. (1988) *Maps and guide to microdissection of the rat brain.*, Elsevier, New York.
335. Palkovits, M., Brownstein, M.J., Saavedra, J.M., and Axelrod, J., Norepinephrine and dopamine content of hypothalamic nuclei of the rat. (1974) *Brain Res.*, 77, 137-149.
336. Palkovits, M., Mezey, E., Skirboll, L.R., and Hokfelt, T., Adrenergic projections from the lower brainstem to the hypothalamic paraventricular nucleus, the lateral hypothalamic area and the central nucleus of the amygdala in rats. (1992) *J. Chem. Neuroanat.*, 5, 407-415.
337. Panagis, G., Hildebrand, B.E., Svensson, T.H., and Nomikos, G.G., Selective c-fos induction and decreased dopamine release in the central nucleus of amygdala in rats displaying a mecamylamine-precipitated nicotine withdrawal syndrome. (2000) *Synapse*, 35, 15-25.
338. Pani, L., Porcella, A., and Gessa, G.L., The role of stress in the pathophysiology of the dopaminergic system. (2000) *Mol. Psychia.*, 5, 14-21.

339. Parkes, D.G., Weisinger, R.S., and May, C.N., Cardiovascular actions of CRH and urocortin: an update. (2001) *Peptides*, 22, 821-827.
340. Parkinson, J.A., Robbins, T.W., and Everitt, B.J., Dissociable roles of the central and basolateral amygdala in appetitive emotional learning. (2000) *Eur. J. Neurosci.*, 12, 405-413.
341. Patchev, V.K., Kalogeras, K.T., Zelazowski, P., Wilder, R.L., and Chrousos, G.P., Increased plasma concentrations, hypothalamic content, and in vitro release of arginine vasopressin in inflammatory disease-prone, hypothalamic corticotropin-releasing hormone-deficient Lewis rats. (1992) *Endocrinology*, 131, 1453-1457.
342. Paxinos, G. and Watson, C. (1986) *The brain in stereotaxic coordinates*. Academic, New York.
343. Pedersen, W.A., McCullers, D., Culmsee, C., Haughey, N.J., Herman, J.P., and Mattson, M.P., Corticotropin-releasing hormone protects neurons against insults relevant to the pathogenesis of Alzheimer's disease. (2001) *Neurobiol. Dis.*, 8, 492-503.
344. Peper, M.K., Aversive learning in patients with unilateral lesions of the amygdala and hippocampus. (2001) *Biol. Psychol.*, 58, 1-23.
345. Pert, A., Moody, T.W., Pert, C.B., Dewald, L.A., and Rivier, J., Bombesin: receptor distribution in brain and effects on nociception and locomotor activity. (1980) *Brain Res.*, 193, 209-220.
346. Petraglia, F., Florio, P., Gallo, R., Salvestroni, C., Lombardo, M., Genazzani, A.D., Di Carlo, C., Stomati, M., D'Ambrogio, G., and Artini, P.G., Corticotropin-releasing factor-binding protein: origins and possible functions. (1996) *Horm. Res.*, 45, 187-191.
347. Petrov, T., Jhamandas, J.H., and Krukoff, T.L., Electrical stimulation of the central nucleus of the amygdala induces fos-like immunoreactivity in the hypothalamus of the rat: a quantitative study. (1994) *Brain Res. Mol. Brain Res.*, 22, 333-340.
348. Petrov, T., Krukoff, T.L., and Jhamandas, J.H., The hypothalamic paraventricular and lateral parabrachial nuclei receive collaterals from raphe nucleus neurons: a

- combined double retrograde and immunocytochemical study, (1992) *J. Comp. Neurol.*, 318, 18-26.
349. Petrov, T., Krukoff, T.L., and Jhamandas, J.H., Convergent influence of the central nucleus of the amygdala and the paraventricular hypothalamic nucleus upon brainstem autonomic neurons as revealed by c-fos expression and anatomical tracing, (1995) *J. Neurosci. Res.*, 42, 835-845.
 350. Piazza, P.V. and Le Moal, M., Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications, (1997) *Brain Res. Brain Res. Rev.*, 25, 359-372.
 351. Pich, E.M., Lorang, M., Yeganeh, M., Rodriguez de Fonseca, F., Raber, J., Koob, G.F., and Weiss, F., Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis, (1995) *J. Neurosci.*, 15, 5439-5447.
 352. Pignatelli, D., Magalhaes, M.M., and Magalhaes, M.C., Direct effects of stress on adrenocortical function, (1998) *Hor. Metab. Res.*, 30, 464-474.
 353. Pisarska, M., Mulchahey, J.J., Welge, J.A., Geraciotti Jr, T.D., and Kasckow, J.W., Age-related alterations in emotional behaviors and amygdalar corticotropin-releasing factor (CRF) and CRF-binding protein expression in aged Fischer 344 rats, (2000) *Brain Res.*, 877, 184-190.
 354. Plamondon, H. and Merali, Z., Push-pull perfusion reveals meal-dependent changes in the release of bombesin-like peptides in the rat paraventricular nucleus, (1994) *Brain Res.*, 668, 54-61.
 355. Plamondon, H. and Merali, Z., Anorectic action of bombesin requires receptor for corticotropin-releasing factor but not for oxytocin, (1997) *Eur. J. Pharmacol.*, 340, 99-109.
 356. Plamondon, H. and Merali, Z., Regulation of ingestion by CRF and bombesin-like peptides: distinct meal-related peptide level changes, (1997) *Am. J. Physiol.*, 272, R268-R274.

357. Plotsky, P.M., Cunningham, E.T.Jr., and Widmaier, E.P., Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. (1989) *Endocr. Rev.*, 10, 437-458.
358. Posener, J.A., Schatzberg, A.F., Williams, G.H., Samson, J.A., McHale, N.L., Bessette, M.P., and Schildkraut, J.J., Hypothalamic-pituitary-adrenal axis effects on plasma homovanillic acid in man. (1999) *Biol. Psychia.*, 45, 222-228.
359. Posener, J.A., Schildkraut, J.J., Williams, G.H., Gleason, R.E., Salomon, M.S., Mecheri, G., and Schatzberg, A.F., Acute and delayed effects of corticotropin-releasing hormone on dopamine activity in man. (1994) *Biol. Psychia.*, 36, 616-621.
360. Potter, E., Behan, D.P., Linton, E.A., Lowry, P.J., Sawchenko, P.E., and Vale, W.W., The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. (1992) *Proc. Nat. Acad. Sci. U.S.A.*, 89, 4192-4196.
361. Prasad, B.M., Sorg, B.A., Ulibarri, C., and Kalivas, P.W., Sensitization to stress and psychostimulants. Involvement of dopamine transmission versus the HPA axis. (1995) *Ann. NY Acad. Sci.*, 771, 617-625.
362. Prewitt, C.M. and Herman, J.P., Lesion of the central nucleus of the amygdala decreases basal CRH mRNA expression and stress-induced ACTH release. (1994) *Ann. NY Acad. Sci.*, 746, 438-440.
363. Raadsheer, F.C., van Heerikhuize, J.J., Lucassen, P.J., Hoogendijk, W.J., Tilders, F.J., and Swaab, D.F., Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. (1995) *Am. J. Psychia.*, 152, 1372-1376.
364. Racine, R.J., Steingart, M., and McIntyre, D.C., Development of kindling-prone and kindling-resistant rats: Selective breeding and electrophysiological studies. (1999) *Epil. Res.*, 35, 183-195.
365. Rex, A., Stephans, S.E., and Fink, H., "Anxiolytic" action of diazepam and abecarnil in a modified open field test. (1996) *Pharmacol. Biochem. Beh.*, 53, 1005-1011.

366. Ricardo, J.A. and Koh, E.J.. Anatomical evidence of direct projections from the nucleus of solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. (1978) *Brain Res.*, 153, 1-26.
367. Richard, D., Rivest, R., Naimi, N., Timofeeva, E., and Rivest, S.. Expression of corticotropin-releasing factor and its receptors in the brain of lean and obese Zucker rats. (1996) *Endocrinology*, 137, 4786-4795.
368. Richardson, N.R. and Gratton, A.. Behavior-relevant changes in nucleus accumbens dopamine transmission elicited by food reinforcement: an electrochemical study in the rat. (1996) *J. Neurosci.*, 16, 8160-8169.
369. Richter, R.M. and Weiss, F.. In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. (1999) *Synapse*, 32, 254-261.
370. Richter, R.M., Zorrilla, E.P., Basso, A.M., Koob, G.F., and Weiss, F.. Altered amygdalar CRF release and increased anxiety-like behavior in Sardinian alcohol-preferring rats: a microdialysis and behavioral study. (2000) *Alcohol. Clin. Exp. Res.*, 24, 1765-1772.
371. Ridgway, E.C., Ladenson, P.W., Cooper, D.S., Daniels, G.H., Francis, G.S., and Maloof, F.. Cardiac function in mild and severe primary hypothyroidism. (1982) *Life Sci.*, 30, 651-658.
372. Rivest, S.. How circulating cytokines trigger the neural circuits that control the hypothalamic-pituitary-adrenal axis. (2001) *Psychoneuroendocrinology*, 26, 761-788.
373. Rivest, S. and Rivier, C.. The role of corticotropin-releasing factor and interleukin-1 in the regulation of neurons controlling reproductive functions. (1995) *Endocr. Rev.*, 16, 177-199.
374. Rivier, J., Rivier, C., and Vale, W.. Synthetic competitive antagonists of corticotropin-releasing factor: Effect on ACTH secretion in the rat. (1984) *Science*, 224, 889-891.
375. Rodriguez de Fonseca, F., Carrera, M.R.A., Navarro, M., Koob, G.F., and Weiss, F.. Activation of corticotropin-releasing factor in the limbic system during cannabinoid withdrawal. (1997) *Science*, 276, 2050-2054.

- 376. Rohmeiss, P., Becker, H., Dietrich, R., Luft, F., and Unger, T., Vasopressin: mechanism of central cardiovascular action in conscious rats. (1986) *J. Cardio. Pharmacol.*, 8, 689-696.
- 377. Romero, L.M., Levine, S., and Sapolsky, R.M., Adrenocorticotropin secretagog release: Stimulation by frustration and paradoxically by reward presentation. (1995) *Brain Res.*, 676, 151-156.
- 378. Roozendaal, B., Koolhaas, J.M., and Bohus, B., Central amygdala lesions affect behavioral and autonomic balance during stress in rats. (1991) *Physiol. Beh.*, 50, 777-781.
- 379. Roozendaal, B., Koolhaas, J.M., and Bohus, B., The role of the central amygdala in stress and adaption. (1997) *Acta Physiologica Scandinavica*, 640, 51-54.
- 380. Ross, C.A., Armstrong, D.M., Ruggiero, D.A., Pickel, V.M., Joh, T.H., and Reis, D.J., Adrenaline neurons in the rostral ventrolateral medulla innervate thoracic spinal cord: a combined immunocytochemical and retrograde transport demonstration. (1981) *Neurosci. Lett.*, 25, 257-262.
- 381. Roth, K.A., Mefford, I.M., and Barchas, J.D., Epinephrine, norepinephrine, dopamine and serotonin: differential effects of acute and chronic stress on regional brain amines. (1982) *Brain Res.*, 239, 417-424.
- 382. Rothschild, A.J., Langlais, P.J., Schatzberg, A.F., Miller, M.M., Saloman, M.S., Lerbinger, J.E., Cole, J.O., and Bird, E.D., The effects of a single acute dose of dexamethasone on monoamine and metabolite levels in rat brain. (1985) *Life Sci.*, 36, 2491-2501.
- 383. Saito, M., Sugimoto, T., Tahara, A., and Kawashima, H., Molecular cloning and characterization of rat V1b vasopressin receptor: evidence for its expression in extra-pituitary tissues. (1995) *Biochem. Biophys. Res. Comm.*, 212, 751-757.
- 384. Sajdyk, T.J., Katner, J.S., and Shekhar, A., Monoamines in the dorsomedial hypothalamus of rats following exposure to different tests of "anxiety". (1997) *Prog. Neuro-Psychopharmacol. Biol. Psychia.*, 21, 193-209.
- 385. Sajdyk, T.J., Schober, D.A., Gehlert, D.R., and Shekhar, A., Role of corticotropin-releasing factor and urocortin within the basolateral amygdala of rats in anxiety and panic responses. (1999) *Beh. Brain Res.*, 100, 207-215.

386. Salacinski, P.R., McLean, C., Sykes, J.E., Clement-Jones, V.V., and Lowry, P.J., Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent. 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen), (1981) *Anal. Biochem.*, 117, 136-146.
387. Sapolsky, R.M., Meaney, M.J., and McEwen, B.S., The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation, (1985) *Brain Res.*, 350, 169-173.
388. Sapolsky, R.M., Romero, L.M., and Munck, A.U., How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions, (2000) *Endocr. Rev.*, 21, 55-89.
389. Sara, S.J. and Bergis, O., Enhancement of excitability and inhibitory processes in hippocampal dentate gyrus by noradrenaline: a pharmacological study in awake, freely moving rats, (1991) *Neurosci. Lett.*, 126, 1-5.
390. Sara, S.J., Devauges, V., Biegon, A., and Blizard, D.A., The Maudsley rat strains as a probe to investigate noradrenergic-cholinergic interaction in cognitive function, (1994) *J. Physiol.*, 88, 337-345.
391. Sara, S.J. and Hervé-Minviell, A., Inhibitory influence of frontal cortex on locus coeruleus neurons, (1995) *Proc. Nat. Acad. Sci. U.S.A.*, 92, 6032-6036.
392. Sara, S.J. and Segal, M., Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition, (1991) *Prog. Brain Res.*, 88, 571-585.
393. Sarnyai, Z., Shaham, Y., and Heinrichs, S.C., The role of corticotropin-releasing factor in drug addiction, (2001) *Pharmacol. Rev.*, 53, 209-243.
394. Sawchenko, P.E., Brown, E.R., Chan, R.K.W., Ericsson, A., Li, H.-Y., Roland, B.L., and Kovács, K.J., The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress, (1996) *Brain Res.*, 107, 201-222.
395. Schatzberg, A.F., Posener, J.A., and Rothschild, A.J., The role of dopamine in psychotic depression, (1995) *Clin. Neuropharmacol.*, 18, S66-S73.

396. Schmidt, E.D., Binnekade, R., Janszen, A.W., and Tilders, F.J., Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats. (1996) *J. Neuroendocrinol.* 8, 703-712.
397. Schneider, F., Gur, R.E., Alavi, A., Seligman, M.E.P., Mozley, L.H., Smith, R.J., Mozley, P.D., and Gur, R.C., Cerebral blood flow changes in limbic regions induced by unsolvable anagram tasks. (1996) *Am. J. Psychia.* 153, 206-212.
398. Schwartz, M.W., Dallman, M.F., and Woods, S.C., Hypothalamic response to starvation: implications for the study of wasting disorders. (1995) *Am. J. Physiol.* 269, R949-R957.
399. Seasholtz, A.F., Burrows, H.L., Karolyi, I.J., and Camper, S.A., Mouse models of altered CRH-binding protein expression. (2001) *Peptides*, 22, 743-751.
400. Selye, H., A syndrome produced by diverse nocuous agents. (1936) *Nature*. 138, 32.
401. Selye, H. (1956) *The stress of life*, McGraw-Hill Book Company, Inc., New York.
402. Senba, E., Umemoto, S., Kawai, Y., and Noguchi, K., Differential expression of fos family and jun family mRNAs in the rat hypothalamo-pituitary-adrenal axis after immobilization stress. (1994) *Mol. Brain Res.* 24, 283-294.
403. Shepard, J.D., Barron, K.W., and Myers, D.A., Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. (2000) *Brain Res.* 861, 288-295.
404. Shiraishi, I., Honma, K., Honma, S., and Hiroshige, T., Ethosecretogram: relation of behavior to plasma corticosterone in freely moving rats. (1984) *Am. J. Physiol.* 247, R40-R45.
405. Shors, T.J., Acute stress rapidly and persistently enhances memory formation in the male rat. (2001) *Neurobiol. Learn. Mem.* 75, 10-29.
406. Shors, T.J. and Servatius, R.J., The contribution of stressor intensity, duration, and context to the stress-induced facilitation of associative learning. (1997) *Neurobiol. Learn. Mem.* 68, 92-96.

407. Shors, T.J., Weiss, C., and Thompson, R.F., Stress-induced facilitation of classical conditioning, (1992) *Science*, 257, 537-539.
408. Simar, M.R., Saphier, D., and Goeders, N.E., Dexamethasone suppression of the effects of cocaine on adrenocortical secretion in Lewis and Fischer rats, (1997) *Psychoneuroendocrinology*, 22, 141-153.
409. Singewald, N., Kaehler, S.T., and Philippu, A., Noradrenaline release in the locus coeruleus of conscious rats is triggered by drugs, stress and blood pressure changes, (1999) *Neuroreport*, 10, 1583-1587.
410. Singewald, N. and Philippu, A., Release of neurotransmitters in the locus coeruleus, (1998) *Prog. Neurobiol.*, 56, 237-267.
411. Singewald, N., Zhou, G.Y., Chen, F., and Philippu, A., Corticotropin-releasing factor modulates basal and stress-induced excitatory amino acid release in the locus coeruleus of conscious rats, (1996) *Neurosci. Lett.*, 204, 45-48.
412. Smagin, G.N., Heinrichs, S.C., and Dunn, A.J., The role of CRH in behavioral responses to stress, (2001) *Peptides*, 22, 713-724.
413. Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W., and Lee, K.F., Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development, (1998) *Neuron*, 20, 1093-1102.
414. Spiegler, B.J. and Mishkin, M., Evidence for the sequential participation of inferior temporal cortex and amygdala in the acquisition of stimulus-reward associations, (1981) *Brain Res.*, 3, 303-317.
415. Spina, M.G., Basso, A.M., Zorrilla, E.P., Heyser, C.J., Rivier, J., Vale, W., Pich, E.M., and Koob, G.F., Behavioral effects of central administration of the novel CRF antagonist astressin in rats, (2000) *Neuropsychopharmacology*, 22, 230-239.
416. Spreux-Varoquaux, O., Alvarez, J.C., Berlin, I., Batista, G., Despierre, P.G., Gilton, A., and Cremniter, D., Differential abnormalities in plasma 5-HIAA and platelet serotonin concentrations in violent suicide attempters: relationships with impulsivity and depression, (2001) *Life Sci.*, 69, 647-657.

417. Steckler, T. and Holsboer, M., Corticotropin-releasing hormone receptor subtypes and emotion. (1999) *Biol. Psychia.*, 46, 1480-1508.
418. Sternberg, E.M., Glowa, J.R., Smith, M.A., Calogero, A.E., Listwak, S.J., Aksentijevich, S., Chrousos, G.P., Wilder, R.L., and Gold, P.W., Corticotropin releasing hormone related behavioral and neuroendocrine responses to stress in Lewis and Fischer rats. (1992) *Brain Res.*, 570, 54-60.
419. Sternberg, E.M., Hill, J.M., Chrousos, G.P., Kamilaris, T., Listwak, S.J., Gold, P.W., and Wilder, R.L., Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. (1989) *Proc. Nat. Acad. Sci. U.S.A.*, 86, 2374-2378.
420. Sternberg, E.M., Young, W.S., III, Bernardini, R., Calogero, A.E., Chrousos, G.P., Gold, P.W., and Wilder, R.L., A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. (1989) *Proc. Nat. Acad. Sci. U.S.A.*, 86, 4771-4775.
421. Stock, G., Rupprecht, U., Stumpf, H., and Schlor, K.H., Cardiovascular changes during arousal elicited by stimulation of amygdala, hypothalamus and locus coeruleus. (1981) *J. Auton. Nerv. Sys.*, 3, 503-510.
422. Stock, G., Rupprecht, U., Stumpf, H., and Schlor, K.H., Cardiovascular changes during arousal elicited by stimulation of amygdala, hypothalamus and locus coeruleus. (1981) *J. Auton. Nerv. Sys.*, 3, 503-510.
423. Stoehr, J.D., Cheng, S.W., and North, W.G., Homozygous Brattleboro rats display attenuated conditioned freezing responses. (1993) *Neurosci. Lett.*, 153, 103-106.
424. Stoehr, J.D., Cheng, S.W., Serlin, M.C., Cramer, C.P., and North, W.G., A physiological role of vasopressin in the expression of conditioned fear. (1993) *Ann. NY Acad. Sci.*, 689, 674-676.
425. Stohr, T., Schulte, W.D., Weiner, I., and Feldon, J., Rat strain differences in open-field behavior and the locomotor stimulating and rewarding effects of amphetamine. (1998) *Pharmacol. Biochem. Beh.*, 59, 813-818.

- 426. Stohr, T., Szuran, T., Welzl, H., Pliska, V., Feldon, J., and Pryce, C.R., Lewis/Fischer rat strain differences in endocrine and behavioral responses to environmental challenge. (2000) *Pharmacol. Biochem. Beh.* 67, 809-819.
- 427. Stokes, P.E., Fluoxetine: A five year review. (1993) *Excerpta Medica*, 15, 216-243.
- 428. Stout, J.C. and Weiss, J.M., An animal model for measuring behavioral responses to anxiogenic and anxiolytic manipulations. (1994) *Pharmacol. Biochem. Beh.* 47, 459-465.
- 429. Stratakis, C.A. and Chrousos, G.P., Neuroendocrinology and pathophysiology of the stress system. (1995) *Ann. NY Acad. Sci.* 771, 1-18.
- 430. Sutton, R.E., Koob, G.F., Le Moal, M., Rivier, J., and Vale, W., Corticotropin releasing factor produces behavioral activation in rats. (1982) *Nature*, 297, 331-333.
- 431. Swanson, L.W. and Hartman, B.K., The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. (1975) *J. Comp. Neurol.* 163, 467-505.
- 432. Swanson, L.W. and Petrovich, G.D., What is the amygdala?. (1998) *Trends Neurosci.* 21, 323-331.
- 433. Swanson, L.W., Sawchenko, P.E., Lind, R.W., and Rho, J.H., The CRH motoneuron: differential peptide regulation in neurons with possible synaptic, paracrine, and endocrine outputs. (1987) *Ann. NY Acad. Sci.* 512, 12-23.
- 434. Swanson, L.W., Sawchenko, P.E., Rivier, J., and Vale, W., Organization of ovine CRF immunoreactive cells and fibres in the rat brain: An immunohistochemical study. (1983) *Neuroendocrinology*, 36, 165-186.
- 435. Swanson, L.W. and Simmons, D.M., Corticosterone produces increased levels of CRF mRNA in the amygdala like it does neurotransmitter CRF cells in the PVN, while it produces major decreases in the neurohormonal cells in the parvocellular PVN. (1989) *J. Comp. Neurol.* 285, 413-435.

436. Swiergiel, A.H., Takahashi, L.K., and Kalin, N.H., Attenuation of stress-induced behavior by antagonism of corticotropin-releasing factor receptors in the central amygdala in the rat. (1993) *Brain Res.* 623, 229-234.
437. Swiergiel, A.H., Takahashi, L.K., and Kalin, N.H., Attenuation of stress-induced behavior by antagonism of corticotropin-releasing factor receptors in the central amygdala in the rat. (1993) *Brain Res.* 623, 229-234.
438. Taber, M.T. and Fibiger, H.C., Activation of the mesocortical dopamine system by feeding: lack of selective response to stress, (1997) *Neuroscience*, 77, 295-298.
439. Tabert, M.H., Borod, J.C., Tang, C.Y., Lange, G., Wei, T.C., Johnson, R., Nusbaum, A.O., and Buschsbaum, M.S., Differential amygdala activation during emotional decision and recognition memory tasks using unpleasant words: an fMRI study. (2001) *Neuropsychologia*, 39, 556-573.
440. Tache, Y., Brown, M., and Collu, R., Effects of neuropeptides on adenohipophyseal hormone response to acute stress in male rats. (1979) *Endocrinology*, 105, 220-224.
441. Takao, K., Nagatani, T., Kitamura, Y., and Yamawaki, S., Effects of corticosterone on 5-HT1A and 5-HT2 receptor binding and on the receptor-mediated behavioral responses of rats. (1997) *Eur. J. Pharmacol.*, 333, 123-128.
442. Tanaka, M., Yoshida, M., Emoto, H., and Ishii, H., Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. (2000) *Eur. J. Pharmacol.*, 405, 397-406.
443. Thierry, A.M., Tassin, J.P., Blanc, G., and Glowinski, J., Selective activation of mesocortical DA system by stress. (1976) *Nature*, 263, 242-244.
444. Tilders, F.J. and Berkenbosch, F., CRF and catecholamines: their place in the central and peripheral regulation of the stress response. (1986) *Acta Endocrinologica*, 276, 63-75.
445. Tilders, F.J., Schmidt, E.D., and de Goeij, D.C., Phenotypic plasticity of CRF neurons during stress. (1993) *Ann. NY Acad. Sci.*, 697, 39-52.

446. Timpl. P., Spanagel. R., Sillaber. I., Kresse. A., Reul. J.M., Stalla. G.K., Blanquet. V., Steckler. T., Holsboer. F., and Wurst. W., Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor. (1998) *Nat. Genet.* 19. 162-166.
447. Tjurmina. O.A., Goldstein. D.S., Palkovits. M., and Kopin. I.J., Alpha2-adrenoceptor-mediated restraint of norepinephrine synthesis, release, and turnover during immobilization in rats. (1999) *Brain Res.* 826. 243-252.
448. Tohyama. M. and Takatsuji. K. (1998) *Atlas of neuroactive substances and their receptors in the rat*. Oxford University Press, New York.
449. Torii. M., Kubo. K., and Sasaki. T., Differential effects of B-endorphin and met- and leu-enkephalin on steroid hormone-induced lordosis in ovariectomized female rats. (1997) *Pharmacol. Biochem. Beh.* 58. 837-842.
450. Turecki. G., Briere. R., Dewar. K., Antonetti. T., Lesage. A.D., Seguin. M., Chawky. N., Vanier. C., Alda. M., Joober. R., Benkelfat. C., and Rouleau. G.A., Prediction of level of serotonin 2A receptor binding by serotonin receptor 2A genetic variation in postmortem brain samples from subjects who did or did not commit suicide. (1999) *Am. J. Psychia.* 156. 1456-1458.
451. Turnbull. A.V. and Rivier. C., Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. (1997) *Proc Soc Exp Biol Med.* 215. 1-10.
452. Ursin. H. and Olff. M. (1993) The stress response. In Stanford.S.C., Salmon.P. (Eds.) *Stress: From Synapse to Syndrome*. Academic Press, San Diego. pp.3-22.
453. Vale. W., Spiess. J., Rivier. C., and Rivier. J., Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and b-endorphin. (1981) *Science*. 213. 1394-1397.
454. Vale. W., Vaughan. J., Yamamoto. G., Bruhn. T., Douglas. C., Dalton. D., Rivier. C., and Rivier. J., Assay of corticotropin releasing factor. (1983) *Met. Enzymol.* 103. 565-577.
455. Valentino. R.J., Curtis. A.L., Page. M.E., Pavcovich. L.A., and Florin-Lechner.S.M., Activation of the locus ceruleus brain noradrenergic system during

- stress: circuitry, consequences, and regulation. (1998) *Adv. Pharmacol.*, 42, 781-784.
456. Valentino, R.J., Pavlovich, L.A., and Hirata, H., Evidence for corticotropin-releasing hormone projections from Barrington's nucleus to the periaqueductal gray and dorsal motor nucleus of the vagus in the rat. (1995) *J. Comp. Neurol.*, 363, 402-422.
 457. Valentino, R.J. and Wehby, R.G., Corticotropin-releasing factor: evidence for a neurotransmitter role in the locus coeruleus during hemodynamic stress. (1988) *Neuroendocrinology*, 48, 674-677.
 458. Van Bockstaele, E.J., Colago, E.E., and Valentino, R.J., Corticotropin-releasing factor-containing axon terminals synapse onto catecholamine dendrites and may presynaptically modulate other afferents in the rostral pole of the nucleus locus coeruleus in the rat brain. (1996) *J. Comp. Neurol.*, 364, 523-534.
 459. Van Bockstaele, E.J., Colago, E.E., and Valentino, R.J., Amygdaloid corticotropin-releasing factor targets locus coeruleus dendrites: substrate for the co-ordination of emotional and cognitive limbs of the stress response. (1998) *J. Neuroendocrinol.*, 10, 743-757.
 460. Van Bockstaele, E.J., Peoples, J., and Valentino, R.J., Anatomic basis for differential regulation of the rostralateral peri-locus coeruleus region by limbic afferents. (1999) *Biol. Psychia.*, 46, 1352-1363.
 461. Van de Kar, L.D., Piechowski, R.A., Rittenhouse, P.A., and Gray, T.S., Amygdaloid lesions: Differential effect on conditioned stress and immobilization-induced increases in corticosterone and renin secretion. (1991) *Neuroendocrinology*, 52, 89-95.
 462. van Dijken, H.H., de Goeij, D.C., Sutanto, W., Mos, J., De Kloet, E.R., and Tilders, F.J., Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats. (1993) *Neuroendocrinology*, 58, 57-64.
 463. Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W., and Sawchenko, P.E., Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. (2000) *J. Comp. Neurol.*, 428, 191-212.

464. Van Raaij, M.T., Dobbe, C.J., Elvers, B., Timmerman, A., Schenk, E., Oortgiesen, M., and Wiegant, V.M., Hormonal status and the neuroendocrine response to a novel heterotypic stressor involving subchronic noise exposure. (1997) *Neuroendocrinology*, 65, 200-209.
465. Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., and Rivier, C., Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. (1995) *Nature*, 378, 287-292.
466. Veening, J.G., Swanson, L.W., and Sawchenko, P.E., The organization of projections from the central nucleus of the amygdala to brainstem sites involved in central autonomic regulation: a combined retrograde transport-immunohistochemical study. (1984) *Brain Res.*, 303, 337-357.
467. Vellucci, S.V. (1997) The autonomic and behavioral responses to stress. In Buckingham, J.C., Gillies, G.E., Cowell, A.-M. (Eds.) *Stress, stress hormones and the immune system*. John Wiley & Sons, New York, pp.49-70.
468. Wada, E., Way, J., Lebacqz-Verheyden, A.M., and Battey, J.F., Neuromedin B and gastrin-releasing peptide mRNAs are differentially distributed in the rat nervous system. (1990) *J. Neurosci.*, 10, 2917-2930.
469. Walker, C.D., Toufexis, D.J., and Burlet, A., Hypothalamic and limbic expression of CRF and vasopressin during lactation: implications for the control of ACTH secretion and stress hyporesponsiveness. (2001) *Prog. Brain Res.*, 133, 99-110.
470. Wallace, D.M., Magnuson, D.J., and Gray, T.S., The amygdalo-brainstem pathway: selective innervation of dopaminergic, noradrenergic and adrenergic cells in the rat. (1989) *Neurosci., Lett.*, 97, 252-258.
471. Wassen, F.W.J.S., Moerings, E.P.C.M., van Toor, H., Hennemann, G., and Everts, M.E., Thyroid hormone uptake in cultured rat anterior pituitary cells: effects of energy status and bilirubin. (2000) *J. Endocrinol.*, 165, 599-606.
472. Watanobe, H. and Takebe, K., Intravenous administration of tumor necrosis factor- α stimulates corticotropin releasing hormone secretion in the push-pull cannulated median eminence of freely moving rats, (1992) *Neuropeptides*, 22, 81-84.

473. Watanobe, H. and Takebe, K., Intrahypothalamic perfusion with interleukin-1-beta stimulates the local release of corticotropin-releasing factor and arginine vasopressin and the plasma adrenocorticotropin in freely moving rats: a comparative perfusion of the paraventricular nucleus and the median eminence. (1993) *Neuroendocrinology*, 57, 593-599.
474. Watanobe, H. and Takebe, K., Effects of intravenous administration of interleukin-1-beta on the release of prostaglandin E2, corticotropin-releasing factor, and arginine vasopressin in several hypothalamic areas of freely moving rats: estimation by push-pull perfusion. (1994) *Neuroendocrinology*, 60, 8-15.
475. Weninger, S.C., Dunn, A.J., Muglia, L.J., Dikkes, P., Miczek, K.A., Swiergiel, A.H., Berridge, C.W., and Majzoub, J.A., Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. (1999) *Proc. Nat. Acad. Sci. U.S.A.*, 96, 8283-8288.
476. Weninger, S.C., Peters, L.L., and Majzoub, J.A., Urocortin expression in the Edinger-Westphal nucleus is up-regulated by stress and corticotropin-releasing hormone deficiency. (2000) *Endocrinology*, 141, 256-263.
477. Wenkster, D., Pfaus, J.G., and Fibiger, H.C., Dopamine transmission increases in the nucleus accumbens of male rats during their first exposure to sexually receptive female rats. (1993) *Brain Res.*, 618, 41-46.
478. Whitnall, M.H., Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. (1993) *Prog. Neurobiol.*, 40, 573-629.
479. Whitnall, M.H. and Smallridge, R.C., Altered thyroid axis function in lewis rats with genetically defective hypothalamic CRH/VP neurosecretory cells. (1997) *Endocr. Res.*, 23, 365-376.
480. Wickelgren, I., Getting the brain's attention. (1997) *Science*, 278, 35-37.
481. Widmaier, E.P. and Dallman, M.F., The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perfused pituitaries in vitro: rapid inhibition by glucocorticoids. (1984) *Endocrinology*, 115, 2368-2374.
482. Wiersma, A., Baauw, A.D., Bohus, B., and Koolhaas, J.M., Behavioural activation produced by CRH but not alpha-helical CRH (CRH-receptor

- antagonist) when microinfused into the central nucleus of the amygdala under stress-free conditions. (1995) *Psychoneuroendocrinology*, 20, 423-432.
483. Wiersma, A., Bohus, B., and Koolhaas, J.M., Corticotropin-releasing hormone microinfusion in the central amygdala diminishes a cardiac parasympathetic outflow under stress-free conditions. (1993) *Brain Res.*, 625, 219-227.
 484. Wiersma, A., Knollema, S., Konsman, J.P., Bohus, B., and Koolhaas, J.M., Corticotropin-releasing hormone modulation of a conditioned stress response in the central amygdala of Roman high (RHA/Verh)-avoidance and low (RLA/Verh)-avoidance rats. (1997) *Beh. Genetics*, 27, 547-555.
 485. Wilensky, A.E., Schafe, G.E., and Ledoux, J.E., The amygdala modulates memory consolidation of fear-motivated inhibitory avoidance learning but not classical fear conditioning. (2000) *J. Neurosci.*, 20, 7059-7066.
 486. Windle, R.J., Wood, S., Shanks, N., Perks, P., Conde, G.L., da Costa, A.P., Ingram, C.D., and Lightman, S.L., Endocrine and behavioural responses to noise stress: comparison of virgin and lactating female rats during non-disrupted maternal activity. (1997) *J. Neuroendocrinol.*, 9, 407-414.
 487. Windle, R.J., Wood, S.A., Lightman, S.L., and Ingram, C.D., The pulsatile characteristics of hypothalamo-pituitary-adrenal activity in female Lewis and Fischer 344 rats and its relationship to differential stress responses. (1998) *Endocrinology*, 139, 4044-4052.
 488. Wise, R.A., Neurobiology of addiction. (1996) *Curr. Opin. Neurobiol.*, 6, 243-251.
 489. Wolkowitz, O., Sutton, M., Koulu, M., Labarca, R., Wilkinson, L., Doran, A., Hauger, R., Pickar, D., and Crawley, J., Chronic corticosterone administration in rats: behavioral and biochemical evidence of increased central dopaminergic activity. (1986) *Eur. J. Pharmacol.*, 122, 329-338.
 490. Wong, M.L., al Shekhlee, A., Bongiorno, P.B., Esposito, A., Khatri, P., Sternberg, E.M., Gold, P.W., and Licinio, J., Localization of urocortin messenger RNA in rat brain and pituitary. (1996) *Mol. Psychia.*, 1, 307-312.
 491. Woodruff, G.N., Hall, M.D., Reynolds, T., and Pinnock, R.D., Bombesin receptors in the brain. (1996) *Ann. NY Acad. Sci.*, 780, 223-243.

492. Wynn, P.C., Hauger, R.L., Holmes, M.C., Millan, M.A., Catt, K.J., and Aguilera, G., Brain and pituitary receptors for corticotropin releasing factor: localization and differential regulation after adrenalectomy. (1984) *Peptides*, 5, 1077-1084.
493. Wyvell, C.L. and Berridge, K.C., Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: enhancement of reward "wanting" without enhanced "liking" or response reinforcement. (2000) *J. Neurosci.*, 20, 8122-8130.
494. Yehuda, R., Biology of posttraumatic stress disorder. (2001) *J. Clin. Psychia.*, 62, 41-46.
495. Yu, B. and Shinnick-Gallagher, P., Corticotropin-releasing factor increases dihydropyridine- and neurotoxin-resistant calcium currents in neurons of the central amygdala. (1998) *J. Pharmacol. Exp. Therapeu.*, 284, 170-179.
496. Zalcman, S., Irwin, J., and Anisman, H., Stressor-induced alterations of natural killer cell activity and central catecholamines in mice. (1991) *Pharmacol. Biochem. Beh.*, 39, 361-366.
497. Zelazowski, P., Patchev, V.K., Zelazowski, E., Chrousos, G.P., Gold, P.W., and Sternberg, E.M., Release of hypothalamic corticotropin-releasing hormone and arginine-vasopressin by interleukin 1b and aMSH: Studies in rats with different susceptibility to inflammatory disease. (1993) *Brain Res.*, 631, 22-26.
498. Zelazowski, P., Smith, M.A., Gold, P.W., Chrousos, G.P., Wilder, R.L., and Sternberg, E.M., In vitro regulation of pituitary ACTH secretion in inflammatory disease susceptible Lewis (LEW/N) and inflammatory disease resistant Fischer (F344/N) rats. (1992) *Neuroendocrinology*, 56, 474-482.
499. Zerbe, R.L. and Feuerstein, G., Cardiovascular effects of centrally administered vasopressin in conscious and anesthetized rats, (1985) *Neuropeptides*, 6, 471-483.
500. Zhang, J.X., Harper, R.M., and Ni, H.F., Cryogenic blockade of the central nucleus of the amygdala attenuates aversively conditioned blood pressure and respiratory responses. (1986) *Brain Res.*, 386, 136-145.
501. Zhang, X., Kindel, G.H., Wulfert, E., and Hanin, I., Effects of immobilization stress on hippocampal monoamine release: modification by mivazerol, a new alpha 2-adrenoceptor agonist. (1995) *Neuropharmacology*, 34, 1661-1672.

502. Zola-Morgan, S., Squire, L.R., Alvarez-Royo, P., and Clower, R.P., Independence of memory functions and emotional behavior: Separate contributions of the hippocampal formation and the amygdala. (1991) *Hippocampus*, 1, 207-220.