Distribution Agreement:

In presenting this dissertation/thesis as a partial fulfillment of the requirements for an advanced degree from Emory University, I agree that the Library of the University shall make it available for inspection and circulation in accordance with its regulations governing materials of this type. I agree that permission to copy from, or to publish this dissertation may be granted by the professor under whose direction it was written, or, in his absence, by the Dean of the Graduate School when such copying or publication is solely for scholarly purposes and does not involve potential financial gain. It is understood that any copying from, or publication of this thesis/dissertation which involves potential financial gain will not be allowed without written permission.

_____

Elizabeth I. Martin

Corticotropin-Releasing Factor Overexpression in the Central Amygdala: Gene Expression, HPA Axis Function, and Behavior

Elizabeth I. Martin
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Neuroscience

Charles B. Nemeroff, MD, PhD
Advisor

Joe Cubells, MD, PhD
Committee Member

Michael J. Owens, PhD
Committee Member

Kerry J. Ressler, MD, PhD
Committee Member

Wiley Vale, PhD
Committee Member

Jay Weiss, PhD
Committee Member

Accepted:

Lisa A. Tedesco, PhD — Date
Dean of the Graduate School

Corticotropin-Releasing Factor Overexpression in the Central Amygdala: Gene Expression, HPA Axis Function, and Behavior

By

Elizabeth I. Martin
BA, Lawrence University 2003

Advisor: Charles B. Nemeroff, MD, PhD

An Abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Program in Neuroscience
Graduate Division of Biological and Biomedical Science

2009

Abstract

Corticotropin-Releasing Factor Overexpression in the Central Amygdala: Gene Expression, HPA Axis Function, and Behavior

By

Elizabeth I. Martin

Mood and anxiety disorders including major depressive disorder and post-traumatic stress disorder have been associated with a disrupted hypothalamic-pituitary-adrenal (HPA) axis response to stress, attributed to corticotropin-releasing factor (CRF) overexpression in the paraventricular nucleus of the hypothalamus (PVN). However, PVN output is determined by summation of signals from limbic and brainstem sources; disruption in one of these regions may result in increased PVN CRF and thus HPA axis hyperactivity. Long-term gene expression changes which confer the chronic nature of these disorders may take place primarily in the PVN or may take place primarily in limbic structures, which then modulate the PVN. The utility of CRFergic circuits as pharmaceutical targets for the treatment of mood and anxiety disorders could be improved with greater knowledge of distinct, regionally-specific CRF expression patterns. The goal of this research is to develop tools to manipulate gene expression within CRF-producing cells. Here we describe a transgenic mouse in which 3.0Kb of the CRF promoter reliably targets transgene expression to CRF-producing neurons. The cell-type specificity of this promoter was also employed in a lentiviral vector to overexpress CRF from CRFergic cells. Because the CeA is known to influence the behavioral stress-response and hypothesized to play a role in HPA axis regulation, this virus was injected bilaterally into the CeA of adult male rats. Chronic CRF overexpression in the CeA increased expression of CRF and vasopressin in the PVN, leading to increased HPA axis activation, and decreased expression of MR in the hippocampus, resulting in HPA axis disinhibition. These gene-expression changes and HPA axis hyperactivity also resulted in an increase in anxiety-like behavior. These data suggest that HPA axis hyperactivity in human patients may be secondary to altered signals from CRF neurons within the CeA. This and future work elucidating the precise mechanisms through which overexpression of CRF precipitates psychopathology may provide useful preventative and therapeutic tools for mood and anxiety disorders.

Corticotropin-Releasing Factor Overexpression in the Central Amygdala: Gene Expression, HPA Axis Function, and Behavior

By

Elizabeth I. Martin
BA, Lawrence University 2003

Advisor: Charles B. Nemeroff, MD, PhD

A dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Program in Neuroscience
Graduate Division of Biological and Biomedical Science

2009

Acknowledgements:

I would like to extend my heartfelt gratitude to my advisor, Charlie Nemeroff, for his mentorship, guidance, encouragement, and understanding through the painful process that is graduate school. I am also grateful for the support and advice of my committee members Mike Owens, Joe Cubells, Kerry Ressler, Jay Weiss, and Wylie Vale. I would also like to thank Kerry Ressler for hosting me in his lab and teaching me about viral vectors. I am also deeply indebted to Jasmeer Chhatwal, recent graduate from the Ressler lab, for helping me to design and develop the 3.0Kb CRF promoter, Susan Plott, research Czar of the Nemeroff lab, and Gretchen Neigh for all of their assistance, patience, and moral support. Most importantly, I would like to thank my family, especially my parents, and my friends, especially Jennifer Foth, Jeff Turriff, and Patrick Flandreau. I could not have done it without you.

TABLE of CONTENTS

Chapter 1: General Introduction and Background

TABLE of CONTENTS

Chapter 2: Viral Vector and Transgenic Tools to Manipulate Gene Expression within CRF-Expressing Cells

TABLE of CONTENTS

Chapter 3: Behavioral effects of lentiviral-vector mediated region and cell-type specific overexpression of CRF within CRF-producing cells of the central amygdala.

TABLE of CONTENTS

Chapter 4: Effects of Region and Cell-Type Specific Overexpression of CRF within CeA CRF cells on HPA Axis Activity

TABLE of CONTENTS

Chapter 5: Chronic CeA CRF-OE Alters Expression of Genes Involved in HPA Axis Regulation

TABLE of CONTENTS

Chapter 6: General Discussion

TABLE of CONTENTS

Appendix A

Keen-Rinehart, E, Michopoulos, V, Toufexis, DJ, **Martin, EI**, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME (2008). Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. *Molecular Psychiatry.*

TABLE of CONTENTS

Appendix B:

Martin EI and Nemeroff CB. 2008. The Role of Corticotropin-Releasing Factor in the Pathophysiology of Depression: Implications for Antidepressant Mechanisms of Action. *Psychiatric Annals April 2008*

TABLE of CONTENTS

Appendix C

Martin EI and Nemeroff CB. 2007. The Biology of Generalized Anxiety Disorder and Major Depressive Disorder: Commonalities and Distinguishing Features. *In* The Future of Psychiatric Diagnosis: Refining the Research Agenda Depression and Generalized Anxiety Disorder, *American Psychiatric Institute Press*

TABLE of CONTENTS

Appendix D

Elizabeth I. Martin, Terrell E. Brotherton, Becky Kinkead, K.V. Thrivikraman, Paul M. Plotsky, Charles B. Nemeroff, Michael J. Owens. Diurnal Regulation of Gene Expression in Select Brain Regions of Adult Male Sprague Dawley Rats *Society for Neuroscience* Annual meeting, November, 2007

CHAPTER 1:

Symptoms and Epidemiology of Mood and Anxiety Disorders

For many mood and anxiety disorders, particularly major depressive disorder (MDD), generalized anxiety disorder (GAD) and post-traumatic stress disorder (PTSD), symptom onset is associated with environmental stress or traumatic events (Kendler et al., 1999; Bale, 2006). The following is a brief description of the diagnostic criteria and epidemiology.

A. Major Depressive Disorder

According to the American Psychiatric Association (*DSM-IV TR* 2000), MDD is a cyclical disorder with alternating depressive episodes and periods of euthymia. A depressive episode is a period longer than two weeks during which a patient experiences depressed mood most of the day every day along with anhedonia and at least five of the following symptoms:

- Weight change
- Change in sleeping patterns
- Psychomotor agitation or retardation
- Fatigue and loss of energy
- Feelings of worthlessness and inappropriate guilt
- Difficulty thinking and concentrating
- Thoughts of death

MDD may be subdivided into typical or atypical depression, melancholic depression, or psychotic depression. Psychotic depression is a particularly severe MDD subtype; these patients experience mood-congruent delusions or hallucinations and many (50-75%) experience cognitive impairments. Compared to the other subtypes, psychotic depression has a stronger association with thoughts of suicide or homicide (DSM-IV TR 2000).

With a lifetime prevalence of 16.2%, and a 12-month prevalence of 6.6%, MDD is one of the most common psychiatric illnesses. The mean duration of a single major depressive episode (MDE) is 16 weeks (Kessler et al., 2003), 50% of patients experience their first depressive episode before the age of 40, and most (50 to 85%) of those patients will experience a second episode. Each subsequent MDE increases the likelihood of continued episodes and decreases the likelihood of a positive response to treatment (DSM-IV-TR 2000; Eaton et al., 2008). It is estimated that only 50% of MDD patients are being actively treated and, of those patients receiving medical attention, treatment was successful for less than half; at any given time only 21.7% of MDD patients receive adequate medical care (Kessler et al., 2003).

Morbidity and mortality rates for MDD patients are high; two thirds of MDD patients contemplate suicide and 10-15% succeed. Furthermore, a diagnosis of MDD is associated with increased risk for cardiovascular disease, slower recovery from surgery, and poorer prognosis in cancer and AIDS patients (Kessler et al., 2003; Eaton et al., 2008). Nearly 60% of depressed patients are also diagnosed with an anxiety disorder (reviewed in (Malhi et al., 2002). The converse is also true, with the majority of anxiety-disorder patients experiencing depression symptoms if not reaching diagnostic criteria for major depressive disorder (MDD) (DSM-IV TR 2000).

B. Anxiety Disorders

Anxiety is a response to an unknown, internal, vague, or chronic threat. Anxiety disorders include GAD, PTSD, panic disorder, social anxiety disorder, specific phobia, and obsessive compulsive disorder. These disorders are characterized by shortness of breath and chest pain, motor tension, autonomic hyperactivity, and increased vigilance (DSM-IV TR 2000). Among the anxiety disorders, GAD and PTSD have the greatest comorbidity with MDD.

1. Generalized Anxiety Disorder

GAD is diagnosed when a patient experiences six or more months of excessive anxiety and worry accompanied by at least three additional symptoms such as restlessness, fatigue, difficulty concentrating, irritability, and muscle tension (DSM-IV TR 2000). Lifetime and 12-month prevalence rates of GAD are 2.8% and 1.2% for men, and 5.3% and 2.7% for women, respectively (Vesga-Lopez et al., 2008).

2. Post-Traumatic Stress Disorder

PTSD is diagnosed when exposure to perceived or actual threat of death or serious injury results in intense fear, helplessness, or horror for at least one month. Combat veterans, victims of natural disasters, and victims of criminal violence are at risk for PTSD. Among these groups lifetime prevalence rates are community and situationally based. PTSD is characterized by persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness to current life events. Associated symptoms may include self-destructive and impulsive behavior, dissociative symptoms, somatic complaints, feelings of ineffectiveness, shame, despair, or hopelessness, loss of

previously sustained beliefs, hostility and social withdrawal, along with a constant sense of being threatened, and changes in personality characteristics (DSM-IV TR 2000).

C. Pharmacotherapy for Depression and Anxiety Symptoms

Current antidepressant drugs were developed based on two serendipitous findings; first, that monoamine depletion with the antihypertensive agent reserpine causes depression in some patients, and second, that the anti-tubercular agent isoniazid, which inhibits monoamine oxidase (MAO), the enzyme responsible for degrading monoamine neurotransmitters intracellularly, was noted to improve patients' mood. As such, researchers hypothesized that decreased monoamine availability is a biological substrate of depression. Drugs were developed to block presynaptic monoamine transporter proteins, to inhibit MAO, and/or to exert differential actions at pre- and post-synaptic monoamine receptors (reviewed in (Nemeroff and Owens, 2002).

1. Tricyclic Antidepressants (TCA)

In the 1950s, researchers attempting to create antipsychotic drugs created the tricyclic molecule imipramine. Imipramine was demonstrated to possess antidepressant properties (Azima and Vispo, 1958), leading to the development of additional tricyclic compounds as antidepressants. It has since been determined that these drugs block presynaptic reuptake transporters for the neurotransmitters (NTs) serotonin and norepinephrine and this is thought to reduce symptoms of depression and anxiety. However TCAs also block postsynaptic receptors for histamine, resulting in sedation, and postsynaptic acetylcholine receptors, resulting in blurred vision, dry mouth, tachycardia (rapid heart rate), and

cognitive distortion. Furthermore TCAs are lethal in overdose, an important consideration for disorders with increased risk of suicide (Wallach et al., 1968).

2. Monoamine Oxidase Inhibitors (MAOI)

MAOIs increase NT availability, allowing a greater effect on post synaptic receptors. The original MAOIs, including phenelzine (Nardil) (Hobbs, 1959), isocarboxazid (Marplan) and tranylcypromine (Parnate) are irreversible and non-selective. The side-effect profile of these drugs is a major limiting factor in their usage. Inhibition of MAO in the liver and intestine and inhibition of other critical metabolic enzymes results in risk of drug-drug and drug-food interactions resulting in a potentially fatal increase in blood pressure. As such, strict dietary restrictions must be adhered to in patients taking MAOIs. Reversible, selective MAOIs such as moclobemide (Aurorix) were developed soon after and have a much improved safety profile (Casacchia et al., 1984). Despite the potential side effects, even the original MAOIs are still prescribed and can be successful in patients who failed to respond to SSRIs and TCAs. This may be particularly true in patients with severe and atypical depression (Baker et al., 1992).

3. Serotonin-Specific Reuptake Inhibitors

Similar to MAO inhibition, blockade of serotonin reuptake increases serotonin availability in the synapse. Side effects of SSRIs include nausea, dizziness, changes in appetite and weight, and sexual side effects such as anorgasmia, erectile dysfunction, and decreased libido. Another potential complication with SSRI administration is serotonin syndrome; disorientation and confusion, motor ataxia and increased reflexes, agitation and restlessness, fever, shivers, chills, sweating, diarrhea, hypertension, and tachycardia due to acute elevations in serotonin availability. The converse, serotonin withdrawal

syndrome, precipitated by abrupt drug discontinuation, is accompanied by flu-like symptoms, dizziness, motor ataxia, sensory disturbances, and sleep disturbances. Serotonin withdrawal can be prevented by gradual tapering off medication (Wernicke, 1985).

Anatomy of Emotion

A. The Limbic System

In 1937 neuroanatomist James Papez hypothesized that brain regions dedicated to motivation and emotion processing formed an interconnected circuit (FIGURE 1-1). In Papez's emotion-processing system, the cingulate gyrus, located at the middle edge ("limbus") of the cerebral cortex, projects to the hippocampus CA fields, which project via the fornix axon tract to the mamillary bodies of the hypothalamus. The mamillary bodies then inform the anterior thalamic nucleus, followed by return of signal to the cingulate cortex. Papez viewed these structures as a closed circuit with the cingulate cortex functioning as a receptive field for emotion. In 1948 a medical doctor, Paul Ivan Yakovlev, added to Papez's circuit the orbitofrontal cortex (OFC), insular cortex, anterior temporal lobe, amygdala, and dorsomedial nucleus of the thalamus. The following year, the physiologist Paul MacLean added the forebrain and coined the term 'limbic system' to define the emotional processing circuit. The limbic system is no longer conceived as a closed circuit but involves many cortical and subcortical regions along with the

connections between them (FIGURE 1-2 and 1-3). For a more detailed history of limbic system research see (Nakano, 1998).

1. Periaqueductal Gray

The periaqueductal grey matter (PAG) is a collection of cell bodies surrounding the cerebral aqueduct in the midbrain. The PAG is an important center in the motor output for the behavioral response to stress.

2. Ventral Tegmental Area and Nucleus Accumbens

The ventral tegmental area (VTA) is a group of cell bodies in the ventral midbrain (tegmentum). These cells contain dopamine (DA) and project via the medial forebrain bundle to the nucleus accumbens (NAc) in the ventral striatum. This mesolimbic dopaminergic pathway is implicated in reward and addiction. (See (Hikosaka et al., 2008) for a recent review on reward processing).

3. Septal Nuclei

The septal nuclei are involved in reward and reinforcement as well as emotional regulation and impulse control, potentially via inhibitory effects on the amygdala. In laboratory rats, septal lesions, which may disinhibit the amygdala, produce extreme aggression towards handlers and cage-mates. In contrast, laboratory rats will self-administer electrical stimulation to this region (Olds and Milner, 1954).

4. Hypothalamus

The hypothalamus is a relatively small brain region located in the ventral diencephalon. It is divided into numerous subdivisions each with a particular role in maintaining homeostasis. Its responsibilities include regulating body temperature, food and water intake, and maintaining the circadian rhythm. Via its connections with the anterior and

posterior pituitary gland, the hypothalamus controls endocrine systems to regulate thyroid hormones, growth hormones, and sex steroids. The hypothalamus is interconnected with the amygdala, and prefrontal cortex (FIGURE 1-3). Furthermore hormones regulated by the hypothalamus influence emotionality (See (Hokfelt et al., 1989) for a review on the hypothalamic neurosecretory system).

5. Limbic Cortex

The limbic cortex is part of the phylogenetically ancient cortex. It includes the insular cortex and cingulate cortex. The limbic cortex integrates the sensory, affective, and cognitive components of pain and processes information regarding the internal bodily state. The dorsal insula has major connections to the somatosensory cortex while the ventral insula is involved in visceral sensation and autonomic responses via connections with the OFC and amygdala (Vogt et al., 1992; Treede et al., 1999).

6. Prefrontal Cortex

The frontal lobe is the most phylogenetically recent brain region, far more extensive in humans than even our closest primate relatives. The prefrontal frontal cortex (PFC) is responsible for executive functions such as planning, decision making, predicting consequences for potential behaviors, and understanding and moderating social behavior. Prefrontal activity is also implicated in personality development; damage to this region, as in the infamous case of Phineas Gage, can remarkably alter personality and behavior (reviewed in (Harlow, 1999).

The PFC is subdivided by anatomy and function with the OFC and medial prefrontal (mPFC) subdivisions having the highest interconnectivity with other limbic system structures. The OFC codes information, controls impulses, and regulates mood.

The ventromedial prefrontal cortex (vmPFC) is involved in reward processing (Keedwell et al., 2005) and in the visceral response to emotions, which are enhanced by the right vmPFC and inhibited by the left vmPFC (Drevets, 2001). The PFC regulates impulses, emotions, and behavior, via inhibitory top-down control of emotional-processing limbic structures (e.g. (Miller and Cohen, 2001).

7. Hippocampus

The hippocampus is located in the temporal lobe dorsal to the amygdala. Although included in Papez's original limbic circuit, the hippocampus has since become better known for its role in spatial working memory and encoding and retrieval of declarative (conscious) memories. Damage to the temporal lobe inclusive of the hippocampus can result in anterograde amnesia, the inability to form new memories. In terms of its role in emotion, the hippocampus is highly interconnected with the amygdala, has tonic inhibitory control over the hypothalamic stress response system, and plays a role in negative feedback for the HPA axis. Hippocampal volume and neurogenesis (growth of new cells) in this structure have been implicated in stress sensitivity and resiliency in relationship to mood and anxiety disorders.

8. Amygdala

The amygdala is an evolutionarily ancient structure responsible for processing emotionally-salient external stimuli and eliciting the appropriate behavioral response. The amygdala is responsible for the expression of fear and aggression as well as species-specific defensive behavior. The amygdala also plays a role in formation and retrieval of emotional and fear-related memories.

The amygdala is a complex structure composed of functionally distinct nuclei and subnuclei that vary in cyto- and chemoarchitecture as well as in connectivity (Asan et al., 2005). There has been some debate regarding the most appropriate division of the amygdala and nomenclature of nuclei, with over a dozen nuclei and subnuclei identified. Most relevant to this review is the central nucleus of the amygdala (CeA).

The CeA is heavily interconnected with cortical regions including the limbic cortex. It also receives input from the hippocampus, thalamus, and hypothalamus, including the paraventricular nucleus of the hypothalamus (PVN). The CeA receives further connections from the lateral septum (LS) and from brainstem monoaminergic nuclei. Efferent connections from the CeA travel to the hypothalamus and to midbrain and brainstem monoaminergic and cranial nerve nuclei (reviewed in (Knapska et al., 2007).

The CeA is also reciprocally connected with the bed nucleus of the stria terminalis (BNST) as part of the extended amygdala. The BNST is located rostral to the CeA in the basal forebrain; much of the effect of the CeA on the endocrine response to stress is due to bisynaptic and multi-synaptic pathways through the BNST. FIGURE 1-4 is a schema of CeA projections in relationship to the endocrine, autonomic, and behavioral stress response.

B. Limbic System Disruptions in Mood and Anxiety Disorders

Symptoms of mood and anxiety disorders are thought to result in part from disruption in the balance of activity in emotional centers of the brain relative to higher cognitive centers (e.g. (Goldapple et al., 2004; Seminowicz et al., 2004). Decreased top-down inhibitory input from executive brain regions may be responsible for the amygdala overactivity patients with mood and anxiety disorders (see (Rauch et al., 2003) for a review).

Remarkably, an increase in resting amygdalar regional cerebral blood flow (rCBF) may be specific to primary mood disorders; patients with obsessive compulsive disorder, phobias, or other neuropsychiatric conditions do not demonstrate increased resting amygdalar activity (Drevets, 2003). The magnitude of increased rCBF and metabolism in the amygdala correlates with the symptom severity of a depressive episode (Drevets, 2001; Anand and Shekhar, 2003; Drevets, 2003). Amygdalar overactivity in MDD patients persists even in the absence of conscious processing as evidenced by sleep studies (Drevets, 2003), or in response to split-second presentation of fearful facial stimuli (Anand and Shekhar, 2003). Both conscious and unconscious amygdalar overactivity normalizes after successful antidepressant treatment, suggesting that amygdala hyperactivity is causally related to the state of a MDE (Drevets, 2003).

Although resting amygdala activation appears to be specific for mood disorders, symptom-provocation paradigms reveal anxiety-induced amygdalar activation, particularly in the right hemisphere (Liotti et al., 2000). In PTSD patients, the amygdala is involved in fear learning, which is associated with PTSD symptoms, as well as extinction learning, which is associated with PTSD treatment and symptom reduction.

Severity of PTSD symptoms predicts the magnitude of amygdala activation when encoding memories (Dickie et al., 2008). TABLE 1-1 describes the major findings regarding the limbic cortex and amygdala activity in depression and sadness compared to normal and pathological anxiety (Mayberg, 1997; Mayberg et al., 1999; Mayberg, 2003).

Neurotransmission in Emotion

Receptors for NTs fall into two general classes: ionotropic and metabotropic. Ionotropic receptors are ligand-gated ion channels. When a NT binds an ionotropic receptor, the ion channel becomes more permeable to influx or efflux of ions which regulate the membrane potential of the postsynaptic cell. Depending on the specific channel, the membrane potential may become closer or further away from the threshold of depolarization for an action potential.

Metabotropic receptors are not bound to ion channels but to signaling molecules called G-proteins. When a NT binds a metabotropic receptor, the G-protein is activated and initiates signal transduction cascades to increase (G_s) or decrease (G_i) production of cyclic adenosine monophosphate (cAMP). cAMP acts as a second messenger and it activates protein kinase A (PKA). Metabotropic receptors coupled to G_q proteins activate phospholipase C (PLC). In each case the resulting signal transduction cascades activate cellular enzymes, modify cell surface ion channels, and influence gene expression in the nucleus. FIGURE 1-5 diagrams a signal transduction cascade initiated by metabotropic receptors coupled to G_s.

A. Amino acid Neurotransmitters

Glutamate is the main excitatory NT of the central nervous system (CNS). The ionotropic glutamate N-methyl-D-aspartate (NMDA) receptor is well known for its role in long-term potentiation, the neurochemical substrate of learning. The most prevalent glutamate receptor is the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. AMPA and a third glutamate receptor, the kainate receptor, are also ionotropic. Several classes of metabotropic glutamate receptors (mGluR) also exist.

The main inhibitory NT in the CNS is γ-amino-butyric-acid (GABA). The ionotropic receptor, $GABA_A$ activation is enhanced by benzodiazepines and barbiturates, which have anti-epileptic and anxiolytic properties. $GABA_B$ receptors are G-protein coupled. Presynaptic $GABA_B$ activation inhibits further GABA release. Postsynaptically, $GABA_B$ receptors are located on some cell bodies and dendrites where they have suppressive effects on the postsynaptic cell (reviewed in (Carlson, 2001; Kent et al., 2002).

B. Monoamines

Monoamines, named for their structure, include serotonin (5-hydroxytryptamine**,** 5-HT), norepinephrine (NE), and DA. In the presynaptic neuron, monoamines are packaged into vesicles by vesicular monoamine transporter (vMAT). After being released into the synapse, they are reclaimed into the presynaptic cell by NT-specific transporters: the dopamine transporter (DAT), norepinephrine transporter (NET) and the serotonin transporter (SERT). 5-HT, NE and DA are degraded by the enzyme MAO. DA and NE,

the catecholamine NTs, are inactivated by the enzyme catecholamine-O-methyltransferase (COMT) as well as MAO.

1. Serotonin

Serotonergic cell bodies are concentrated in the medial and dorsal raphe nucleus (MRN, DRN) in the hindbrain. These nuclei have widespread projections throughout the neocortex and limbic system as well as the cerebellum. DRN 5-HT cells are highly interconnected with DA and NE nuclei. Numerous 5-HT receptors have been cloned and identified. Mediated by an impressive pre- and post-synaptic receptor diversity, 5-HT is able to have expansive regional and cell-type specific effects (see (Kent et al., 2002) for a review of 5-HT receptors and their role in anxiety).

2. Catecholamines

a) Dopamine

Dopamine cell bodies are contained in two midbrain nuclei, the substantia nigra (SN) and VTA. Dopaminergic projections from the SN travel to the striatum. This nigrostriatal DA pathway plays an important role in movement; damage to SN DA cells or blockade of DA receptors in the caudate/putamen results in Parkinsonian symptoms. The mesolimbic DA pathway from the midbrain VTA to the NAc in the ventral striatum is involved in emotion and reinforcement. The mesocortical DA pathway also originates in the VTA and projects throughout the cortex

b) Norepinephrine

Norepinephrine-producing cells (termed noradrenergic) are positioned in the hindbrain locus coeruleus (LC). NE neurons project from the LC throughout the cortex and limbic system and to the cerebellum. Noradrenergic projections from the LC to other hindbrain

nuclei and the spinal cord play an important role in the autonomic nervous system (ANS). Heavy interconnections between the LC, DRN, and CeA mediate the role of NE in emotion.

C. Neuropeptides

Peptide signaling molecules are an evolutionarily ancient method of cellular communication and exist throughout the animal kingdom from hydra to humans. The over 50 NPs identified range in size from three amino acids to 43. Most NPs were discovered in the periphery for their role in maintenance of homeostasis and have been identified centrally where they often moderate central homeostatic pathways related to their peripheral roles.

NPs colocalize with, and in some cases are packaged and released with each other and with classical NTs. Many NPs are expressed in limbic regions, particularly the hypothalamus, where they can influence stress and emotion circuitry. The functional implications of these limbic colocalizations have been addressed in numerous reviews (e.g. (Honkaniemi et al., 1992; Watts, 1996; Palkovits, 2000; Cole and Sawchenko, 2002; Holmes et al., 2003; Gysling et al., 2004; Barrera et al., 2005). Below is a brief description of select neuropeptides.

1. Angiotensin II (Ang-II)

In the periphery AngII regulates blood pressure and salt retention from the kidney. Centrally it is expressed in the hypothalamus where it moderates salt homeostasis and drinking behavior.

2. Cholecystokinin (CCK)

CCK in the gastrointestinal system plays a role in digestion. In the CNS it is located in the amygdala, hippocampus, PAG, SN and DRN. Among other things, it is known to play a role in feeding behavior (Fink et al., 1998).

3. Enkepahlin (Enk)

Both centrally and in the periphery, Enk regulates pain processing. It is positioned in the dorsal root ganglia of the spinal cord and in the periphery is released from the adrenal gland and immune system cells (Miller and Pickel, 1980).

4. Galanin (Gal)

Gal is also located in the dorsal root ganglia of the spinal cord but in contrast to Enk, Gal is pro-nociceptive. Peripherally Gal plays a role in inflammatory pain. Gal is also colocalized with monoamines in brainstem nuclei. In addition to nociception, Gal influences feeding behavior and regulates neuroendocrine and cardiovascular systems (Bedecs et al., 1995; Liu and Hokfelt, 2002; Lang et al., 2007).

5. Neuropeptide Y (NPY)

NPY is an important component of the sympathetic nervous system in the periphery. Centrally it is expressed in the hypothalamus, hippocampus, and amygdala and is often found colocalized with NE. It is best known for its role in feeding behavior and ravenous hunger (Leibowitz, 1990).

6. Neurotensin

Peripheral neurotensin in the intestine and other peripheral organs plays a role in gastrointestinal motility. Centrally neurotensin is expressed by the hypothalamus,

amygdala, and dopaminergic nuclei. Central neurotensin has been implicated in feeding behavior and nociception (Binder et al., 2001).

7. Oxytocin (OT)

OT is expressed peripherally in reproductive organs and is known to modulate labor and parturition. OT in the cardiovascular system influences heart rate and blood pressure. Centrally OT regulates reproductive, maternal, and affiliative behavior (Gimpl and Fahrenholz, 2001; Meyer-Lindenberg, 2008)

8. Vasopressin (AVP)

In the periphery, AVP is also known as antidiuretic hormone. It increases water retention from the kidneys and in high concentrations constricts blood vessels. Central AVP also regulates fluid homeostasis. AVP in the hypothalamus is often colocalized with OT and influences affiliative behavior (Egashira et al., 2006).

In addition to the aforementioned roles, each of these peptides is also involved in the stress-response system, psychopathology, and/or the mechanism of action of antidepressant drugs, often via interactions with corticotropin-releasing factor (CRF), the NP responsible for coordinating the endocrine, autonomic, and behavioral responses to stress (TABLE 1-2).

CRF Mediates the Endocrine, Autonomic, and Behavioral Response to Stress

CRF is a 41 amino acid peptide discovered in 1981 by Vale and colleagues for its role in initiating the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1981) and has since been identified as a key mediator of the endocrine, autonomic and behavioral response to stress. CRF belongs to a family of NPs including urocortin (UCN), UCN II, and UCN III. CRF and its related peptides are the natural ligands for two G protein-coupled receptors, CRF_1 and CRF_2. CRF and UCN share a high affinity for CRF_1. UCN exhibits an equal affinity for both receptors (Donaldson et al., 1996), while CRF has a much greater affinity for CRF_1. UCNII and III bind almost exclusively to CRF_2 (reviewed in (Bale and Vale, 2004)). Both CRF receptors are class-B G-protein-coupled receptors and are thought to most commonly, though not exclusively, couple to G_s (Grammatopoulos et al., 2001). The neurobiology of CRF has been extensively reviewed (e.g. (Owens and Nemeroff, 1991; Sawchenko et al., 1993; Bale and Vale, 2004).

The CRF system is highly conserved through evolution. In humans and laboratory animals, CRF is expressed centrally in the cortex, limbic system, and brain stem (FIGURE 1-6). Hypothalamic CRF initiates the endocrine response to stress while extrahypothalamic CRF is largely responsible for the autonomic and behavioral stress response. Extrahypothalamic CRF-producing regions include the PFC, cingulate, and insular cortex, hippocampus, extended amygdala, and LC. CRF is produced in a variety of cell types including neurons and glia (Kapcala and Dicke, 1992) and is colocalized with a variety of other NTs and NPs both centrally and in the periphery (e.g. (Wolter, 1985; Hisano et al., 1987; Palkovits, 2000; Valentino et al., 2001; Smialowska et al., 2002).

CRF actions on the CRF_1 receptor, more so than CRF_2, have been implicated in the initial CRF-mediated response to stress. In the rat and mouse, CRF_1 is present throughout the cortex and limbic regions with high expression in the anterior pituitary gland, basolateral amygdala (BLA), CeA, BNST, and cerebellum (Asan et al., 2005). In contrast, expression of CRF_2 is more circumscribed. The CRF_{2A} subtype is present in the septum, the ventromedial and supraoptic nuclei of the hypothalamus, DRN, and some regions of the amygdala. CRF_{2B} is expressed in high concentrations in the choroid plexus and cerebral arterioles. In the periphery, CRF_{2B} is located in skeletal muscle and cardiac smooth muscle.

CRF_{2A} is expressed in some regions that contain CRF_1. These include the PVN, BNST, and hippocampus (Chalmers et al., 1995; Van Pett et al., 2000). It has been suggested that CRF_2 activation represents a slower, secondary response to stress which is responsible for returning the system to homeostasis after HPA axis activation. Distribution of CRF_1 and CRF_2 receptors in the rat brain is shown in FIGURE 1-7. See (Bale and Vale, 2004) for a more detailed review of the CRF receptors.

A. <u>CRF and the Endocrine Response to Stress</u>

CRF cell bodies located in the medial parvocellular division of the paraventricular nucleus of the hypothalamus (PVNmp) initiate the HPA stress axis, which results in increased plasma concentration of stress steroid hormones and, therefore, mobilization of energy sources to respond to threatening stimuli.

More specifically, PVNmp CRFergic neurons terminate within the median eminence (ME) of the hypophysial portal system, which connects the hypothalamus with

the anterior pituitary gland. In the anterior pituitary, CRF peptide activates CRF_1 receptors, eliciting a G_s-mediated signal transduction cascade: activation of the enzyme adenylyl cyclase, conversion of adenosine-tri-phosphate (ATP) to cAMP, and activation of PKA (FIGURE 1-5). Within pituitary corticotrophs PKA can phosphorylate ion channels in the cell membrane, thereby modulating neuronal excitability. Ion channel modulation by PKA has been implicated in the mechanism through which CRF_1 activation elicits release of adrenal corticotropic hormone (ACTH). With continued receptor activation, PKA also activates transcription factors to modify gene expression. This effect of PKA is attributed to CRF_1-activation-induced increase in the transcription of pro-opiomelanocortin (POMC), the precursor to ACTH (reviewed in (Aguilera, 1994).

The effect of CRF on pituitary corticotrophs can be potentiated by AVP and, to a lesser degree, OT. Much research has demonstrated that stress increases expression of AVP in parvocellular PVN neurons and increases the percentage of CRF-containing PVN neurons which coexpress AVP (Ma et al., 1999). In this way, HPA axis activation continues even in the presence of high circulating glucocorticoids (GC), which provide negative feedback to PVN CRF but have less of an effect on AVP, particularly after chronic increases in plasma GC (Hwang and Guntz, 1997), reviewed in (Owens and Nemeroff, 1991; Scott and Dinan, 1998).

Once released from the pituitary corticotrophs, ACTH enters the body's general circulation. ACTH receptors are concentrated on the adrenal cortex and, like CRF receptors, are coupled to Gs. ACTH receptor-induced signal-transduction cascades result in the synthesis and secretion of androgens, mineralocorticoids, and GCs. The primary GC is cortisol in humans and corticosterone in rodents.

Circulating GCs act on receptors located in the cytoplasm of numerous central and peripheral cell types. There are two main receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR receptors in the CNS are mostly restricted to the hippocampus; these receptors have a high affinity for GCs and are 80% occupied under basal conditions. GC-induced MR activation in the hippocampus provides important tonic inhibition of PVN CRF cells and helps to regulate the circadian rhythm of the HPA axis. GR are present in cortical and subcortical regions in addition to the CA1/2 fields and the dentate gyrus (DG) of the hippocampus. These receptors have a much lower affinity for GC and are activated only after HPA axis activation when high concentrations of GC are available.

GCs diffuse through the plasma membrane and bind to the receptors. Upon ligand binding, GRs dimerize and translocate to the nucleus where they regulate gene transcription. The immediate actions of GCs under stressful conditions serve to mobilize energy stores in preparation to fight or flee from the source of danger. More specifically the effects of GC include:

1. Increased blood glucose concentrations
2. Increased circulating free amino acids
3. Inhibition of inflammatory cytokines
4. Decreased retention of water from the kidney
5. Increased arousal and improved memory
6. Elevated blood pressure

High circulating concentrations of GC also provide negative feedback to the HPA axis to restore homeostasis after an acute stress. GC-mediated negative feedback decreases CRF gene expression in the PVNmp and decrease the release of CRF peptide into the ME. GCs also decrease transcription of POMC and inhibit release of ACTH from the anterior pituitary (Dallman et al., 1987). Disruptions in negative feedback and overall dysregulation of the HPA axis are associated with numerous physiological and psychological disorders (see below).

B. CRF and the Autonomic Response to Stress

The ANS is composed of two branches: the sympathetic nervous system and the parasympathetic nervous system. These two branches exert opposing physiological effects; parasympathetic stimulation slows heart rate and blood pressure and increases energy storage. In contrast, the sympathetic nervous system expends energy. Sympathetic activation increases blood flow to skeletal muscles and increases heart rate and blood pressure; it is this branch which is activated by stress. Like increased energy availability due to HPA axis activation, the elevated heart rate and blood supply to skeletal muscles prepare the organism for a behavioral response to stress.

The ANS is regulated by cranial nerve nuclei and other brainstem nuclei. For example, the nucleus of the solitary tract (NST) receives visceral afferent input from cranial nerves and uses this information to regulate motor output to the stomach and heart, and to regulate blood flow (Monk et al., 2006). In addition to these direct effects on the ANS, the NST also transmits visceral sensory information to the parabrachial nucleus (PBN). The PBN then projects to the PAG and amygdala. The PAG interprets

the sensory information from the both NST and PBN and projects to the reticular formation, which produces coordinated patterns of autonomic activation.

Extrahypothalamic CRF sources, particularly the amygdala, also project to the PBN and reticular formation where they decrease parasympathetic output. From the PVN, a small percentage (<1%) of CRF neurons project, not to the ME, but to brainstem autonomic centers (Reyes et al., 2005).

C. CRF and the Behavioral Response to Stress

Ultimately initiating the appropriate behavioral response to stress determines survival. Stress-induced behaviors include freezing, fighting, or fleeing from danger. The impact of CRF on the behavioral response to stress is attributed to CRF produced in and released from the CeA and BNST, regions with the highest concentrations of extrahypothalamic CRF. Site-specific injections of CRF into the CeA mimic the effects of overall CNS CRF activation. Similarly, the behavioral effects of global CRF_1 agonist or antagonist administration can be duplicated by site-specific injection into the CeA (Liebsch et al., 1995; Bakshi et al., 2002; Daniels et al., 2004; Asan et al., 2005). In response to chronic stress, activation of this circuit may become pathological and lead to symptoms of anxiety and depression (see below).

CRF in Psychopathology and Response to Antidepressant Drugs

A. CRF in the Psychopathology of Mood and Anxiety Disorders

1. Major Depressive Disorder

In many depressed patients, the HPA axis is hyperactive, as evidenced by elevated plasma ACTH and cortisol concentrations and by altered ACTH and cortisol responses in standardized endocrine challenge tests including the dexamethasone suppression test (DST) and the CRF stimulation test. In the DST, systemic administration of dexamethasone, a synthetic GC, decreases (i.e. suppresses) plasma ACTH and cortisol concentrations via negative feedback at the level of the pituitary gland. It is well established, however, that many MDD patients are DST non-suppressors, suggesting that the HPA axis is hyperactive and/or that the negative feedback mechanism is desensitized in these patients (reviewed in (Ising et al., 2005).

In the CRF stimulation test, intravenously administered CRF (which does not enter the CNS) elevates plasma ACTH and cortisol concentrations by stimulating CRF_1 receptors in the anterior pituitary. However, MDD patients as a group demonstrate a blunted ACTH response in this test. Decreased CRF_1 expression in the anterior pituitary secondary to chronic overexpression of CRF likely explains the blunted pituitary response to exogenous CRF; CRF concentrations and CRF mRNA expression is elevated in the hypothalamus in postmortem tissue from depressed patients (reviewed in (Mitchell, 1998).

A combination of the DST and the CRF stimulation test, the Dex/CRF test, developed by Holsboer and colleagues, is generally considered to be the most sensitive

measure of HPA axis activity. In this test, many MDD patients exhibit elevated plasma ACTH and cortisol concentrations relative to healthy control subjects, suggesting that both GC insensitivity and elevated CRF contribute to HPA axis hyperactivity in depression (reviewed in (Ising et al., 2005).

There also exists considerable evidence for hyperactivity of extrahypothalamic CRF-containing circuits in depressed patients. Chronically elevated activity of extrahypothalamic CRF and associated increased synaptic availability of CRF is thought to be responsible for the decreased density of cortical CRF_1 receptors in depressed suicide victims. Elevated cerebrospinal fluid (CSF) concentrations of CRF, observed in many MDD patients, and are also thought to reflect the hyperactivity in extrahypothalamic CRF-producing regions. In particular, CRF mRNA expression in the CeA has been posited to be upregulated in MDD patients. As one main output of the amygdala, CRF projections from the CeA travel to cortical and brainstem regions including to noradrenergic cells in the LC. Overactivity in this CeA-LC projection could explain the observations of elevated CRF concentrations in the LC in MDD patients (reviewed in (Arborelius et al., 1999).

2. Generalized Anxiety Disorder

Available data show neither hypercortisolism, DST non-suppression, nor increased CSF CRF concentrations in GAD patients (Fossey et al., 1996; Nutt, 2001). That CRF and the HPA axis appear to play a less prominent role in GAD than in other anxiety disorders or in MDD is surprising given that a plethora of studies describe a role for CRF and the HPA axis in anxiety-like behavior in experimental animals and that CRF antagonists have been demonstrated to possess anxiolytic effects. It is possible that the lack of evidence

for a pathophysiological role for CRF circuits in GAD is an artifact of the paucity of endocrine studies in these patients.

3. Post Traumatic Stress Disorder

Numerous studies have identified HPA axis disruption in PTSD patients (Yehuda et al., 1991; Fossey et al., 1996; Nutt, 2001; Yehuda, 2001; de Kloet et al., 2006; Risbrough and Stein, 2006). Compared to healthy control subjects, and in contrast to MDD patients, PTSD patients exhibit decreased plasma cortisol concentrations (Yehuda et al., 1990). The degree of decrease in plasma cortisol was negatively correlated with PTSD symptom severity (Olff et al., 2006). However, there have also been studies showing no difference in cortisol in PTSD patients (e.g. (Rasmusson et al., 2001; Lipschitz et al., 2003).

As opposed to DST non-suppression observed in MDD patients, subjects with PTSD exhibit hyper-suppression of plasma ACTH and cortisol in response to challenge with dexamethasone (Yehuda et al., 2002), although negative findings have also been reported (Kudler et al., 1987). Like MDD patients, CSF concentrations of CRF were reported to be higher in PTSD patients than comparison subjects in two studies (Bremner et al., 1997; Baker et al., 2001). DST-hypersuppression in PTSD patients may result from sensitized central GR receptors, secondary to chronic elevations in CRF. That is in opposition to MDD patients in whom chronic CRF hyperactivity is thought to result in GR desensitization and reduced negative feedback. The observed CRF and HPA-axis disruption could result from insufficient GC signaling due to decreased hormone bioavailability or could be due to decreased hormone receptor sensitivity.

The underlying cause of HPA axis disruption is not well understood. To achieve tight control over the basal, circadian, HPA axis rhythm and the HPA axis responsivity to stress, PVN output is regulated by the coordination of numerous limbic structures. Plasticity in one or more of these regions may result in enhanced PVN CRF output and subsequent HPA axis dysfunction. Neuroplasticity refers to the physical restructuring of existing dendrites and synaptic contacts. Neuroproliferation includes neurogenesis and expansion of existing neurons via growth of new synapses, axonal sprouting, and dendritic branching. On the other end of the spectrum is neuronal degeneration, atrophy, and apoptosis (programmed cell death). Chronic stress decreases proliferation in the hippocampus (e.g. (Tanapat et al., 1998); reviewed in (McEwen, 1994) and hippocampal volume is decreased in patients with mood and anxiety disorders (Sheline et al., 1996). Cumulative results of numerous studies indicate that antidepressant drugs increase neuroplasticity in the brain and that this increase is temporally associated with the onset of drug efficacy (reviewed in (Duman et al., 1999; Malberg, 2004).

These data have led to the neuroproliferation hypothesis of depression. Recent research has been aimed at identifying substrates within neuroproliferation signal transduction cascades as safe and effective targets for novel antidepressant and anxiolytic pharmaceuticals. One such potential target is brain-derived neurotrophic factor (BDNF). BDNF-mediated signal transduction cascades increase the expression of anti-apoptotic genes. Severe stress has been shown to decrease hippocampal BDNF expression (Smith et al., 1995b). Increased apoptosis secondary to diminished BDNF expression during chronic stress may be associated with decreased hippocampal volume observed in MDD and PTSD patients.

An alternative hypothesis is that chronic GC elevation is the primary cause of symptom onset and maintenance and increased CRF and decreased BDNF result from insufficient GC negative feedback to the PVN (for a review see (Holsboer, 2000). Disruption of GR-dependent negative feedback to the PVN has been attributed to decreased BDNF in the hippocampus (Barbany and Persson, 1992; Chao and McEwen, 1994) and decreased BDNF in the hippocampus has been attributed to increased GCs (Schaaf et al., 1998) providing a potential link between these two regulatory elements.

Importantly, extrahypothalamic CRF, via direct and indirect connections to the hippocampus and the hypothalamus has the ability to disinhibit PVNmp CRF-producing cells, thereby eliciting HPA axis activity. In this way, elevations in extra hypothalamic CRF may cause both the disruption of GR-dependent negative feedback and decreased BDNF in the hippocampus (Ziegler, 2002; Herman et al., 2003).

B. CRF in the Mechanism of Action of Antidepressant Drugs

Currently available antidepressants, anxiolytics, and mood stabilizers have been shown to reduce the overall responsiveness of the HPA axis, and the activity of hypothalamic and extrahypothalamic CRF neurons (Grigoriadis et al., 1989; Skelton et al., 2000; Stout et al., 2001; Gilmor et al., 2003). Importantly, non-pharmacological antidepressant treatments such as electroconvulsive therapy also normalize HPA axis reactivity (e.g. (Yuuki et al., 2005)), and reduce the elevated CSF CRF concentrations observed in depressed patients. These data support the hypothesis that HPA axis normalization is associated with symptom resolution.

HPA axis normalization following successful treatment may reflect normalization of CRF signaling. This hypothesis is supported by the fact that changes in CRF mRNA expression and CRF concentrations as well as CRF_1 mRNA expression and binding have been demonstrated following chronic antidepressant administration in laboratory animals. At the level of the brain stem, chronic but not acute administration of the TCA imipramine increases CRF binding in rats (Grigoriadis et al., 1989; Owens et al., 1989). Chronic imipramine and desipramine administration to rats also showed a trend toward increased CRF binding in the striatum, cerebellum and frontal cortex, but not in the parietal/temporal cortex, hippocampus, or anterior pituitary gland (Grigoriadis et al., 1989). Such increases in the density of CRF_1 receptor binding sites are likely secondary to antidepressant-induced reductions in CRF neuronal activity. These data are concordant with the observations that both normal controls and depressed patients exhibit reductions in CSF CRF concentrations after treatment with desipramine (Veith et al., 1993) and fluoxetine (De Bellis et al., 1993). These changes also roughly follow the time course of symptom resolution, supporting the hypothesis that normalization of CRF neurotransmission plays a causal role in the mechanism of action of antidepressant drugs.

Antidepressants of different classes reduce CRF responsiveness to stress. Administration of the SSRI sertraline or the MAOI phenelzine can enhance the signal-to-noise ratio of rat LC neuronal activity, which is decreased by exogenous CRF administration (Valentino and Curtis, 1991). Both the MAOI tranylcypromine and the serotonin-norepinephrine reuptake inhibitor (SNRI) venlafaxine, when administered chronically, reduce chronic variable stress-induced increases in CRF heteronuclear (hn) RNA expression in the PVN. Because there were no changes in baseline CRF or HPA

axis measures in antidepressant-treated rats, these results suggested that chronic antidepressant treatment decreases CRF neuronal sensitivity to stress (Stout et al., 2002).

Neuroanatomical connections between CNS monoamine and NP circuits provide numerous opportunities by which antidepressants can influence CRFergic neurotransmission. One hypothesized mechanism of action of SSRIs is that by increasing 5-HT availability, SSRIs decrease activation of the LC noradrenergic pathways to the CeA, potentially normalizing CRF expression in this region, and thus decreasing symptom severity (FIGURE 1-9). Several thorough reviews discuss interactions between CRF and other neuromodulators in psychopathology (Ressler and Nemeroff, 2000; Nutt, 2001) and in the antidepressant and anxiolytic effects of pharmaceuticals (Kent et al., 2002).

That CRF plays such a strong role in antidepressant response suggests that direct manipulations of the CRF system may provide more efficient and effective treatments for mood and anxiety disorders. A burgeoning database from preclinical (e.g. (Gutman and Nemeroff, 2003) and clinical (e.g. (Zobel et al., 2000) research has revealed that CRF_1 receptor antagonists possess antidepressant and anxiolytic properties and likely represent a novel class of antidepressants. Many such agents have been developed and are in various stages of testing from the laboratory to the clinic. A better understanding of CRF regulation and circuitry from extrahypothalamic and hypothalamic sources will be critical in understanding the mechanism of action of CRF_1 antagonists and other antidepressants and may provide additional insight into the underlying pathology of mood and anxiety disorders.

FIGURE 1-1: Papez's Limbic Circuit

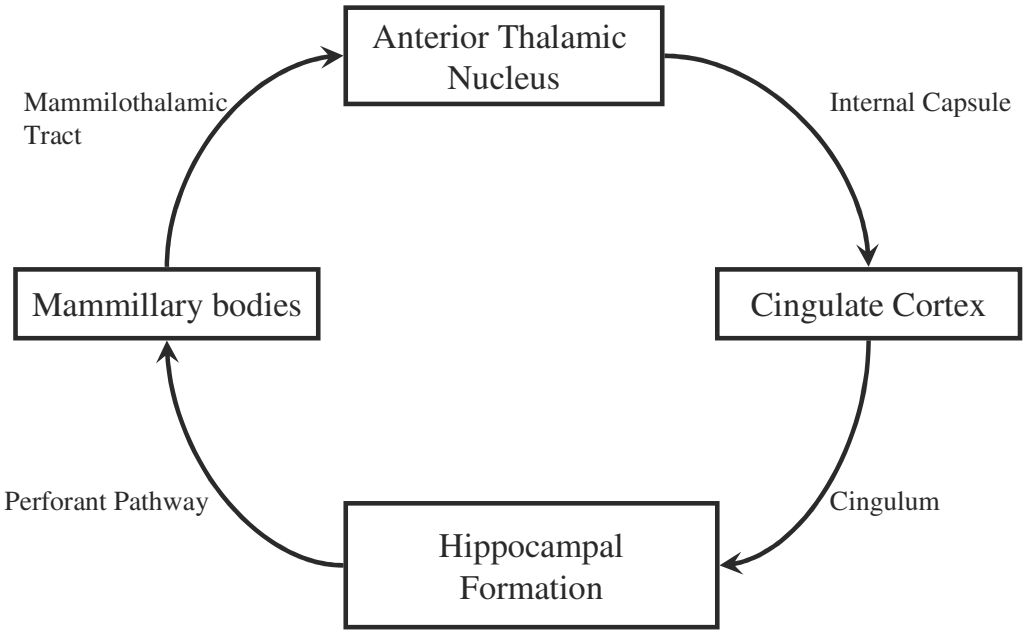
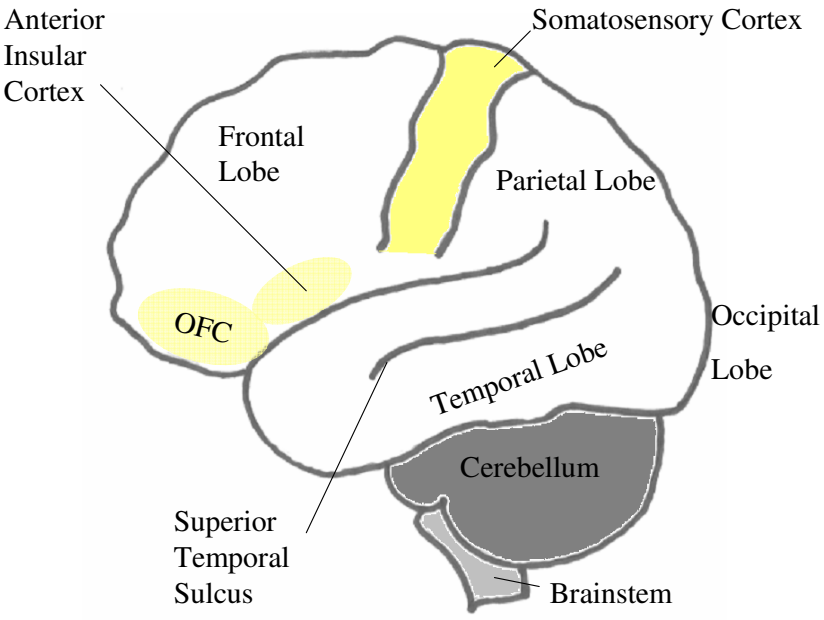


FIGURE 1-2: The Limbic System in the Human Brain
(NAc, Nucleus Accumbens; OFC, Orbital Frontal Cortex; VTA, Ventral tegmental area; PAG, periaqueductal Gray)

A. Lateral view of cortex



B. Sagittal slice through midline

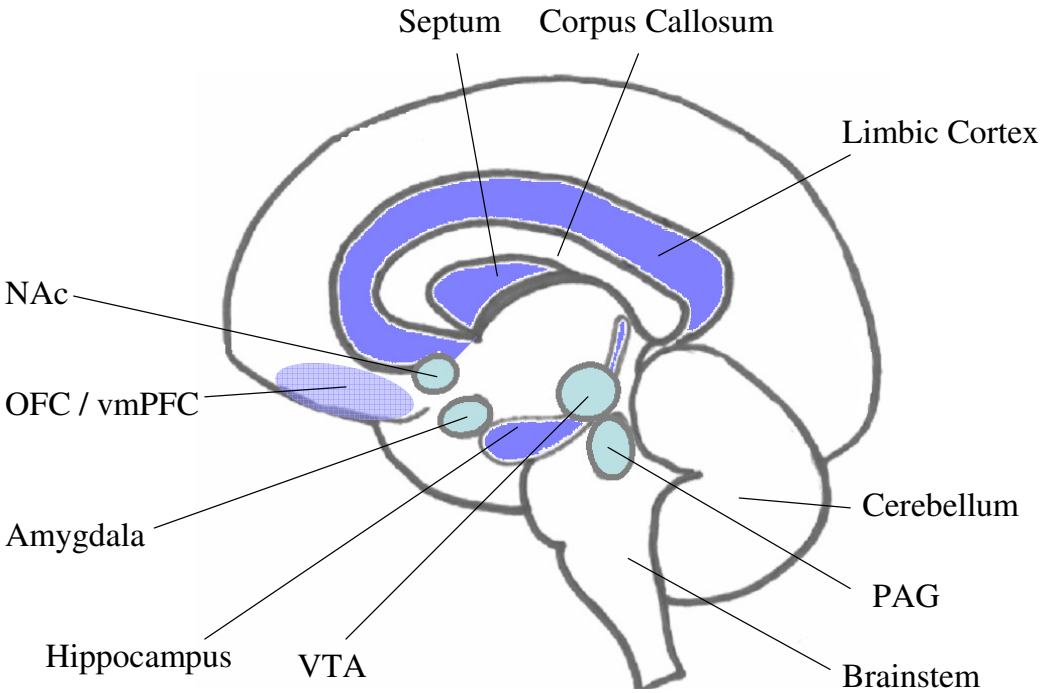


FIGURE 1- 3: The limbic system in the rat brain; regulation of hypothalamic output to the pituitary gland.
(PFC, prefrontal cortex; LS, lateral septum; SFO, subfornical organ; BNST, bed nucleus of the stria terminalis; Amyg, Amygdala; PVN, paraventricular nucleus of the hypothalamus; mPOA, medial preoptic area of the hypothalamus; ME, median eminence; LC, locus coeruleus; NST, nucleus of the solitary tract)

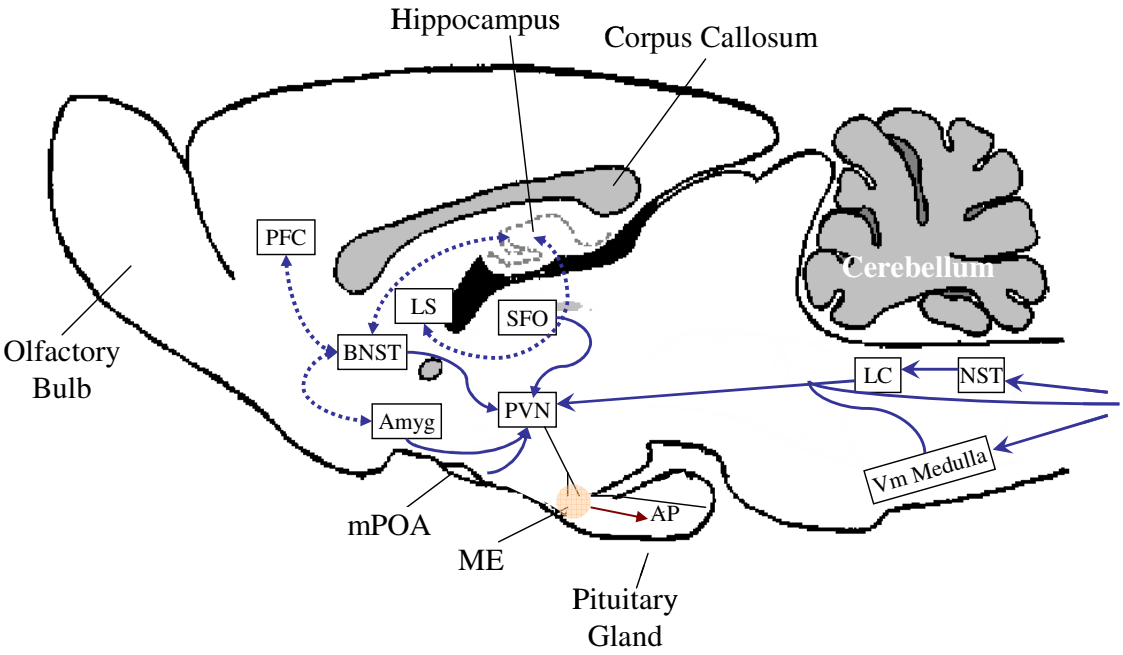
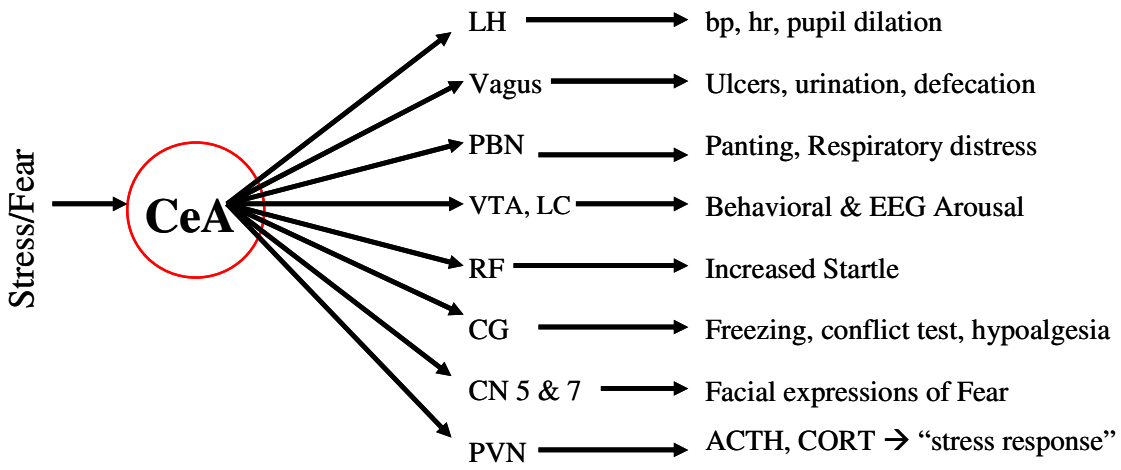


FIGURE 1-4: Efferent Projections from the CeA
(LH, lateral hypothalamus; Vagus, motor nucleus for the 10th cranial nerve; PBN, parabrachial nucleus; VTA, ventral tegmental area; LC, locus coeruleus; RF, reticular formation; CG, cingulate gyrus; CN 5 & 7, motor nuclei for cranial nerves 5 and 7; PVN, paraventricular nucleus of the hypothalamus).



Modified from Aggleton, 2000

TABLE 1-1: Anatomy of Mood and Anxiety Disorders

	MDD and sadness	**Anxiety disorders, and normal anxiety**
Insular Cortex	•Acute sadness activates dorsal insula	•Acute anxiety activates ventral insula
Cingulate Cortex	•Pregenual ACC deactivated in euthymic MDD •Pregenual ACC activated in acute MDD •Subgenual ACC normal in acute MDD but hypoactive in remitted MDD patients •ACC and PCC activated by acute sadness	•Acute anxiety has no effect on ACC but deactivates the PCC
Amygdala	•Overactive at rest in primary mood disorders •Magnitude of activity correlates to severity •Overactivity without conscious perception •Normal activity after treatment •Smaller volume of left amygdala vs. controls	•Not overactive at rest •Overactive during symptom provocation •Right amygdala most relevant to anxiety

FIGURE 1-5: G_s-mediated Signal Transduction Cascades
Ligand binding the receptor signals Gs α to activate adenylyl cyclase (AC), which then converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which acts as a second messenger to activate phosphokinase A (PKA). Alerations in on channels, other signaling molecules, and transcription factors sensitive to cAMP and/or PKA can influence gene expression as well as membrane potential.

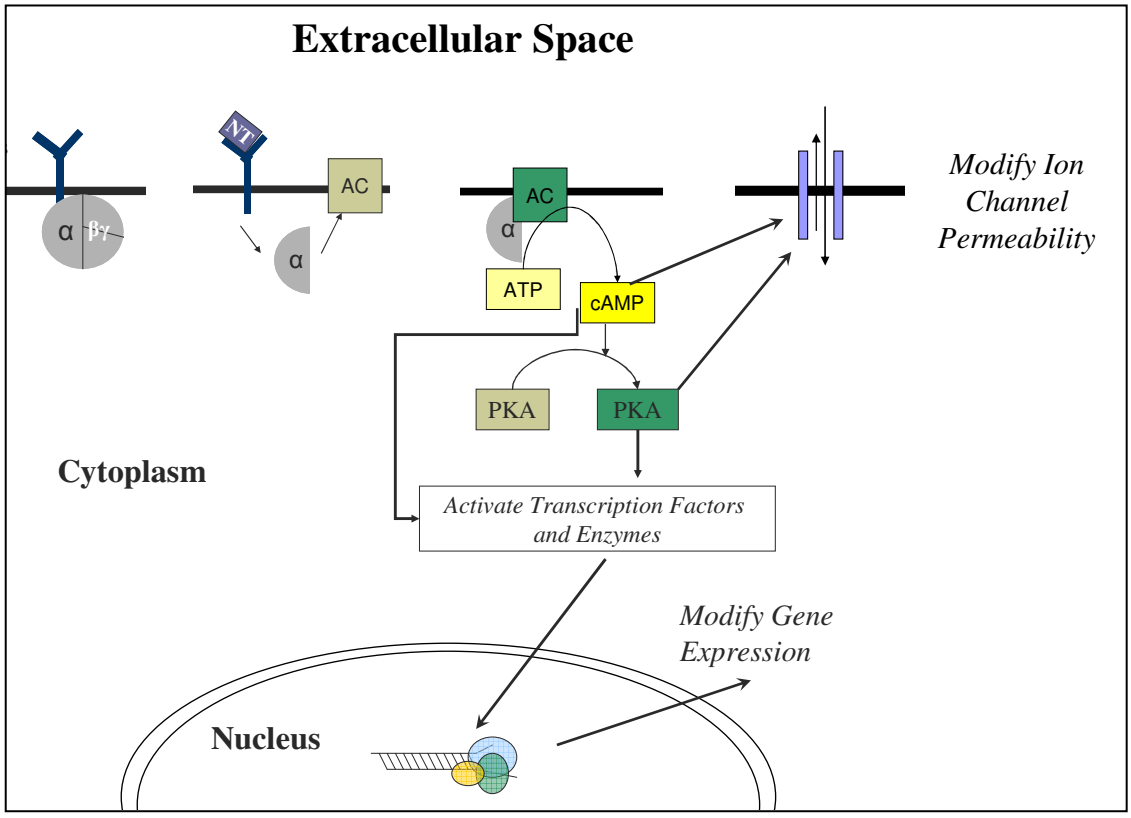


TABLE 1-2: Neuropeptides in Stress and Interactions with CRF

Neuropeptide	Colocalization with CRF	Role in stress-neurobiology	Role in Psychopathology
Angiotensin II (Ang-II) (Lenkei et al., 1997)	Extensive	Increased by physiological stress Weak ACTH secretagogue Increases CRF	Anxiogenic
Cholecystokinin (CCK) (Brawman-Mintzer et al., 1997; Koszycki et al., 2004)	Extensive	Weak ACTH secretagogue	Anxiogenic Exogenous CCK evokes anxiety; Patients with anxiety-disorders are hypersensitive
Enkepahlin (Enk) (Ma et al., 1999)	Extensive	Increased by stress and pain. Tonic inhibition of CRF	
Galanin (Gal) (Barrera et al., 2005; Karlsson and Holmes, 2006)	Moderate	Increased by physiological and psychological stress and pain	Depressogenic. Galanin antagonists are being developed and possess antidepressant properties
Neuropeptide Y (NPY) (Hashimoto et al., 1996; Heilig, 2004; Martin, 2004; Sajdyk et al., 2004; Hou et al., 2006; Yehuda et al., 2006; Karl and Herzog, 2007)	Extensive	Increased during stress. Endogenous alarm system. Stress-induced increase in feeding. Modulate behavior to cope with chronic stress.	Antidepressant and anxiolytic in laboratory animals. Depressed patients have low plasma concentrations of NPY especially in first episode. Plasma NPY concentration is normalized by antidepressants
Neurotensin (Binder et al., 2001; Zhao and Pothoulakis, 2006)	Slight	Stress-induced anti-nociception Stress-induced behavior via PAG	Implicated in schizophrenia and mechanism of action of anti-psychotic drugs.
Oxytocin (OT) (Gimpl and Fahrenholz, 2001)	Slight	Weak ACTH secretagogue	Low OT in CSF is associated with depression in women.
Vasopressin (AVP) (van Londen et al., 1997; Ma et al., 1999; Wigger et al., 2004; Goekoop et al., 2006)	Extensive	Increased by stress Moderate ACTH secretagogue synergize to stimulate ACTH production and release	Potentially elevated in depression

FIGURE 1- 6: CRF Peptide Distribution in the Rat Brain
(BNST, bed nucleus of the stria terminalis; Hip, hippocampus; LS, lateral septum; CeA, Central Amygdala; mPOA, medial preoptic area of the hypothalamus; LH, lateral hypothalamus; PVN, paraventricular nucleus of the hypothalamus; CG, central grey; LDT, laterodorsal tegmental nucleus; MAN, motor nucleus of the vagus nerve; DRN, dorsal raphe nucleus). Modified from (Swanson et al., 1983).

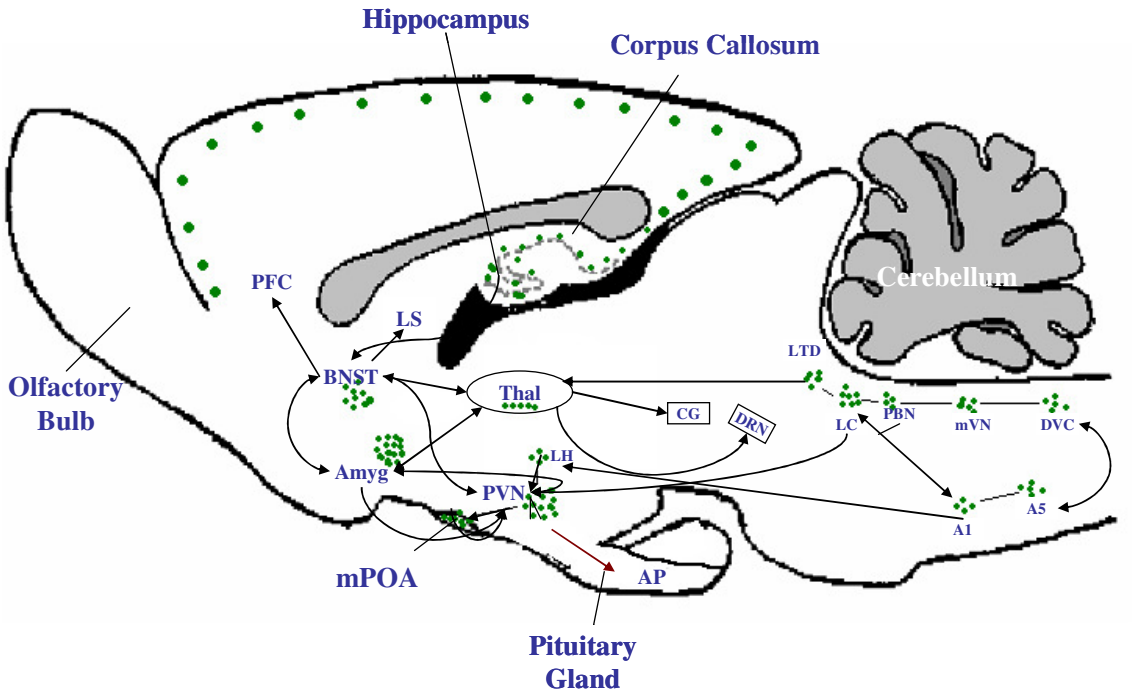


FIGURE 1-7: CRF Receptor Distribution in the Rat Brain
(AON, anterior olfactory nucleus; APit, anterior pituitary; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1-CA4,hippocampal areas CA1 to CA4; CeA, central nucleus of the amygdala; Cereb, cerebellum; CoA, cortical nuclei of the amygdala; CingCx, cingulate cortex; DBB, diagonal band of Broca; DG, dentate gyrus; FrCx, frontal cortex; IC, inferior colliculus; IO, inferior olive; LC, locus coeruleus (based on primate data); LDTg, laterodorsal tegmental nucleus; LS, lateral septum; MA, medial amygdala; MS, medial septum; NV, trigeminal nuclei; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaqueductal gray; ParCx, parietal cortex; PPTg, pedunculopontine tegmental nucleus; PVN, paraventricular hypothalamic nucleus; R, red nucleus; RN, raphe nuclei; SC, superior colliculus; SN, substantia nigra; Thal, thalamus; VMH, ventromedial hypothalamus).
From (Steckler and Holsboer, 1999)

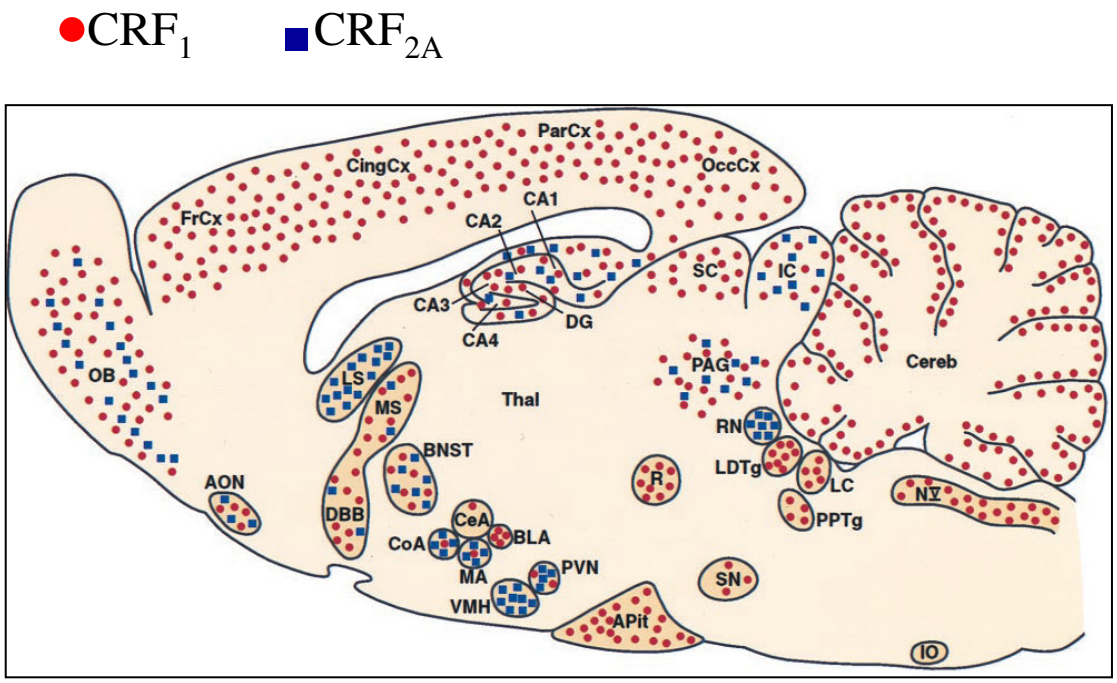
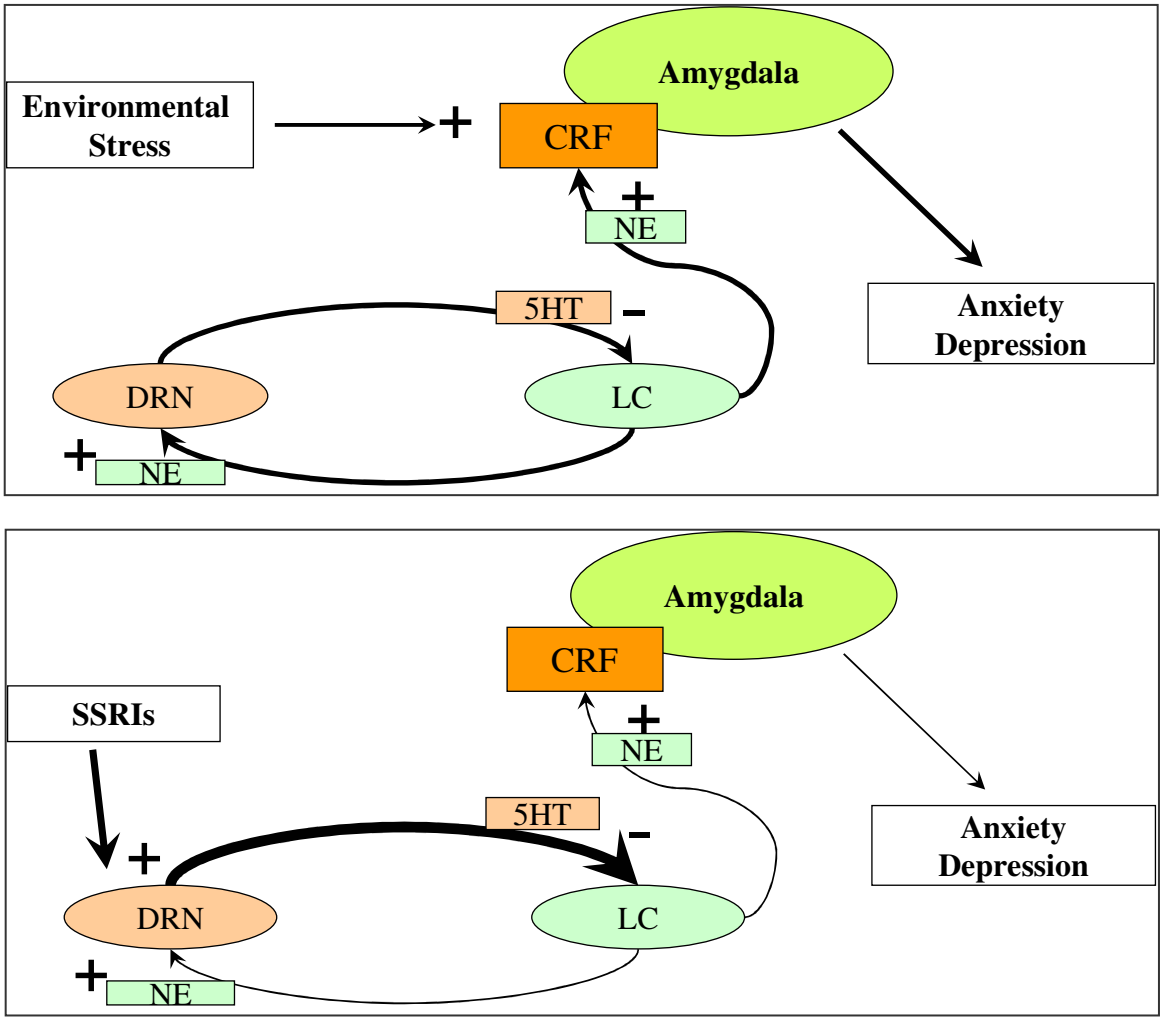


FIGURE 1-8: CRF in a Hypothesized Mechanism of Action of SSRIs



CHAPTER 2:

Viral Vector and Transgenic Tools to Manipulate Gene Expression within CRF-Expressing Cells

INTRODUCTION

CRF is the preeminent regulator of the mammalian endocrine, autonomic, and behavioral stress response and has been implicated in the pathophysiology of a variety of illnesses ranging from irritable bowel syndrome (Tache and Brunnhuber, 2008) to MDD (Nemeroff, 1988) and PTSD (Nemeroff et al., 2005; Risbrough and Stein, 2006).

CRF is expressed in high quantities in the CeA and PVN where it is produced and released from a variety of cell types, making it difficult to distinguish the specific role of CRF from other signaling molecules expressed in the same region and, in fact, the same cells.

In general, it is accepted that the stress response is mediated by activation of CRF_1 receptors, which has a higher affinity for CRF (Owens and Nemeroff, 1991) wheras CRF_2 receptor activation may initiate a secondary stress-response circuit to return the system to homeostasis (reviewed in (Bale and Vale, 2004; Gysling et al., 2004). The differential role of the CRF_1 and CRF_2 receptors likely reflect, at least in part, activation of distinct central sources of CRF; the endocrine response to stress is initiated by the HPA axis while the role of CRF in the behavioral stress response is generally attributed

to CRF expression and release from the extended amygdala. The autonomic stress response is most directly mediated by CRF acting in hindbrain nuclei (Wiersma et al., 1993; Reyes et al., 2005; Tache and Brunnhuber, 2008)(reviewed in (Owens and Nemeroff, 1991; Claes, 2004).

Importantly, CRF expression is differentially regulated in hypothalamic and extrahypothalamic regions. For example, there are numerous partial glucocorticoid-responsive elements (GRE) in the promoter regions for CRF, allowing GCs to provide negative feedback to PVN CRF cells, but increase CeA and BNST CRF mRNA expression dependent on cell-type specific presence of other GR cofactors (Makino et al., 1994a; Vamvakopoulos and Chrousos, 1994; King et al., 2002). Importantly, GRE in the proximal CRF promoter are evolutionarily conserved, as is the region-specific effects of GC on CRF expression, supporting a critical role for GC in the CRF-mediated stress response (Yao et al., 2008).

Increased hypothalamic and extrahypothalamic CRFergic signaling have been reported in patients with mood and anxiety disorders including MDD and PTSD (e.g. (Arato et al., 1989; Bremner et al., 1997; Heim et al., 1997a, b; Holsboer, 2003). Although it is well established that hypothalamic and extrahypothalamic CRF-expressing regions contribute to the stress response, but currently available technology is insufficient to allow the degree of specificity required to distinguish the relationship between these CRF systems, especially given the complex differential regulation of CRF in distinct brain regions.

To improve the understanding of CRF, its interaction with other NTs and NPs, and its role in the pathophysiology of mood and anxiety disorders, tools must be

developed to identify and isolate diverse populations of CRF-containing cells. Our group has previously outlined a method of screening promoters for cell-type-specific expression *in vitro* and *in vivo* utilizing lentiviral vectors (LV) (Chhatwal et al., 2007). Using the previously described method, we amplified 1.3Kb and 3.0Kb promoter lengths upstream of the ATG start site for the CRF coding sequence (cds). The promoters were inserted into a lentiviral vector backbone (FIGURE 2-1) and used to drive expression of Cre-recombinase, a 35-kDa enzyme that recognizes specific 34 base pair (bp) sequences known as LoxP sites (reviewed in (Lewandoski, 2001; Wilson and Kola, 2001). The 34bp LoxP sequence is composed of a 13bp inverted repeat separated by an 8bp spacer (reviewed in (Lewandoski, 2001; Wilson and Kola, 2001). Cre-recombinase will excise DNA flanked by LoxP sites (‘‘floxed”) via intrachromosomal recombination (FIGURE 2-2). The Cre-recombinase coding sequence used in this vector includes a 5′ nuclear localization sequence (translated sequence: MAPKKKRKV) to enhance Cre-recombinase-mediated recombination efficiency.

The following studies describe the ability of both the 1.3Kb and 3.0Kb promoters to produce functional Cre-recombinase enzyme in *vitro* and *in vivo* and the differential specificity of the two promoter lengths at targeting CRF-expressing regions *in vivo*. Finally we describe a novel transgenic mouse containing the LVCRFp3.0Cre construct and, when crossed with fluorescent reporter strains, its use to identify and isolate CRF-expressing cells for electrophysiological recording.

MATERIALS and METHODS

A. Designing and Creating CRF-Cre Vectors

Primers were designed to amplify 1.3Kb or 3.0Kb regions upstream of the ATG start site for the CRF cds. FIGURE 2-1A depicts the regions of the mouse CRF gene used as the promoters in this study. Importantly, both promoter lengths include CRF exon 1 and intron 1, regions that contain key regulatory elements for CRF expression (e.g. (Seth and Majzoub, 2001). Both promoter regions were amplified from the CRF gene in bacterial artificial chromosome (BAC) clone #129A14 using polymerase chain reaction (PCR). The resulting amplicons were topo-cloned into pCR2.1-topo, (Invitrogen Corp., Carlsbad, CA, USA), according to manufacturers' instructions. Incorporated into the original primers were custom restriction sites used for later subcloning steps (*CRF 5'p3.0-Cla1* GCCTATCGATGGAAAGAAAGCACAAAGGATGCCG; *CRF 5'p1.3 Cla1* GCTGAGGCATCGATAAATGTCCAGATCCACCCC; *CRF 3'BamHI* CCAGCGGATCCAGCCGCATGTTAGGGGCGCTCTCTGAA).

To create the final LVCRFp-Cre constructs, the lentiviral vector packaging construct, pCMV-GFP-dNhe (Tiscornia et al., 2003) (kind gift of Inder Verma, Salk Institute, La Jolla, CA, USA) was digested with *Cla1* and *Sal1* to remove the CMVGFP sequence, treated with antarctic phosphotase (New England Biolabs) to prevent re-ligation and band-purified from a 0.75% agarose gel using a DNA-purification kit (Epicentre Biotechnologies, Madison, WI). The linearized, phosphotased lentivirus backbone with *Cla1/Sal1* ends was combined in solution with the CRF promoter, a *Cla1/BamHI* fragment, as well as the coding sequence for Cre-recombinase, a *BamH1/Sal1* fragment. The two inserts were ligated together and into the lentiviral

backbone using T4 DNA ligase according to the manufacturers' instructions (New England Biolabs, Ipswich, MA).

Products of ligation were transformed into ElectroTen-Blue® Electroporation-Competent Cells (Stratagene, La Jolla, CA). In a microcentrifuge tube on ice, 1µl of the ligation reaction was added to 50µl competent cells. The bacteria and DNA were transferred to an electroporation cuvette and shocked at 1700V using an electroporator. The cuvette was removed and 900µl warm SOC media was added. After shaking for 1 hour at 37 °C, solution was transferred to 5ml of Luria broth (LB) containing 1:100 ampicillin. After 5hrs of shaking at 37 °C DNA plasmids were isolated using a miniprep kit according to manufacturer's instructions (Qiagen Hilden, Germany). Correct clones were identified via diagnostic digests of restriction sites within and outside of the promoter and Cre-recombinase inserts. Clones identified as correctly oriented were packaged into virus particles (see below).

B. Producing LVCRFp-Cre Virus

1. Purifying and Concentrating DNA

100µl of LB containing the correctly oriented clones was added to 500ml LB with 1:100 ampicillin and grown overnight at 37 °C with 225 RPM shaking. The following morning, the LB was centrifuged for 12min in 250ml centrifuge bottle, using the JLA16.200 rotor, at 7000 RPM. Supernatant was decanted and bacterial pellets resuspended in 4ml of 10mM EDTA. To lyse the cells, 8ml 0.2N NaOH with 1.0% SDS was added and the mixture incubated for 5min at room temperature. 6ml 3M KOAc, pH 5.5 was added to neutralize the reaction and precipitate genomic DNA and protein. The

precipitate was then filtered out and supernatant containing plasmid DNA was dispensed into centrifuge tubes. An equal volume of isopropanol was added to precipitate the plasmid DNA. The mixture was then spun for 10min at 10,000 RPM in a tabletop centrifuge (Fisher Scientific). Supernatant was discarded and the pellet containing RNA and plasmid was dissolved in 500µl TE and transferred to a microcentrifuge tube.

To eliminate RNA, 5µl of 20mg/ml RNase A was added and incubated 30min at RT. Plasmid DNA was then extracted with Phenol: Chloroform (1:1). DNA was again precipitated with an equal volume of isopropanol, incubated for 5min at room temperature, and then centrifuged at maximum speed for 10min in a microcentrifuge. After aspirating supernatant, the pellet was washed with 70% EtOH and centrifuged for another 5min at maximum speed. The pellet was then resuspended in 100-500µl of TE buffer and quantified using spectrophotometry as a 1:500 dilution. From the 500ml culture, approximately 4mg was obtained.

2. Production of Recombinant Lentiviral Vectors:

Virus-production procedures have been described in detail (Rattiner et al., 2004; Chhatwal et al., 2006) and follow from procedures initially outlined by Verma and co-workers (Naldini et al., 1996c; Miyoshi et al., 1998; Zufferey et al., 1998; Pfeifer et al., 2001).

One day prior to transfection, 293T cells were split and counted with a hemocytometer to achieve a concentration of 1-1.2 x 10^7 cells per 100mm round Plexiglas plate. Cells were grown overnight in DMEM with 10% FBS and 1x penicillin with streptomycin (pen/strep) antibiotics. The next day, 30min prior to transfection,

serum was removed, cells were rinsed with Optimem-I and serum was replaced with fresh Optimem.

Each 100mm plate was transfected with 5μg of the packaging construct pDelta8.91, 2μg of the VSV-G pseudotyping construct and 10μg of the Cre-recombinase expression vector (CMVCre, CRFp1.3Cre, or CRFp3.0Cre). Transfections were performed with Lipofectamine per the manufacturer's protocol (Invitrogen).

After approximately 4hrs, 5ml Optimem-I containing 10% FBS and 1x pen/strep was added. The cells were returned to 37 °C for overnight incubation. The following morning, the medium was changed to DMEM containing 10% FBS and 1x pen/strep. Serum containing the packaged virus was collected from the cells over a period of 5 days post-transfection.

Virus particles were concentrated by first spinning in a tabletop centrifuge at 4500 RPM for 15 min. Supernatant was filtered with 0.45μm filters and spun in refrigerated ultracentrifuge (SW-28 Beckman ultracentrifuge rotor) at 28,000 for 90min. Supernatant was discarded and pellet resuspended in 1ml phosphate-buffered saline (PBS). The PBS containing viral particles was spun on a refrigerated microcentrifuge for 30min at the maximum speed. The resulting pellet was then brought up in 100-500μl sterile PBS with 1% BSA. Virus was stored in 10μl aliquots at -80^{o}C. The resulting titer was assessed in HEK293T cells, and the observed titer of the virus used here was at least 5×10^{8} infectious particles per ml.

C. Animals Subjects

All procedures used are approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and are in compliance with National Institutes of Health

(NIH) guidelines for the care and use of laboratory animals. Mice were group housed in polycarbonate cages (30 x 20 x 16cm) on corn dust bedding and maintained on a 12hr light/dark cycle with food and water available *ad libitum*. Breeding pairs for transgenic Cre-recombinase reporter mice were obtained from Jackson Labs (Bar Harbor, ME, USA).

1. Surgery and Injection of Virus:

Male mice carrying the Rosa26-floxed LacZ insert (RosaLacZ, (Soriano, 1999) and aged 6–10 weeks received an intraparitoneal (IP) injection of the anesthetic, a 4:5 mixture of ketamine and Domitor, and placed in a stereotaxic frame. To maintain aseptic technique, the surgical area was cleaned three times with alternating betadine and 70% EtOH. A midline incision was made in the scalp and holes were drilled in the skull. Mouse Injection Coordinates Relative to Bregma (A/P, anterior/posterior; D/V, dorsal/ventral; M/L, medial/lateral):

	PVN	CeA
– A/P:	-1.0	-1.3
– D/V:	-5.5	-5.3
– M/L:	-1.0 (left)	+2.9 (right)
Angle:	9 °	0°

Injections were performed using a 5µl Hamilton syringe with a 22 gauge beveled-tip needle that had been sterilized with EtOH, rinsed with sterile saline, and coated with sterile 1% BSA prior to virus loading. The needle was lowered slightly ventral to and then brought up to the D/V target. 1µl of virus was injected per hemisphere at a rate of 0.1µl per minute using an automatic micro pump (Ultramicropump II, World Precision

Instruments, Sarastoa, CA). After the injection, the needle was left in place for 2min, then lifted just dorsal to the injection site and left for another 5min before being slowly withdrawn. The skin was closed using a 6-0 Vicryl suture (Ethicon: Johnson & Johnson, Piscataway, NJ).

D. Histological Analysis

1. X-Gal Staining:

Animals were killed 7-10 days post-infection. Brains were obtained following intra-cardiac perfusion (PBS, 4% paraformaldehyde). A brief post-fixation step (2h, 4% paraformaldehyde), and cryoprotection (20% sucrose in PBS, ~16 h at 4°C) preceded sectioning and storage at −80°C. Slides were rinsed in PBS and then incubated in X-gal solution (5mM K4 [Fe (CN_6)], 5mM K_3 [Fe (CN_6)], 0.5mM $MgCl_2$, and 0.5 mg/ml X-gal in 0.1 M Tris–HCl) over night. Slides were rinsed in PBS, dehydrated through ethanol, and cover slipped with DPX.

2. *In situ* Hybridization

a) Tissue Preparation

Animals were killed 7-10 days post-infection. Brains were rapidly dissected, snap-frozen and sectioned at 20μM onto SuperFrost Plus slides, which were then stored at −80°C until processing.

b) Probe Preparation

^{35}S-UTP labeled riboprobes were prepared from linearized clones as previously described (Ressler et al., 2002). Templates were prepared by linearizing the plasmid with an appropriate restriction endonuclease (*PvuII* for CRF; *Not1* for Cre-recombinase). Probe labeling was

performed using a riboprobes combination system with the SP6 RNA polymerase for both CRF and Cre-recombinase following the manufacturer's instructions (Promega, Madison, WI). Labeled riboprobes were digested for 30min at 60^{o}C in hydrolysis solution containing dithiothreitol (DTT), $NaHCO_3$, Na_2CO_3 and sterile water. The reaction was neutralized with a solution containing DTT, NaAc, acetic acid, and sterile water. Riboprobes were purified by sephadex column filtration (Roche Diagnostics, Indianapolis, IN, USA).

c) Tissue Prehybridization

In preparation for hybridization, slides were warmed to room temperature and dried followed by 30min fixation in 4% paraformaldehyde. After washing in PBS for 5min, slides were treated for 8min with Proteinase K (PK) (20µg/ml) dissolved in PK buffer (10mM Tris, 5mM EDTA), rinsed for 5min in PBS, then fixed with 4% paraformaldehyde for 20min. Slides were dipped in H_2O, and immersed for 10min in 0.1mM triethanolamine containing 0.25% acetic anhydride. After 5min rinses with 1x PBS and 0.85% NaCl, slides were dehydrated through increasing concentrations of EtOH and air-dried in a desiccator.

d) Hybridization

Riboprobe hybridization mix was made to a concentration of 10,000 CPM/µl in hybridization buffer consisting of 50% deionized formamide, 10mm DTT, 20mm Tris, 300mM sodium chloride, 5mM EDTA, 10% dextran sulfate, 1× Denhardt's solution, 0.5mg/ml yeast RNA and 10mM NaH_2PO_4. Sections were then incubated overnight in humid chambers at 50°C with at least 100µl/slide of hybridization solution covered with a Parafilm cover slip.

e) *Washing*

Following hybridization, slides were incubated in prewarmed 5× standard sodium citrate (SSC) until cover slips floated off. Sections were washed in 5× SSC/10mM DTT at 50°C for 30min and treated with 50% formamide/ 2× SSC/10mM DTT at 55°C for 30min. Next, slides were washed twice in wash buffer (in mM: Tris, 10; EDTA, 5; NaCl, 100) for 10min at 37°C, then treated with RNase A in wash buffer for 30min at 37°C. Sections were dehydrated for 2min in 30%, 50%, 70%, 85% and 95% ethanol containing 300mM ammonium acetate, followed by 100% ethanol. Slides were air dried and apposed to Biomax MR autoradiographic film (Eastman Kodak, Rochester, NY, USA) for 1–5 days.

E. Production and Testing of CRFp3.0Cre Transgenic

1. DNA preparation and Injection

The LVCRFp3.0Cre construct was linearized with *Nhe1* and *Sph1*. The resulting 6.4Kb segment was band-purified from the agarose gel as described previously. At the Emory University transgenic core facility, DNA was further purified by electroelution and diluted to a 2ng/µl concentration for pronuclear microinjection.

2. Genotyping

Tail clips of resulting offspring were dissolved in 500µl 0.05M NaOH with 5µl 20mg/ml PK at 60^{o}C, with agitation. DNA was purified with phenol:chloroform and precipitated with isopropanol. DNA pellets were brought up in 100µl TE. Potential transgenics were genotyped for the presence of the Cre-recombinase gene using the following PCR primers (SNS primer: GCATTACCGGTCGATGCAACGAGTGATGAG; AS primer:

GAGTGAACGAACCTGGTCGAAATCAGTGCG). Additional primers were used to amplify a region of genomic DNA as a positive control for DNA quality (SNS primer: CGTATCTGCAACTCCAGTC; AS primer: GGAGCGGGAGAAATGGATATG).

All PCR reactions were run using FailSafe™ PCR System with reaction buffer I as per the manufacturer's instructions (EPICENTRE® Biotechnologies, Madison, WI). As a negative control, one tube in each PCR reaction series was run without Taq Polymerase and a second tube was run without template DNA. One tube in each PCR reaction series was also run with DNA containing the Cre-recombinase plasmid as a positive control for the Cre PCR reaction. Amplicons were run on a 1% agarose gel.

3. Development of Mouse Line

PCR-screening identified two male mice (Founders #86 and #136) as positive for Cre-recombinase. These founders were moved from the transgenic facility and were housed and maintained in the laboratory facility as described for Experiment 1. Founder mice were paired with female B6 mice. F1 generation offspring from founder #136 were crossed either with C57/BL6 WT mice, or Rosa26 reporter mice. Offspring of the CRFp3.0Cre/Rosa26 mating were genotyped for the presence of Cre-recombinase and LacZ. The other founder (#86) failed to reproduce.

4. Assessment of Cre-recombinase Expression Specificity

At the time of weaning (approximately 21 days), several F2 offspring from matings with C57/BL6 were sacrificed with CO_2. Brains were collected and fresh-frozen on dry ice for analysis of Cre-recombinase expression. Brains were sectioned on a cryostat and *in situ* hybridization for Cre-recombinase and CRF were performed on serial slides as described previously. F2 offspring from matings with the Rosa26 line were sacrificed with chloral

hydrate overdose and perfused; brains were sectioned and stained for LacZ as described above.

C. Electrophysiological Analysis of Putative CeA CRF Neuron

1. Tissue collection and preparation

Transgenic mice were decapitated under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL, USA). Brains were rapidly removed and placed in ice-cold kynurenic acid-based artificial cerebrospinal fluid ($ACSF_{KA}$), which contained (in mM): NaCl (130), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (6.0), $CaCl_2$ (1.0), $NaHCO_3$ (30.0), glucose (10.0) and kynurenic acid (2.0) as described previously (Rainnie et al., 1994; McDonald et al., 2005; Rainnie et al., 2006). A block of tissue containing the CeA was then mounted on the chuck of a Leica VTS-1000 vibrating microtome (Leica Microsystems Inc., Bannockburn, IL, USA), and 350μm coronal slices were cut. Slices were then hemisected and transferred to a holding chamber containing $ACSF_{KA}$ at room temperature and gassed with a 95–5% oxygen/carbon dioxide mixture for 1 h before being placed in oxygenated control ACSF containing (in mM): NaCl (130), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (1.3), $CaCl_2$ (2.5), $NaHCO_3$ (30) and glucose (10). Experiments started a minimum of 30min following the transfer of slices into the control ACSF.

2. Recording procedures

Thick-walled borosilicate glass patch electrodes (WPI, Sarasota, FL, USA) were pulled on a Flaming/Brown micropipette puller (Model P-97), and had resistances ranging from 4 to 8 MΩ, when filled with a standard patch recording solution that contained 0.3% biocytin and (in mM): K-gluconate (138), KCl (2), $MgCl_2$ (3), phosphocreatine (5), K-

ATP (2), NaGTP (0.2), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (10) and 0.3% biocytin. The patch recording solution was adjusted to a pH of 7.3 with KOH and filtered through a 0.2μm filter (Altech Associates, Inc., Deerfield, IL, USA). Whole-cell patch clamp recordings were obtained with standard techniques, as described previously (Rainnie et al., 1994; McDonald et al., 2005; Rainnie et al., 2006). Briefly, recordings were made with an Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA, USA) using a Digidata 1320A A–D interface and pClamp 8.2 software (Molecular Devices). In cell-attached mode, patch electrode seal resistance was considered acceptable if it was >1.5 GΩ. Whole-cell patch clamp configuration was established in neurons in current-clamp mode, and all data were filtered at 5kHz. The membrane input resistance (R_m) and intrinsic currents, activated at membrane potentials more negative than −60mV, were assessed by determining the voltage response to transient (750mS) current injection ranging from −40 to +23pA.

RESULTS

A. Assessment of promoter activity *in vitro*

To assess promoter activity, the CRF-Cre vectors were transfected into HEK-293 cells along with a reporter plasmid designed to detect Cre-recombinase activity as described previously (Heldt et al., 2007). The reporter plasmid (CX1-LEL-dsRed) contains the GFP gene and its stop codon flanked by LoxP sites upstream of the coding sequence for dsRed, a red fluorescent protein. In the absence of Cre-recombinase, this reporter plasmid expresses high levels of GFP but no dsRed; in the presence of Cre-recombinase,

the GFP cds and its stop codon are excised, resulting in a loss of GFP expression and emergence of red fluorescence. In cells transfected with CX1-LEL-DsRed and infected with LVCRFp1.3Cre or LVCRFp3.0Cre, we observed a time and dose-dependent shift from GFP to dsRed expression (FIGURE 2-3B), demonstrating that both the 1.3 and 3.0Kb promoter lengths produce detectable levels of Cre-recombinase activity *in vitro*.

B. Assessment of Promoter Selectivity *in vivo*

In vivo promoter activity was assessed by injecting LVCMVCre, LVCRF1.3Cre, or LVCRF3.0Cre viral particles into the CeA and PVN of WT or RosaLacZ mice**.** The CeA and PVN regions were selected because they are sites of high levels of CRF expression. In the WT mice, *in situ* hybridization analysis showed remarkable overlap of Cre-recombinase and CRF mRNA expression in the mice injected with LVCRFp3.0Cre but not the other two viruses (FIGURE 2-4a).

The RosaLacZ transgenic mouse strain expresses the LacZ reporter in cells in which a floxed stop codon has been excised by Cre-recombinase (Soriano, 1999). Because a single Cre-recombinase molecule is sufficient to excise the stop codon, the expression of LacZ in these animals serves as an extremely sensitive measure of functional viral Cre-recombinase expression. If the 1.3Kb or 3.0Kb CRF promoter lengths successfully target Cre-recombinase expression to CRF-producing cells, the expression pattern of LacZ should mirror that of CRF. We observed that LacZ staining closely paralleled CRF mRNA expression in the CeA of animals injected with LVCRFp3.0Cre but not LVCRFp1.3Cre or LVCMVCre. Non cell-type specific LacZ expression seen in animals infected with LVCMVCre and LVCRFp1.3Cre viruses strongly suggest that the selectivity of Cre-recombinase expression with LVCRFp3.0Cre

was due to the 3.0Kb promoter in particular rather than innate viral tropism. Representative sections of LacZ expression in virally infected animals are depicted in FIGURES 2-4B and 2-4C.

C. Generating a Transgenic Mouse with the CRFp3.0Cre Construct

Because the 3.0 but not 1.3Kb promoter length was able to target gene expression to CRF-producing regions when injected into WT and Rosa26 mice, the LVCRFp3.0Cre DNA construct was linearized and the approximately 6.4Kb portion of the plasmid containing the 3.0Kb CRF promoter and Cre-recombinase coding sequence was used to create a transgenic mouse (CRFp3.0Cre). Serial sagittal brain slices stained for either CRF mRNA or Cre-recombinase mRNA via ^{35}S-labeled *in situ* hybridization shows remarkable similarity between CRF and Cre-recombinase expression in the transgenic mice (FIGURE 2-5).

D. Crossing CRFp3.0Cre with Cre-recombinase Reporter Strains

By crossing the CRFp3.0Cre transgenic with the aforementioned RosaLacZ strain, we were able to examine functional Cre-recombinase expression. As with the *in situ* hybridization analysis and LacZ staining in virally injected mice, in transgenic mice identified as PCR-positive for both the Cre-recombinase and LacZ constructs, expression patterns of LacZ are essentially identical to endogenous CRF gene expression (FIGURE 2-6), suggesting that the CRFp3.0Cre line is suitable for use in generating selective gene knockouts within CRF cells.

The CRFp3.0Cre transgenic mouse was also crossed with mT/mG, a double-fluorescent Cre-recombinase reporter strain. mT/mG constitutively expresses membrane-

targeted tandem dimer Tomato (mT), a red fluorescent protein. Following Cre-recombinase-mediated excision of mT and its stop codon, this reporter expresses membrane-targeted green fluorescent protein (EGFP) (Muzumdar et al., 2007). Thus cells expressing Cre-recombinase will fluoresce green and cells not expressing Cre-recombinase will fluoresce red. Moreover, each of these fluorescent labels provide high-resolution outline of cell morphology, membrane structures, and fine cellular processes (Muzumdar et al., 2007). FIGURE 2-7 shows region-specific expression of EGFP in cells expressing CRFp3.0Cre and expressing Td-Tomato in cells not expressing Cre-recombinase.

E. Fluorescence-guided Electrophysiological Recording

The CRFp3.0Cre-mT/mG double transgenic mice express EGFP in CRF-expressing cells, allowing us to visualize CRF-containing neurons in real time and target them for electrophysiological recordings. Acute brain slices were prepared and electrophysiological recordings were obtained from visually identified CRF neurons of the mouse CeA. An example of such a recording is shown in FIGURE 2-8 and demonstrates the viability of using cell-type-selective promoters to label and characterize the electrophysiological properties of a sub-population of neurons. (Data from Dr. Rainnie, Emory University, Atlanta, GA).

DISCUSSION

Hyperactivity in hypothalamic and extrahypothalamic stress-responsive CRF systems has been implicated in the pathophysiology of mood and anxiety disorders (e.g.(Koob and Bloom, 1985; Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Nestler et al., 2002; Claes, 2004). Furthermore, CRF_1 receptor antagonists have been demonstrated to reduce stress-sensitivity in experimental animals (reviewed in (Smagin et al., 2001) and improve symptoms of depression in humans (Zobel et al., 2000) but see (Binneman et al., 2008) for negative findings).

The utility of CRFergic circuits as novel targets for the development of putative antidepressants and/or anxiolytics is limited by our lack of understanding of differential, region-specific, CRF regulation. This gap in the literature is largely due to our limited ability to identify and selectively manipulate CRF-containing cells. Achieving restricted transgene expression within CRF-expressing cells in this novel CRFp3.0Cre mouse has the potential to rapidly expand our understanding of CRF regulation in a region-specific manner and perhaps contribute to the identification of relevant targets within selective CRF circuits.

A. Design of CRF-Cre Vectors

The specific location for 5' primers at 3.0 and 1.3Kb upstream of the CRF cds were chosen to minimize the risk for primer-dimers or secondary structure during the PCR process and to limit the number of base pair miss-matches when inserting restriction enzyme recognition sites for later cloning steps.

The promoter lengths were determined based on the distribution of known enhancer and repressor regions in the CRF promoter. Both the 1.3 and 3.0 Kb promoter

lengths contain exon one and intron one of the CRF gene, regions which are known to regulate CRF expression. Within intron one, approximately 140bp upstream of the CRFcds is a highly conserved 21bp recognition region for repressor element-1/neuron-restrictive silencing factors (REST/NRSF) which inhibits CRF transcription via a mechanism partially dependent on histone deacytlyation (Seth and Majzoub, 2001). CRF transcription is also modulated by intracellular cAMP and CRE binding (FIGURE 2-9) (Yao and Denver, 2007). The proximal CRF promoter contains a consensus site for cAMP-responsive element (CRE) located between 238 and 180bp 5' to the putative CRF mRNA cap site (Thompson et al., 1987; Seasholtz et al., 1988; Spengler et al., 1992). A region within 1.1 Kb of the 5' CRF regulatory region is strongly activated by calmodulin kinase (CaMK) IV (Yamamori et al., 2004). A portion of the CRF promoter between -1.7 and -3.3 Kb upstream of the CRFcds contains eight TATA box regions (core promoter sequences, reviewed in (Vamvakopoulos and Chrousos, 1994). It is presumably the effect of the more distal (i.e. upstream of 1.3Kb) regulatory elements which confer the superior cell-type specificity of the CRFp3.0 construct.

B. Assessment of Promoter Selectivity *In Vivo*

As the first step in characterizing this novel strain, mRNA expression of Cre-recombinase and CRF were compared in serial brain sections from F2 generation mice identified as PCR positive for the Cre-recombinase transgene. Coronal brain slices show Cre-recombinase expression in the PVN, BNST, and CeA; regions well known to express CRF. Sagittal slices show Cre-recombinase expression in cortical and hippocampal regions. In each case, the pattern of Cre-recombinase expression corresponded to expression of CRF in a serial slice (FIGURE 2-5).

For further characterization, the CRF3.0Cre mouse was crossed with two reporter mouse strains. The Rosa26 strain contains a floxed-stop-LacZ construct. Histological analysis from the Rosa26 cross demonstrated remarkable specificity in LacZ expression in CRF-producing regions. The overlapping expression patterns and overall lack of ectopic Cre-recombinase expression is evidence that the CRFp3.0 promoter was able to target transgene expression to regions expressing CRF (FIGURES 2-6).

The fluorescent reporter mouse, mT/mG, express red fluorescent protein under baseline conditions and fluoresce green only in cells that express Cre-recombinase. By crossing CRFp3.0Cre with mT/mG, Cre-recombinase-producing cells could be observed in greater resolution. In this cross, green fluorescing cells in the CeA are presumably CRFergic. The absence of Cre-mediated excision in neighboring cells improved our certainty that Cre-recombinase expression is specific to CRF producing cells themselves as opposed to the entire region (FIGURE 2-7). More specific double-labeling analysis is currently underway in the Ressler lab.

C. Fluorescence-guided Electrophysiological Recordings

In addition to their usefulness in characterizing the specificity of the transgene expression, the CRFp3.0Cre-mT/mG cross are themselves an important tool for identifying the electrophysiological profile of CRF-producing cells. The electrophysiological trace shown in FIGURE 2-8 is a representative example of the physiological properties of putative CRF neurons in the CeA. A detailed electrophysiological characterization will be the subject of another paper.

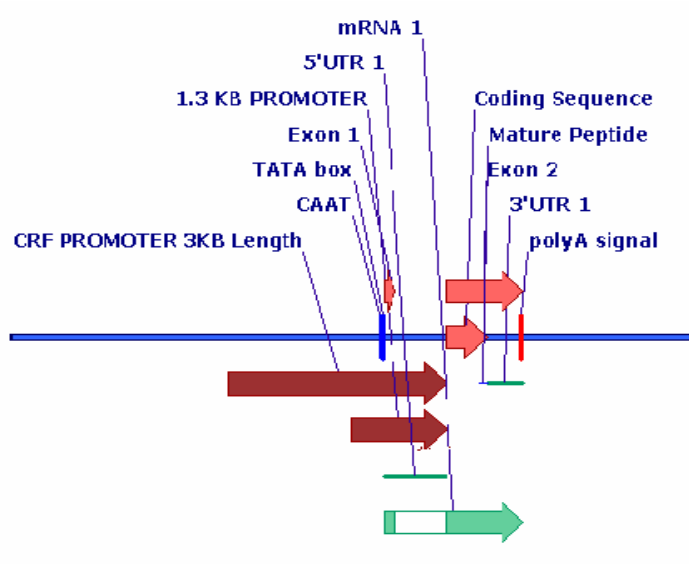
From this first impression, the cellular characterization is consistent with previous analysis of CeA cytoarchitecture. CRF expression in the CeA is concentrated in

intermediate-sized spiny neurons in the lateral subdivision of the CeA (Akmaev et al., 2004; Treweek et al., 2009). In terms of the electrophysiological properties, these putative CRFergic cells have input resistances around 300 mega-ohms; they receive a high level of spontaneous excitatory synaptic input, and can be directly excited by electrically stimulating the BLA. The relatively high input resistance means that these cells will easily summate incoming excitatory synaptic input and hence may fire action potentials relatively easily in response to input from the BLA. They also seem to form action potentials in a relatively rhythmic pattern and they do not seem to accommodate.

The ability to record from neurons identified *a priori* as CRFegic will be an essential tool in further characterizing central CRF circuits. To date, in depth-analysis of CeA neurons has relied heavily on expensive and time consuming double-labeling analysis and very few studies have examined the electrophysiological properties of CeA neurons (e.g. (Rainnie et al., 1992; Schiess et al., 1993). While the majority of CRF producing cells in the CeA are concentrated to medium sized spiny neurons in the lateral CeA, CRF expression is distributed across cytoarchitectonic boundaries. Future studies may identify important physiological distinctions between medium-spiny CRF neurons and other CRF-producing cell types in the CeA and other CRF-producing regions.

FIGURE 2-1: Design of LVCRFp3.0CRF

(A) Mouse Annotated CRF Gene
Maroon arrows depict 1.3 and 3.0Kb promoter lengths used in CRF-Cre viral vectors; promoters include 5' UTR and Exon1.



(B) Map of LVCRFp3.0Cre Viral Vector:
CRF promoter in lentiviral vector backbone drives expression of Cre-recombinase. Contained in the construct are viral sequences required for packaging, reverse transcription, and integration of viral genome. (LTR, Viral long-terminal repeats; RRE, rev response element; cPPT, central polypurine tract; WPRE, woodchuck posttranscriptional regulatory element (WPRE).

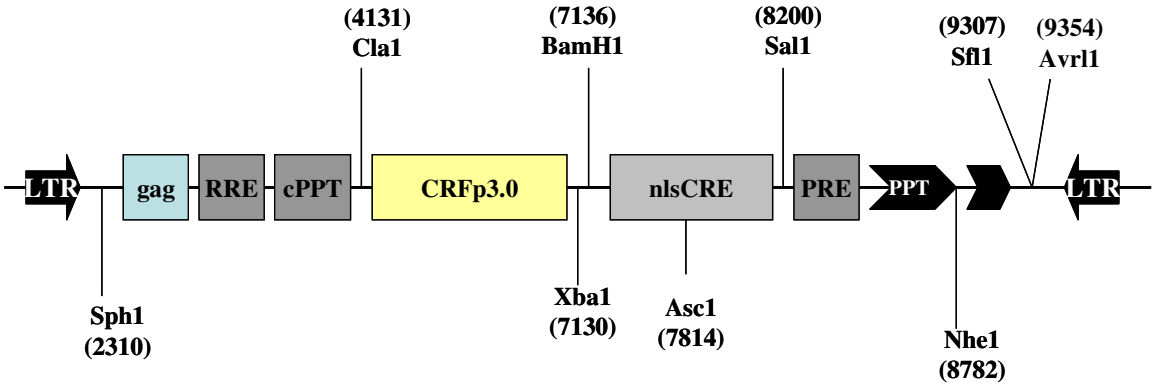
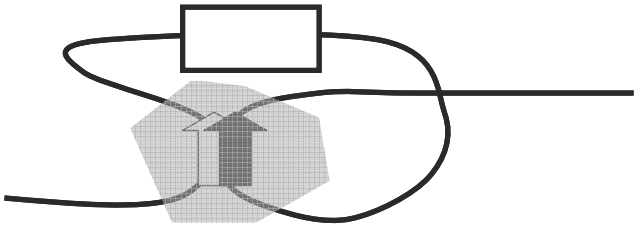


FIGURE 2-2: Cre-recombinase mechanism of action

1. Gene of Interest Flanked by LoxP Sites

2. Chromosomal recombination by the enzyme Cre-Recombinase



3. “Floxed” DNA is excised when LoxP sites are recombined

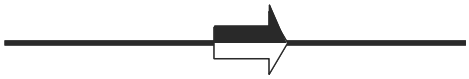


FIGURE 2-3: *In Vitro* Functional Assay

A. Diagram of a reporter vector, Cx-Lel (Chhatwal et al., 2007) using the CMV constitutive promoter to drive expression of fluorescent proteins.

B. Actions of decreasing concentrations of LVCRFp3.0Cre (1µl virus; 1µl of 1:10 dilution; 1µl of 1:100 diluion or 1µl of 1:1000 dilution) on HEK293 cells transfected with the novel reporter vector Cx-Lel. Cells express a floxed green fluorescent reporter protein (GFP) until infected with Cre-expressing lentivirus, causing deletion of the GFP-stop codon and leads to the subsequent expression of dsRed.

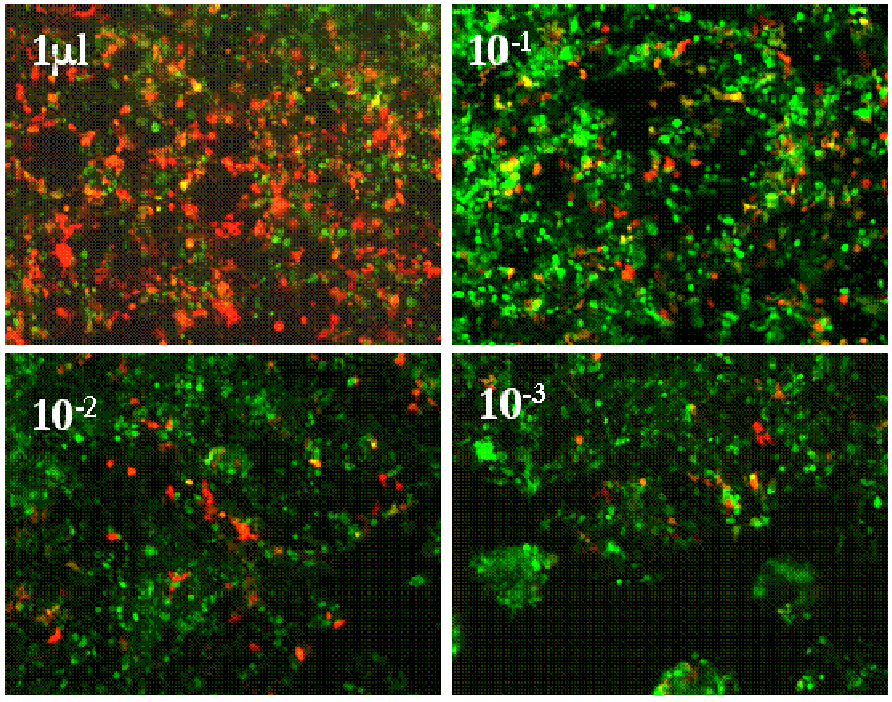


FIGURE 2-4 *In vivo* Functional Assay
(A) Rats were injected unilaterally in the PVN and CeA of WT male mice with LVCMVCre, LVCRFp1.3Cre or LVCRFp3.0Cre lentivirus. Slides are anterior (top) to posterior (bottom). Compared to endogenous CRF expression (right column) only the 3.0Kb promoter length targets transgene expression to the CRF-expressing regions. **(B)** LVCRFp3.0 demonstrates greater specificity for the CRF-expressing PVN (outlined in black) compared to LVCMVCre or LVCRFp1.3Cre. **(C)** LVCRFp3.0Cre expression demonstrated by LacZ staining with X-Gal corresponds to CRF expression throughout the anterior to posterior extent of the PVN as shown by *in situ* hybridization. This experiment demonstrates the ability of the LV-Cre viruses to infect cells and cause genomic rearrangement *in vivo*. Furthermore, it suggests that, compared to LVCMVCre or LVCRFp1.3Cre, the 3.0 Kb promoter region preferentially expresses in CRF-expressing regions.

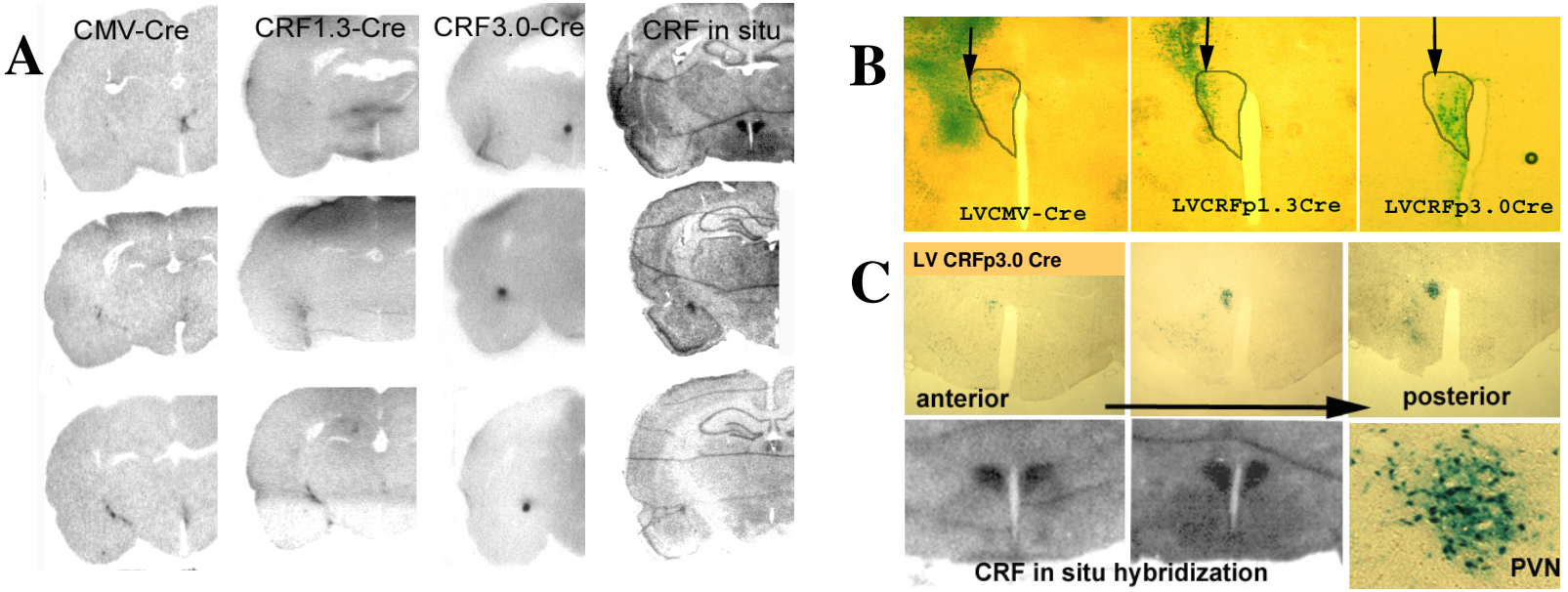


FIGURE 2-5: Cre-recombinase Expression in CRFp3.0Cre Transgenic mice.
In situ hybridization of Cre-recombinase expression (top) matches endogenous CRF mRNA expression (bottom) in PVN-containing coronal sections (left) or serial sagittal sections (right) of F2 generation CRFp3.0Cre transgenic mice.

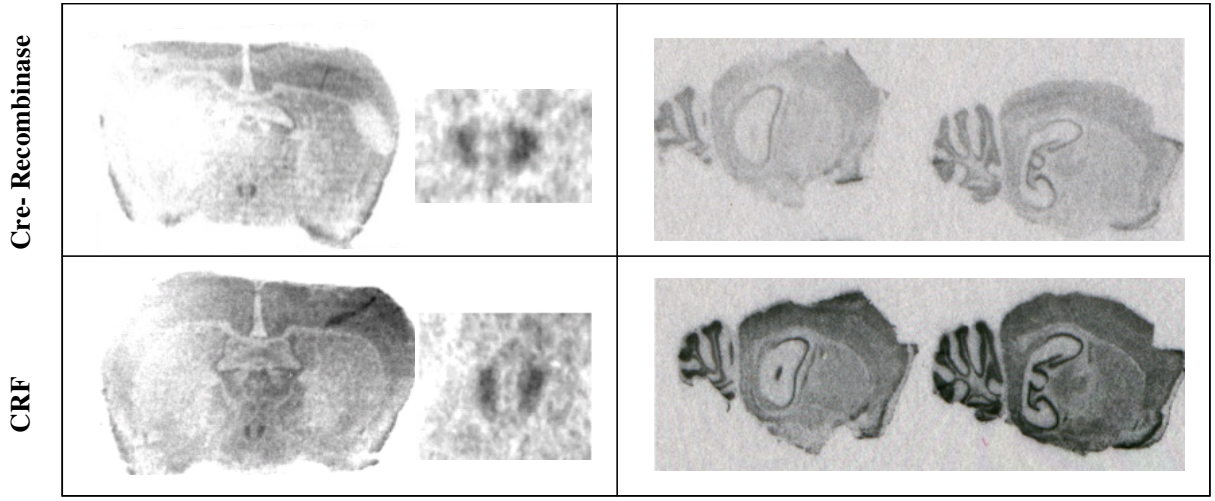
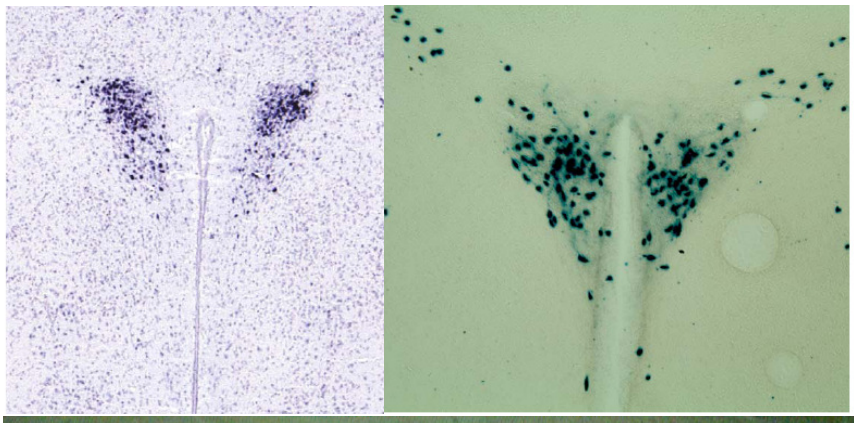


FIGURE 2-6: Functional Cre-recombinase expression in CRFp3.0Cre/Rosa26 Transgenic Mice.

(A) LacZ staining in PVN. Left: the corresponding CRF *in situ* hybridization from the Allen Brain Atlas. **(B)** LacZ staining in the CeA Brain tissue sections were prepared from mice verified by PCR and CRF-Cre + /LacZ+.

(A)



(B)

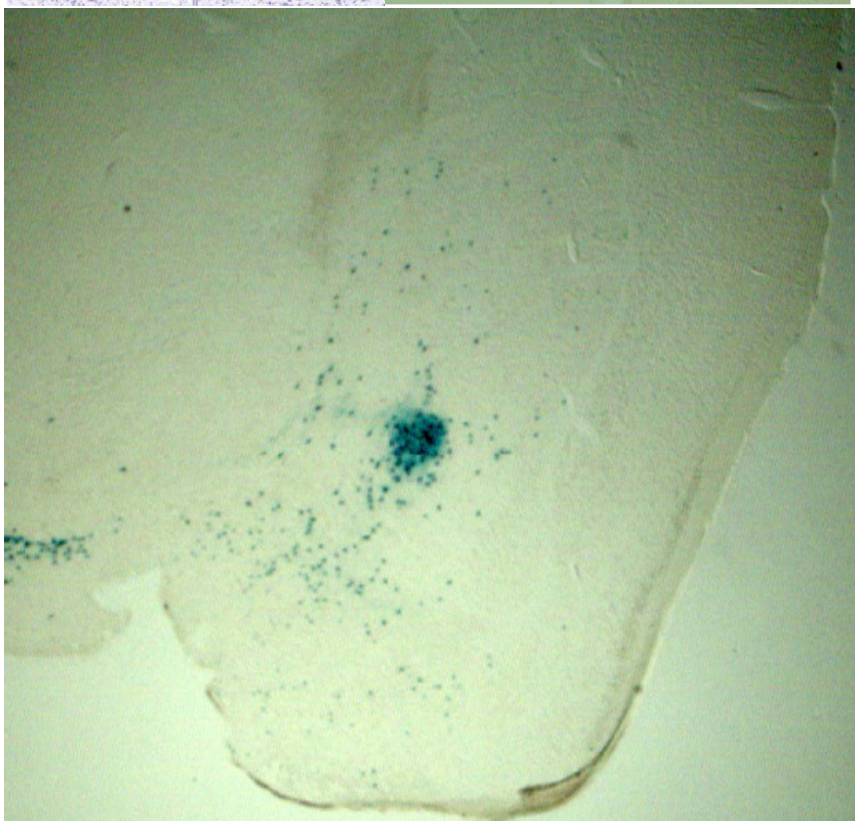
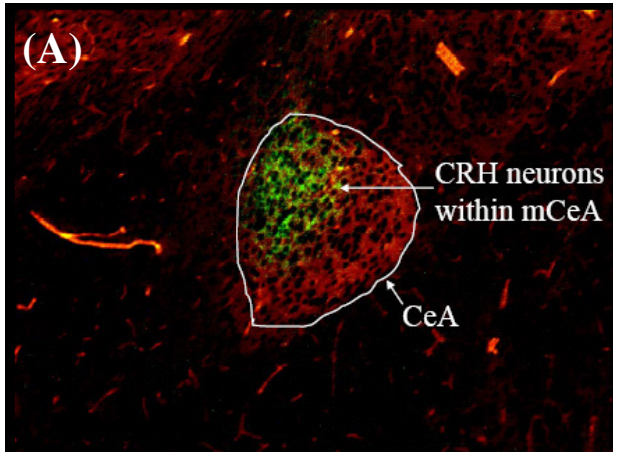


FIGURE 2-7: CRFp3.0Cre/ Td-EGFP reporter transgenic mice.
(A) CeA Regional specific expression of EGFP mediated by CRF-promoter driven Cre-recombinase. Merged image demonstrating overlapping expression of CRFp3.0Cre leading to GFP expression, with Td-Tomato expression in non-floxed cells. 20x objective, 18µm brain slice from a 4-week-old mouse.



(B) BNST Regional specific expression of EGFP mediated by CRF-promoter driven Cre-recombinase.
Left: Td-Tomato expression, Middle: EGFP expression, Right: Merged image. Images were taken under 10x objective, 18µm brain slice from a 4-week old mouse.

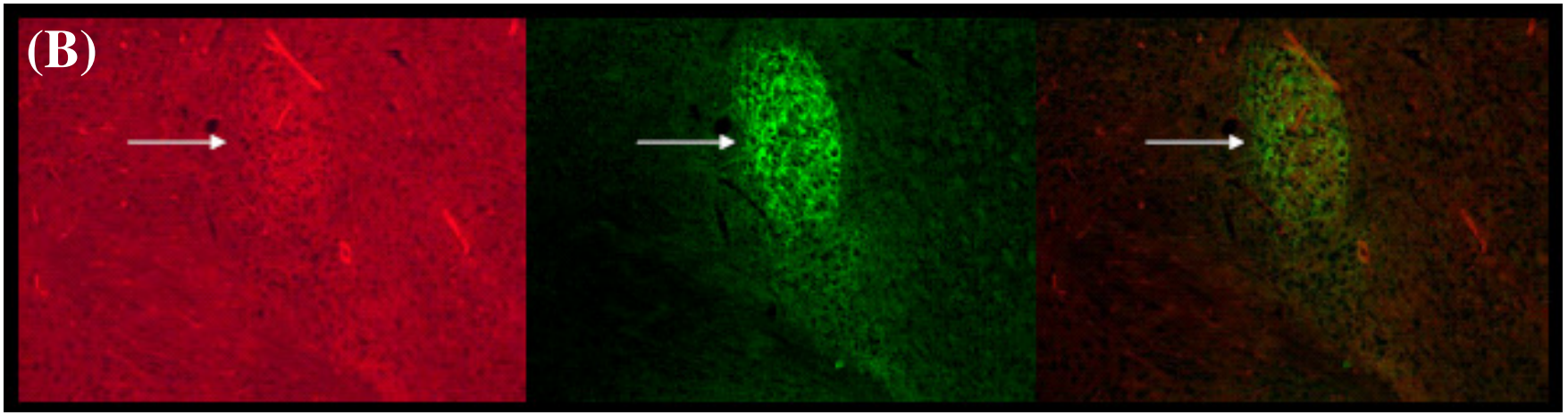


FIGURE 2-8: Physiological properties of CRF-containing neurons in the CeA
(A) Voltage response of a CRF-containing neuron to transient (750 MS) hyperpolarizing and depolarizing current injection. **(B)** CRF neurons are driven by a continuous input of spontaneous EPSCs. **(C)** Photomicrograph showing the expression of CRF-driven GFP expression in the BLA. Arrow indicates neuron illustrated in A and B. Stars indicate CRF negative neurons.

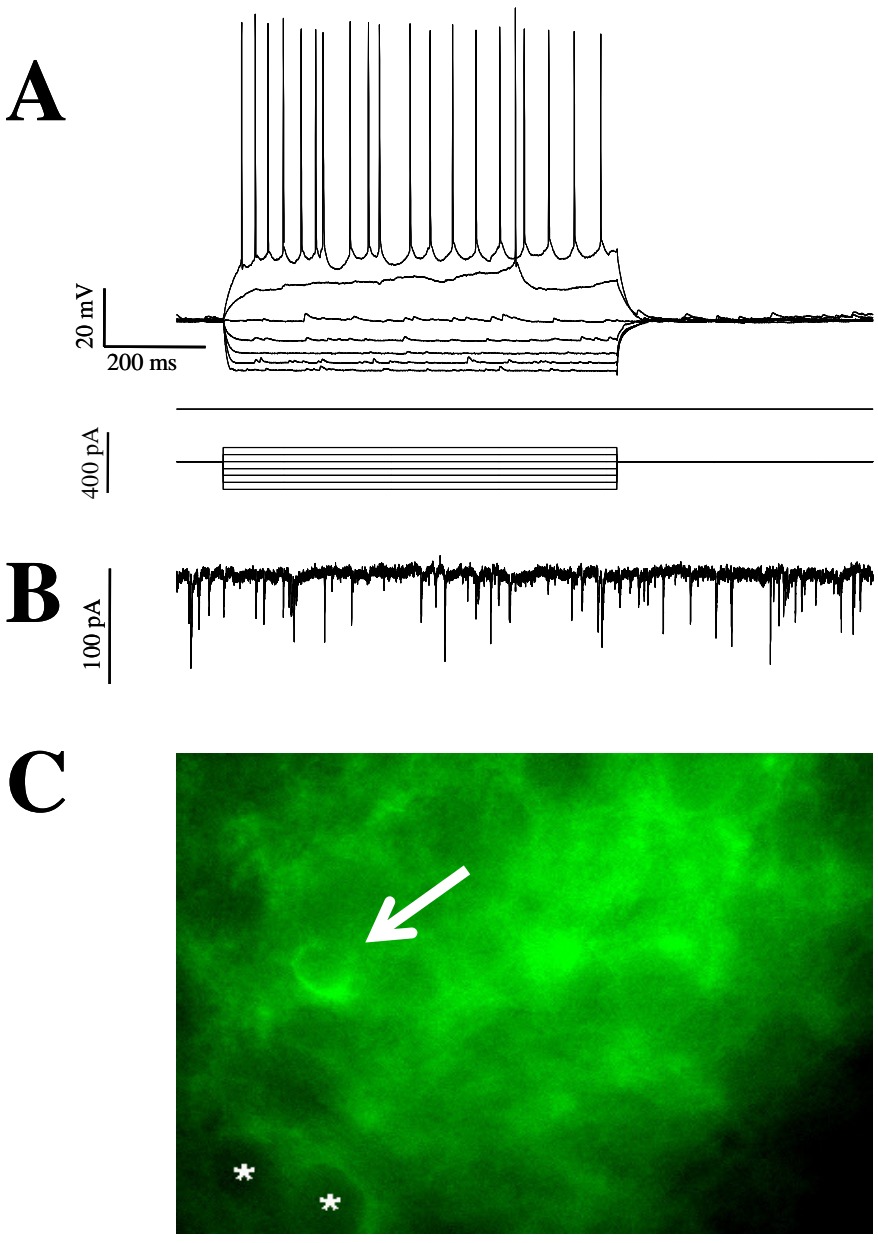
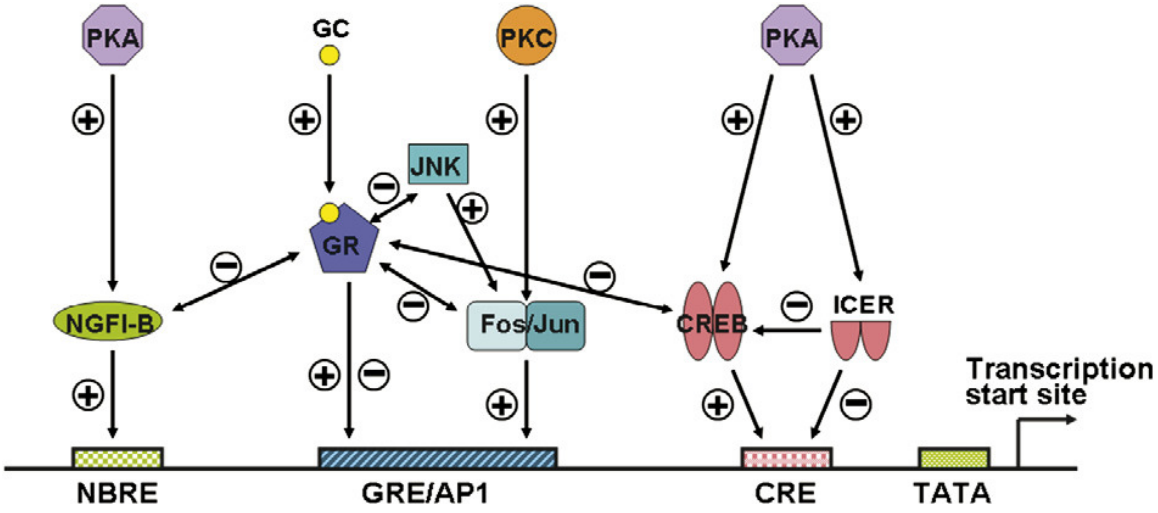


FIGURE 2-9: C*is* and *trans* elements regulate CRF gene transcription.
A highly conserved cAMP response element (CRE) TGACGTCA is present in the human CRF gene promoter centered at 224bp upstream of the transcription start site. (PKA, protein kinase A; PKC, protein kinase C; GC, glucocorticoid; GR, glucocorticoid receptor; GRE/AP1, composite glucocorticoid response element and AP1 (Fos/Jun) binding sites; NGFI-B, nerve growth factor-induced gene B; NBRE, NGFI-B-response element; CRE, cyclic AMP-response element; CREB, CRE binding protein; ICER, inducible cAMP early repressor; JNK, Jun N-terminal kinase). TATA box and transcription start site are indicated. Positive and negative regulation are indicated as (+) and (-), respectively. (From (Yao and Denver, 2007).



CHAPTER 3:

Behavioral effects of lentiviral-vector mediated region and cell-type specific overexpression of CRF within CRF-producing cells of the central amygdala.

INTRODUCTION

With a lifetime prevalence of over 17%, MDD is one of the most common psychiatric illnesses but current treatments are inadequate for half of these patients (Kessler et al., 2003). Symptoms of mood and anxiety disorders are thought to result in part from disruption in the functional balance of emotional centers of the brain relative to higher cognitive centers. Decreased inhibitory control of subcortical limbic structures leads to overactivity in the amygdala (e.g. (Liotti et al., 2000; Drevets, 2003; Dickie et al., 2008).

In particular, overactivity in the CeA, which plays an important role in coordinating the behavioral, endocrine, and autonomic response to stress (e.g. (Prewitt and Herman, 1998; Curtis et al., 2002; Akmaev et al., 2004; Asan et al., 2005) has been associated with enhanced stress sensitivity. CRF is expressed in high quantities in the CeA. It has been suggested the CRF is responsible for the effect of the CeA on the stress response. Elevated CSF CRF concentrations, attributed to hyperactivity of extrahypothalamic CRF sources, have been observed in nonhuman primates with endogenous enhanced sensitivity to stress and fearful temperament (Kalin et al., 2000).

Increased CSF CRF has also been observed in human patients with MDD (Nemeroff et al., 1984) and PTSD (Bremner et al., 1997) and in victims of suicide (Arato et al., 1989). Decreased density of cortical CRF_1 receptors has also been observed in depressed suicide victims, attributed to CRF_1 transcript downregulation secondary to chronically elevated synaptic availability of extrahypothalamic CRF (Nemeroff et al., 1988) reviewed in (Arborelius et al., 1999).

Perhaps the best evidence for the link between CRF, stress, and depression is that both stress and CRF administration to laboratory animals lead to symptoms remarkably similar to those observed in MDD patients (reviewed in (Nemeroff, 1988). Specifically, stress and CRF induce anxiety-like behavior such as decreased exploration in a novel environment (Butler et al., 1990), decreased sleep (Sherman and Kalin, 1987), increased response to fearful stimuli (Butler et al., 1990), decreased eating (Morley and Levine, 1982), decreased body weight that cannot be accounted for solely by the decrease in food intake (Hotta et al., 1991) and suppressed sexual receptivity and behavior (Sirinathsinghji et al., 1983; Keen-Rhinehart et al., 2009).

These anxiety- and depressive-like behaviors are attributed to CRF within the CeA; stress increases CRF in the CeA, (Chappell et al., 1986) and this increase is correlated to increased anxiety-like behavior (Merlo Pich et al., 1995). Other research has provided a less clear analysis of CeA CRF in behavioral symptoms of depression and anxiety. In CRF-knock out (KO) transgenic mice, stress-induced behavioral alterations typically associated with extrahypothalamic CRF systems were unaltered (Dunn and Swiergiel, 1999) despite the fact that CRF_1 antagonists still block these stress-related

behaviors in CRF-KO mice. These data could suggest that other CRF-like molecule(s) may be responsible for these behaviors (Gysling et al., 2004).

Site-specific manipulations help to clarify the impact of particular CRF-producing brain regions on stress-induced physiological and behavioral changes. CRF administration to the CeA increases stress-like behaviors (Buwalda et al., 1997; Buwalda et al., 1998). For example, five days of daily CRF injections into the BLA increased rats' grooming behavior (an anxiety-related phenotype) in an open field after restraint stress (Daniels et al., 2004). CRF injections into the CeA block parasympathetic outflow under basal conditions (Wiersma et al., 1993). Furthermore, CeA-specific reduction in CRF transmission produces an anxiolytic-like phenotype, (e.g. (Rassnick et al., 1993; Bakshi et al., 2002). CeA CRF_1 knock down resulted in an anxiolytic phenotype in rats (Liebsch et al., 1995; Owens et al., 1995; Liebsch et al., 1999). Unfortunately, site-specific injections cannot distinguish whether CRF or an alternative CRF-like ligand accounts for similar *endogenous* effects nor can they distinguish the actions of pre- and post- synaptic receptors. Furthermore, none of the available techniques effectively assess *chronic* CRF dysregulation. The following experiments utilize a novel lentiviral vector to assess anxiety- and depressive- like behavior in rats overexpressing CRF from within CRF-expressing cells in the CeA.

MATERIALS and METHODS

A. Design and Creation of the LVCRFp3.0CRF Construct

Primers were designed to amplify a 3.0Kb region upstream of the ATG start site for the CRFcds. FIGURE 3-1 depicts the region of the mouse CRF gene used as the promoter. The 3.0Kb promoter was PCR amplified from the mouse CRF gene in BAC clone #129A14. The resulting amplicon was topo-cloned into pCR2.1-topo, (Invitrogen Corp., Carlsbad, CA, USA), according to manufacturers' instructions. Incorporated into the original primers were custom restriction sites used for later subcloning steps (*CRF 5'p3.0-Cla1* GCCTATCGATGGAAAGAAAGCACA-AAGGATGCCG; *CRF 3'BamHI* CCAGCGGATCCAGCCGCATGTTAGGGGCGC-TCTCTGAA).

To create the final LVCRFp3.0CRF construct, the lentiviral vector packaging construct, pCMV-GFP-dNhe (Tiscornia et al., 2003), kind gift of Inder Verma, Salk Institute, La Jolla, CA, USA) was digested with *Cla1* and *BamH1* to remove the CMVGFP sequence, treated with antarctic phosphotase (New England Biolabs) to prevent re-ligation, and band-purified from a 0.75% agarose gel using a DNA-purification kit (EPICENTRE® Biotechnologies, Madison, WI). The CRFcds plasmid, a generous gift from Wylie Vale (Salk Institute) was digested with *BglII,* resulting in compatible cohesive ends with *BamHI* digests. The *BglII* CRF fragment was band-purified from a 0.75% gel. The linearized, phosphotased lentivirus backbone with *Cla1/BamH1* ends was combined in solution with the CRF promoter, a *Cla1/BamHI* fragment, and the coding sequence for CRF, a *BglII/BglII* fragment. The two inserts were ligated together and into the lentiviral backbone using the Fast-Link™ DNA Ligation Kit according to the manufacturers' instructions (EPICENTRE®

Biotechnologies, Madison, WI). Correct clones were identified via diagnostic digests of restriction sites within and outside of the promoter and CRFcds inserts. Clones identified as correctly oriented were packaged into virus particles as described in Chapter 2.

B. Determining Virus Titer

HEK293T cells, approximately 50% confluent, were infected with serial dilutions of the CRFp3.0CRF virus (LVCRFp3.0CRF) up to a dilution of 1:1,000,000. 48 hours later, cells were fixed in 4% paraformaldehyde with DMEM (1:1). Cells were rinsed twice for 5min in 1x PBS followed by a 1min incubation in 100% methanol. To eliminate endogenous peroxidases, cells were incubated for 5min in 1% H_2O_2 in PBS then rinsed for 5min in 1x PBS. To prevent non-specific staining, cells were blocked for 10min in 1% nonfat dry milk in PBS. Cells were incubated in 1:1000 Polyclonal anti-CRF made in goat (Santa Cruz Biotechnologies, Santa Cruz, California) overnight at 4^{o}C. The following day, cells were rinsed twice for 5min in 1x PBS followed by a 1hr room-temperature incubation in 1:500 biotinylated bovine anti goat-IgG (Santa Cruz Biotechnologies, Santa Cruz, California). After two 5min rinses in 1x PBS, cells were incubated for 1hr in Avidin DH and biotinylated enzyme (VECTASTAIN Elite Standard ABC Kit, Vector Laboratories, Burlingame, CA). For visualization, the cells were incubated in the dark at room-temperature in DAB Substrate (3, 3'-diaminobenzidine DAB kit, Vector Laboratories Burlingame, CA). After rinsing thoroughly with PBS, CRF-immunopositive cells were counted. Virus used in the following experiments reached at least $1x10^7$ infectious units/µl.

C. *In vivo* Functional Analysis of LVCRFp3.0CRF

Pilot studies verified lentiviral vector expression in control LVCMVGFP and LVCRFp3.0CRF-injected subjects using *in situ* hybridization for the viral WPRE sequence (data not shown). Verification of CeA CRF overexpression (CRF-OE) was assessed with *in situ* hybridization for the CRF transcript. Previous work from our department has shown that a similar virus (LVCMVCRF) increased CRF peptide expression (Keen-Rhinehart et al., 2008). In the present study, CRF peptide was not measured due to incompatible tissue-processing requirements for immunohistochemistry and *in situ* hybridization.

D. Animal Subjects

All animal protocols are approved by the Emory IACUC and the "Guide for Care and Use of Laboratory Animals. Adult male Wistar rats weighing approximately 300g at the time of surgery were pair housed with cage-mates receiving the same virus. For one week following surgery, they were weighed daily and assessed for signs of distress.

In compliance with changes in Emory University biosafety requirements for use of replication-deficient lentiviral vectors, Experiment 1 rats were housed in a cubicle in the animal BSL-2 facility while Experiment 2 rats remained in the cubicle only for a three-day mandatory quarantine period before transfer to the psychiatry department facility. The cubicle and the psychiatry department facility were both maintained on a 0700 on - 1900 off 12-hr light/dark cycle with food and water available *ad libitum*. To minimize stress, researchers rather than animal facility staff were responsible for all cage changes as well as food and water administration.

E. Surgery and Injection of Virus

Rats were anesthetized with ketamine, xylazine, and acepromazie maleate (Welberg et al., 2006), the surgery site was shaved and the rat secured in a stereotaxic frame. To maintain aseptic technique, the surgical area was cleaned three times with alternating betadine and EtOH. A midline incision was made in the scalp and holes were drilled in the skull (coordinates from Bregma: *A/P* -2.1; *M/L* +/- 4.0; *D/V* -8.0).

Injections were performed using a 5µl Hamilton syringe with a 30 gauge beveled-tip needle that had been sterilized with EtOH, rinsed with sterile saline, and coated with sterile 1% BSA prior to virus loading. The needle was lowered to the D/V target. 1µl of control (LVCMVGFP) or CRF-overexpressing (LVCRFp3.0CRF) virus was injected bilaterally into the CeA. An automatic micropump (Ultramicropump II, World Precision Instruments, Sarastoa, CA) was used to inject the virus at a rate of 0.1µl/min. After the injection, the needle was left in place for 2min, then lifted dorsal to the injection site and left for another 5min before being slowly withdrawn. The skin was closed using a 6-0 Vicryl suture (Ethicon: Johnson & Johnson, Piscataway, NJ).

F. Experiment 1 Behavior

The timeline for Experiment 1 is shown in FIGURE 3-2. Injection placement was assessed by post-mortem *in situ* hybridization for CRF. Placement of LVCRFp3.0CRF in the CeA was verified in 12 of the 18 rats who received the LVCRFp3.0CRF injection in Experiment 1 and 25 out of 28 rats who received the LVCRFp3.0CRF injection in Experiment 2. Only those subjects are included in the behavioral, endocrine, and gene-

expression analyses. The eliminated rats either had tissue damage from the surgery, misplaced injection sites, or absence of virus altogether.

Behavioral testing began at least 14 days after the last LV injection surgery; prior testing has demonstrated maximum LV expression after 7-10 days. Behavioral tests were carried out in the center of the ABSL-2 room such that rats were not transferred for testing but were removed from the home cage to a transport cage for the duration of the test and then immediately returned to the home cage.

Pilot studies assessed behavior in the morning (0900) and evening (2100) and determined that there was often a basement effect in behavior in the morning while evening tests provided a greater range of behavior (data not shown). Based on these pilot data, all behavioral tests were run under red lights beginning approximately 2hrs into the active cycle (2100). Video recordings were scored by two reviewers blind to treatment conditions. To minimize effects of multiple testing, only one test was run per week and cage-mates were tested on consecutive days (McIlwain et al., 2001; Paylor et al., 2006). Cage changes were performed two days prior to behavioral tests. Between each animal in the elevated plus, open field, or defensive withdrawal tests, equipment was cleaned with 70% ethanol and thoroughly dried.

1. Open Field

The open field (OF) apparatus was a 75×75cm box with 50cm high walls made of white Plexiglas. Rats were placed into the center the open field. In the 5min test number of edge and center squares, number of rears, time in the center, time rearing, and time grooming were measured.

2. Elevated Plus Maze

The elevated plus maze (EPM) was constructed of black Plexiglas and consisted of two 50cm enclosed arms (i.e., arms with 40cm high walls at the sides), and two 50cm open arms (i.e., arms with no walls at the sides). The four arms were arranged in a cross pattern extending out from a common 10cm x 10cm platform at the center where all arms met. The maze was mounted at a height of 50cm above the floor of the room. Rats were set into the middle square facing an open arm. In the 5min test, open and closed arm entries, time in open and closed arms, rears, and time grooming were measured.

3. Defensive Withdrawal

For the defensive withdrawal (DW) test, rats were placed in a black PVC withdrawal box (10cm diameter tube× 21cm in length, closed at one end) and the withdrawal box was then placed into the OF at a distance of 20cm from a corner with the open end facing the corner. In the 10min test, time in box and number of box entries were measured along with edge and center squares crossed, number of rears, and time spent grooming.

4. Forced Swim Test

The Porsolt methodology was followed to assess time spent climbing or swimming versus time spent immobile in the forced swim test (FST) (Porsolt et al., 1978b). Briefly, the rats were placed individually in a translucent container (40 x 24 x 60cm) filled with water (~24°C) to a depth of approximately 22cm so that the animals could not rest on the bottom nor reach the top of the container. As with the other behavioral measures, day 1 of the forced swim test began 2 hours into the dark phase. Rats were placed into the swim tank for 15min. Cage mates were run at the same time in individual tanks separated by a darkly colored divider. At the end of the 15min test, rats were removed

from the tank and dried with clean cloth towels then returned to their home cage. The following day, rats were again placed in the swim tank this time for 5min, beginning shortly after lights off (1930). Floating debris was removed after every rat and water was changed every other rat. An observer blind to experimental condition assessed time spent climbing, swimming, or immobile in each of the 15 and 5min trials.

5. Sucrose Preference

In a 24hr 2-bottle choice test, rats could drink from tap water or 1% sucrose solution. At 12 hours, the bottle location was switched. Sucrose preference is displayed as percentage of sucrose solution consumed out of total volume consumed.

G. Experiment 2 Behavior

FIGURE 3-3 shows the timeline for Experiment 2. The primary focus of Experiment 2 was the endocrine testing (see Chapter 4); as a measure of anhedonia, the SPT was performed in the fourth week after injection surgery. Animals were individually housed in clean cages with bottles containing tap water and 1% sucrose. Bottle placement was switched at 12hrs. After the 24hr test, rats were returned to pair housing and were subjected to no other behavioral testing for the duration of the Experiment. There were no significant differences in sucrose-preference (TABLE 3-6). These rats were exposed to no other behavioral tests and unhandled between the SPT and the end of the Experiment such that the endocrine analysis (Chapter 4) is not influenced by prior manipulations.

H. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). The unpaired student T-test or two-factor analysis of variance (ANOVA) were used where appropriate. The Grubbs test for outliers was run on all data sets. Significance is indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

RESULTS

A. *In Vitro* and *In Vivo* Functional Assay of LVCRFp3.0CRF

The 3.0Kb promoter length used in this vector is identical to that described in the viral vectors and transgenic mouse discussed in Chapter 2. The ability of LVCRFp3.0CRF to express functional CRF peptide was assessed *in vitro* using immunocytochemistry (FIGURE 3-1). LVCRFp3.0CRF-induced CRF-OE *in vivo* was assessed with *in situ* hybridization for each batch of virus (data not shown). Previous work with a similar viral vector has demonstrated virus-induced CRF peptide expression *in vivo* (Keen-Rhinehart et al., 2009).

B. Behavioral Effects of Chronic CRF-OE from the CeA

Previous research in our lab and others has shown that lentiviral vectors reach maximum expression approximately 7 to 10 days after injection. As such, behavioral testing for this project did not begin until at least 14 days after the last surgery. The timeline and design of this study is shown in FIGURE 3-2.

1. Open Field

The OF test was run in week 5 of the Experiment; at least 28 days after the lentiviral vector injection surgery. In the 5min open field test, we expected CeA CRF-OE rats to spend less time exploring the center of the open field arena. However, there was no significant difference in the number of center squares crossed as a percentage of total locomotion and no significant decrease in the time spent in the center of the open arena as a percentage of the total test time. Time spent grooming was also recorded and did not differ between the control and CeA CRF-OE groups. Chronic overexpression of CeA CRF did decrease total locomotion (total # squares crossed = 103.78 +/- 7.86 in control rats vs. 67.78 +/- 9.66 in CeA CRF-OE subjects; $p < 0.01$). Vertical locomotion was also decreased in the CeA CRF-OE group (23.36 +/-1.52 in control rats vs. 17.5 +/- 2.08 $p < 0.05$ in LVCRFp3.0CRF-injected subjects) (TABLE 3-1).

2. Elevated Plus Maze

In the EPM, CRF-OE within the CeA was expected to increase time spent in closed arms vs. open arms. Using a one-tailed T-test, there was a significant decrease in time spent in the open arm as a percentage of the total test time (21.43 +/- 4.08% in control rats vs. 12.06 +/- 2.64% in CeA CRF-OE subjects; $p < 0.05$) and a trend towards decreased number of open arm entries as a percentage of total arm entries (30.50 +/- 4.19% in control rats vs. 20.85 +/- 4.01% in CeA CRF-OE subjects; $p = 0.055$) in rats chronically overexpressing CeA CRF (FIGURE 3-4). There was no difference in the number of closed arm entries or number of rears in CeA CRF-OE rats, suggesting that the variance in open arm activity is not due to decreased locomotion (two-tailed T-tests; $p > 0.05$) (TABLE 3-2).

3. Defensive Withdrawal

CeA CRF-OE rats were expected to spend a greater amount of time withdrawing and less time exploring the center of the open field arena. In fact, the CeA CRF-OE rats as a group exhibited a significant increase in latency to emerge from the withdrawal box (57.92 +/- 24.0s for controls vs. 259.17 +/- 69.11s in the CeA CRF-OE group; $p < 0.01$ in a one-tailed T-test) and in total spent more time withdrawing than did control subjects (LVCMVGFP = 371.32 +/- 36.65s vs. LVCRFp3.0CRF = 550.72 +/- 21.24s $p < 0.001$ in a one-tailed T-test) (FIGURE 3-5). Of the time spent in the open arena (i.e. not withdrawing) there was no difference in total locomotion. There was perhaps a slight trend towards a decrease in the number of center squares crossed per minute out of the box as a percentage of the total number of squares crossed per minute in the open field (3.82 +/- 1.56% vs. 1.09 +/- 0.73% in a one-tailed T-test; $p = 0.08$) (TABLE 3-3).

4. Forced-Swim Test

Because antidepressant drug administration increases time spent swimming and climbing in the FST, we expected to find a decrease in time immobile in the rats overexpressing CeA CRF. However, there was no group difference in the 15 or 5min trial (TABLE 3-4).

5. Sucrose-Preference Test

The sucrose preference test was performed twice. The first SPT occurred in week 4 before any other behavioral tests. Based on previous research on stress-induced behavior in rats, we expected chronic overexpression of CeA CRF to increase anhedonia as measured by a decreased preference for a solution of 1% sucrose. Although there was a slight decrease in the percentage of 1% sucrose solution consumed out of the total volume, the difference was not significant. Interestingly, there was a slight trend towards

an increase in total volume consumed (196.82 +/- 25.43g vs. 279.72 +/- 26.02; p = 0.06 in a two-tailed T-test) but there was no other evidence for difference in water consumption, suggesting this trend is not meaningful. The second SPT occurred in week 9, after completing the behavioral tests. Increased anhedonia was expected in the CeA CRF-OE group but there was no difference in volume or percentage of 1% sucrose solution consumed (TABLE 3-5; 3-6).

DISCUSSION

Region & Cell-Type Specific Overexpression of CRF within CeA CRF cells decreases central tendency in behavioral tests of anxiety

Behavioral tests for anxiety in rodents were designed to assess the natural desire to explore compared to natural fear of novel, or exposed areas (i.e., a conflict between fear and exploratory drive (Lowry et al., 1996). What defines a behavioral test as a measure of anxiety is its responsiveness to anxiolytic and anxiogenic manipulations. Benzodiazepines and barbiturates used to treat anxiety increase exploratory behavior in the OF, EPM, and DW tests; conversely, pharmaceuticals that increase exploratory behavior in these novel-environment tests have been demonstrated to possess anxiolytic effects in humans (for a review see (Crawley, 1985). In addition to the predictive validity of these measures, chronic stress increases anxiety-like behavior in these paradigms; this increase is associated with elevated CRF expression in the CeA and increases PVN CRF as well as HPA axis activation, suggesting that rodent tests of anxiety reflect biological

alterations which correspond with anxiety disorders in humans (e.g. (Pellow et al., 1985; Merlo Pich et al., 1995), reviewed in (Hogg, 1996).

Increased anxiety-like behavior observed following LVCRFp3.0CRF-mediated chronic CRF-OE in the CeA is concordant with previous work demonstrating that intracerebroventricular (i.c.v.) injections of CRF (Campbell et al., 2004) or CRF injection directly into the amygdala (Daniels et al., 2004) and LC (Butler et al., 1990) increase anxiety-like behavior, and contradicts research identifying a dissociation between CRF activity in the CeA and anxiety-like behavior (e.g. (Merali et al., 2004). Furthermore, recent work from our group (Keen-Rhinehart et al., 2009) and others (Lu et al., 2008) has demonstrated increased anxiety-like behavior in models of CRF-OE in rats and mice, respectively.

In addition to the expected anxiogenic effects of CeA CRF-OE, rats in the LVCRFp3.0CRF group also exhibited decreased total locomotion in the OF test (TABLE 3-1). This is contrary to other studies in which CRF administration increases locomotion in rats (Sutton et al., 1982; Veldhuis and De Wied, 1984), mice (e.g. (Contarino et al., 2000), and amphibians (Lowry et al., 1996) reviewed in (Lowry and Moore, 2006). However, there were no differences in total locomotion in the EPM or DW tests, suggesting that the OF results do not demonstrate a locomotor deficit. Rather, decreased locomotion in LVCRFp3.0CRF-injected rats may represent psychomotor retardation in the novel environment.

Overall, the increase in anxiety-like behavior after chronic CRF-OE from endogenous CRF-producing neurons in the CeA demonstrates that LVCRFp3.0CRF produces functional CRF peptide which elicits behavioral effects similar to those initiated

by chronic stress, supporting the hypothesis that the CeA behavioral stress response is mediated by CRF.

Region & Cell-Type Specific Overexpression of CRF within CeA CRF cells demonstrated no significant effects in behavioral tests of depressive-like behavior

As with behavioral tests of anxiety-like behavior, behavioral assessments of depressive-like behavior are defined by their responsiveness to antidepressant drugs. Decreased consumption of sucrose or saccharin in the SPT is interpreted as a correlate of anhedonia in depressed humans. Administration of imipramine can block stress-induced decreased consumption of sucrose/saccharine solution in rats (Katz, 1982).

The FST is known for its ability to screen potential antidepressant drugs. In the original FST design (Porsolt et al., 1978a), animals are subjected to 15min of forced swimming on day one, administered an antidepressant, and tested for 5min in the FST on day two. Pharmaceuticals that increase 5-HT have been shown to increase swimming behavior while pharmaceuticals that increase NE increase climbing behavior (Cryan et al., 2005).

Based on previous work in animal models of chronic stress, LVCRFp3.0CRF-mediated increased CRF production in CeA CRF cells was expected to decrease consumption of 1% sucrose solution in the SPT, and increase immobility in the FST. In the first SPT there was a trend towards decreased sucrose preference; there was no such trend in the second SPT. While this result is contrary to the effects of chronic mild stress (Kompagne et al., 2008), other research has shown that sucrose preference after

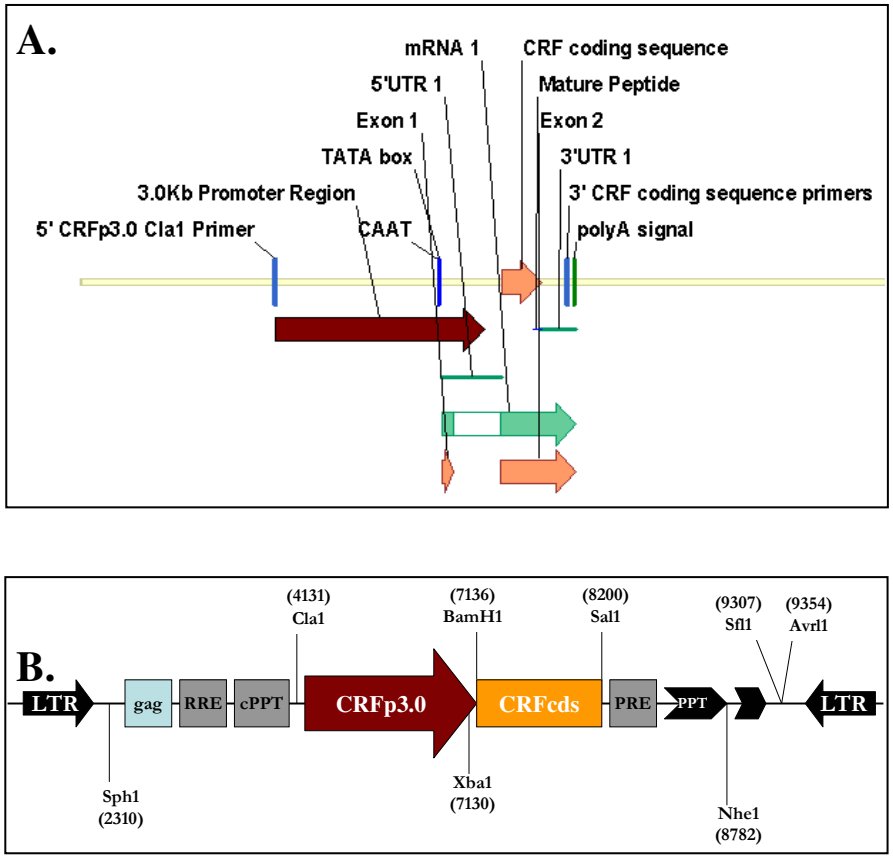
exogenous central CRF administration is increased or decreased dependent on other contextual factors and on the dose of CRF (Heinrichs et al., 1991).

There was also no evidence for an effect of CeA CRF-OE in the FST, which was performed eight weeks after injection of LVCRFp3.0CRF or control virus. These data are contradictory to research demonstrating increased time immobile after chronic mild stress (Kompagne et al., 2008) or region (but not cell-type) selective increases in CeA CRF (Keen-Rhinehart et al., 2009). However, CRF_1 agonists have been demonstrated to increase swimming/climbing behavior in the FST (Tezval et al., 2004). Other recent research has shown that i.c.v. CRF administration to rats (Garcia-Lecumberri and Ambrosio, 2000) or CRF-OE transgenic mice (Lu et al., 2008) increased time swimming and climbing in the FST and describe these data in terms of increased *active stress-coping behaviors* (i.e. decreased immobility) in the FST.

The lack of effect of LVCRFp3.0CRF in the FST here may conjecture that chronic stress and CRF-OE elicit opposing rather than overlapping effects on behavior in the FST, potentially due to unique alterations of monoaminergic signaling.

Importantly, the interpretation that increased swimming/climbing represents an anti-depressant effect is increasingly coming into question and the functional significance of the lack of effect in the SPT and FST in this study remains to be determined.

FIGURE 3-1: LVCRFp3.0CRF Design
(A) Annotated CRF gene; maroon bar shows the 3.0Kb promoter region used in this study. **(B)** Lentiviral vector construct for cell-type specific CRF overexpression. **(C)** *In vitro* expression of CRF-expressing construct (LVCRFp3.0CRF)



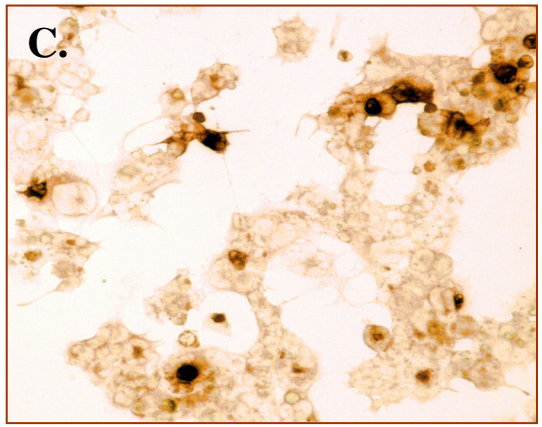


FIGURE 3-2: Timeline for Experiment 1

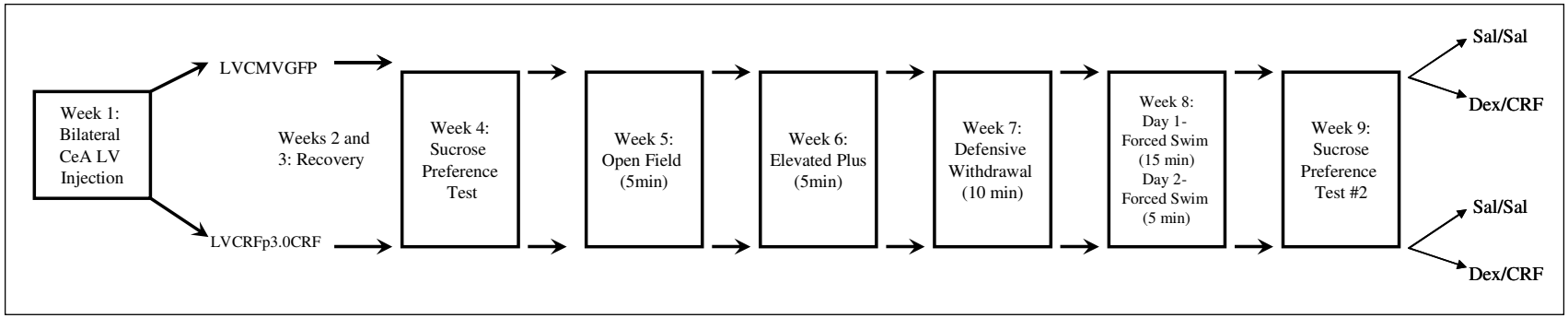


FIGURE 3-3: Timeline for Experiment 2

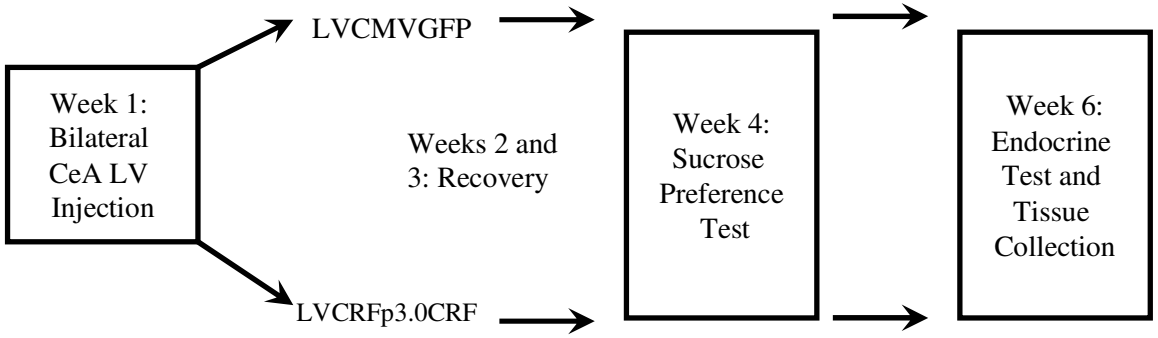


TABLE 3-1: Open Field Test in Experiment 1
Chronic CeA CRF-OE decreases total locomotion in the open field test of behavior in a novel environment. Data are displayed as mean +/- SEM; p-values reflect results of two-tailed T-test analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	LVCMVGFP	**LVCRFp3.0CRF**
Total Locomotion (# Squares Crossed)	103.78 +/- 7.86	67.78 +/- 9.66 $p < 0.01$ **
% Center Squares Crossed	6.43 +/- 1.55	6.18 +/- 1.34 $p > 0.05$
% Time in Center	6.31+/- 1.14	4.87 +/- 1.12 $p > 0.05$
Time Grooming (s)	1.51 +/- 0.81	1.42 +/- 0.74 $p > 0.05$
# Rears	23.36 +/- 1.52	17.5 +/- 2.08 $p < 0.05$ *

FIGURE 3-4: Elevated Plus Maze in Experiment 1
Chronic CeA CRF-OE increases anxiety-like behavior in the EPM. There was a trend towards a decrease ($p = 0.06$) in the percentage of open arm entries out of total arm entries **(A)**, and a significant decrease in the percentage of time spent in the open arm during the 5min trial **(B)**. Graphs display means +/- SEM; p-values reflect results of one-tailed T-test analysis based on the *a priori* hypothesis that CeA CRF-OE would decrease exploration in the open arm.

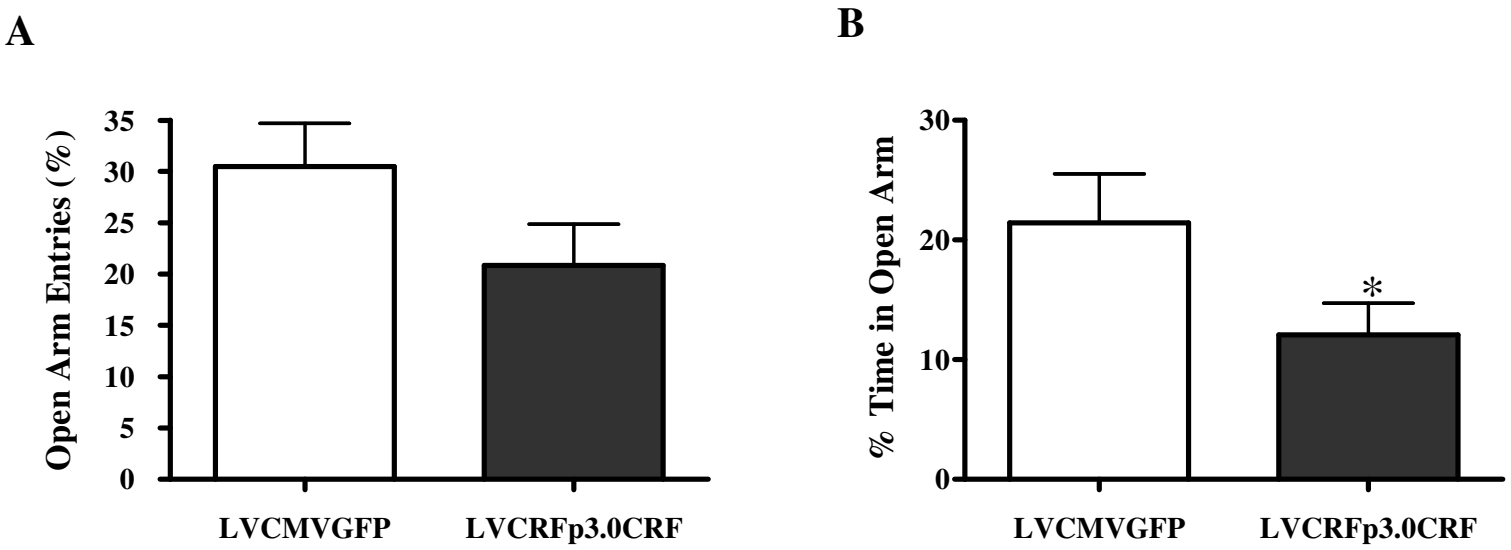


TABLE 3-2: Additional measures of behavioral activity in the EPM. Data are displayed as mean +/- SEM; p-values reflect results of two-tailed T-test analysis.

	LVCMVGFP	**LVCRFp3.0CRF**
Total Arm Entries	18.29 +/- 1.01	15.25 +/- 1.71 $p > 0.05$
Closed Arm Entries	12.58 +/- 0.84	11.56 +/- 1.15 $p > 0.05$
Time Grooming (s)	1.69 +/- 0.96	3.94 +/- 1.03 $p > 0.05$
# Rears	22.5 +/- 1.38	23.83 +/- 0.87 $p > 0.05$

FIGURE 3-5: Defensive Withdrawal in Experiment 1
Rats overexpressing CRF from the CeA emerge from the withdrawal box later than the control animals **(A)** and spend more total time withdrawing **(B)**. Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis based on the *a priori* hypothesis that increased CeA CRF expression would decrease exploration in the center of the open arena (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

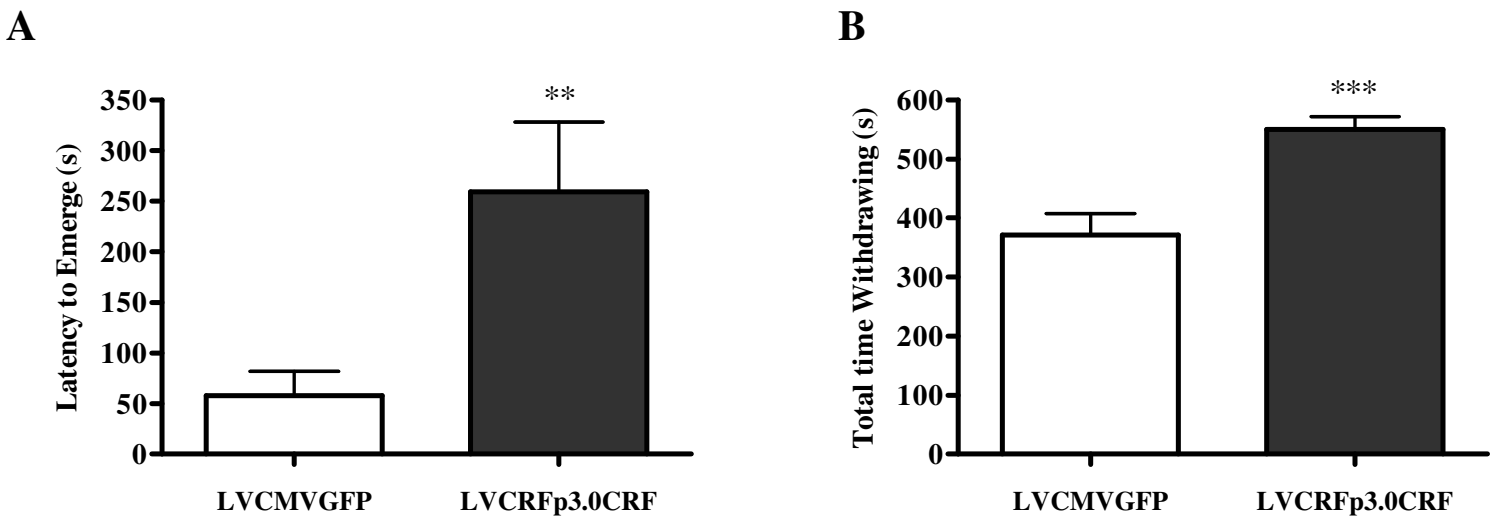


TABLE 3-3: Additional measures of behavior in the DW test
Data are displayed as mean +/- SEM; p-values reflect results of two-tailed T-test analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	LVCMVGFP	**LVCRFp3.0CRF**
Average time in box / box entry (s)	36.83 +/- 5.69	272.35 +/-63.87 $p < 0.01$ **
Total locomotion / min out of box	22.96 +/- 1.83	15.3 +/- 3.52 $p > 0.05$
% Center squares per min out of box / total locomotion per min out of box	3.82 +/- 1.56 %	1.09 +/- 0.73 $p = 0.08$ (one-tailed)
Supported rears / min in open field	0.124 +/- 0.01	0.111 +/- 0.042 $p > 0.05$

TABLE 3-4: Forced Swim Test in Experiment 1
Behavioral activity in the FST is unaltered by chronic CeA CRF-OE. Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis ($p < 0.05$ is significant).

	LVCMVGFP	**LVCRFp3.0CRF**
% Time Immobile 15min trial	30.54 +/- 3.98	25.48 +/- 2.07 $p > 0.05$
% Time Immobile 5min trial	28.42 +/- 5.46	26.06 +/- 3.80 $p > 0.05$

TABLE 3-5: Experiment 1 Sucrose Preference Tests #1 and #2
Data are displayed as mean +/- SEM; p-values reflect results of two-tailed T-test analysis ($p < 0.05$ is significant).

	LVCMVGFP	**LVCRFp3.0CRF**
Test #1		
% Sucrose Solution Consumed	89.94 +/- 1.99	77.77 +/- 8.85 p = 0.07
Total Liquid Consumed (g)	196.82 +/- 25.43	279.73 +/- 26.02 p = 0.06
Test #2		
% Sucrose Solution Consumed	79.37 +/- 4.53	84.82 +/- 3.20 p > 0.05
Total Liquid Consumed (g)	207.02 +/- 22.62	259.43 +/- 23.80 p > 0.05

TABLE 3-6: Experiment 2 Sucrose Preference Test

	LVCMVGFP	LVCRFp3.0CRF
% Sucrose consumed	80.8 +/- 4.06 %	84.5 +/- 2.88 % $p > 0.05$
Total Volume (g)	90.5g +/- 4.38	99.8 +/- 7.42 $p > 0.05$

CHAPTER 4:

Effects of *Region & Cell-Type Specific* Overexpression of CRF within CeA CRF cells on HPA Axis Activity

INTRODUCTION

The stress-responsive HPA axis is comprised of CRF in the PVN, ACTH from the anterior pituitary and GC from the adrenal cortex. In many MDD patients, particularly those with severe or psychotic depression, the HPA axis exhibits marked hyperactivity as evidenced by the following:

1. At rest, plasma ACTH and cortisol concentrations are elevated compared to healthy volunteers.
2. Plasma ACTH and cortisol (and other GCs) are not suppressed by dexamethasone, suggesting that HPA axis negative feedback is disrupted in MDD patients.
3. When CRF is administered in a standard CRF-stimulation test, plasma ACTH concentrations are blunted in MDD patients compared to healthy control subjects.
4. Administration of dexamethasone followed by CRF (the Dex/CRF test), generally considered the most sensitive measure of HPA axis activity, results in elevated plasma ACTH and GC concentrations in MDD patients compared to control subjects (reviewed in (Holsboer, 2000).
5. Depressed patients exhibit elevated CSF CRF and cortisol concentrations.

6. Concentrations of CRF peptide and CRF mRNA expression are elevated in the PVN of depressed patients.
7. CNS CRF_1 mRNA expression is decreased in depressed suicide victims, interpreted to reflect receptor downregulation secondary to chronic elevations in CRF peptide.

HPA axis hyperactivity in MDD has been hypothesized to result from decreased sensitivity to GC negative feedback and increased activity of hypothalamic CRF neurons. Evidence suggests that during a depressive episode, CRF is overexpressed in both hypothalamic and extrahypothalamic regions, the latter including the CeA and BNST. Elevated CSF CRF concentrations and HPA axis hyperactivity normalize upon recovery from depression, suggesting that these are state markers for a depressive episode rather than trait markers for MDD (Plotsky et al., 1998). It has been hypothesized that return to normal HPA axis function is a shared property of all antidepressant treatments (e.g. (Holsboer and Barden, 1996; Owens and Nemeroff, 1999; Stout et al., 2002).

Importantly, the tests that assess HPA axis function in humans are also used to detect HPA axis disturbances in laboratory animal models. CRF manipulation in experimental animals leads to HPA axis alterations observed in these tests. Transgenic mice chronically overexpressing CRF peptide (CRF-OE) developed a 5-fold increase in plasma ACTH, 10-fold increase in plasma GC, increased anxiety-like behavior, learning deficits, and a blunted HPA axis response to stress, likely due to desensitization (Stenzel-Poore et al., 1994). However, as with any conventional transgenic, developmental compensation has likely occurred, making it difficult to interpret these results (Peeters et al., 2004).

Conversely, mice lacking the CRF gene (CRF-KO) are not able to mount a HPA axis response to stress, but contrary to initial expectations, baseline ACTH was normal in

CRF-KO mice, possibly due to compensation by AVP (reviewed in (Venihaki and Majzoub, 2002)).

While human studies are limited to these endocrine tests, experimental animal models provide a more in-depth analysis. HPA axis homeostasis is clearly essential given the psychological and physiological complications associated with its dysregulation. Numerous brain regions and NT systems coordinate to influence the PVN and fine-tune the HPA axis. The CeA may be particularly important in PVN regulation; CeA stimulation results in spikes of plasma ACTH and GC (Feldman et al., 1995b; Feldman et al., 1995a; Feldman and Weidenfeld, 1998), CeA lesions block the normal HPA axis response to stressful stimuli (reviewed in (Herman and Cullinan, 1997), and CeA lesions in non-human primates diminish species-specific fear behavior and decrease CSF CRF as well as plasma ACTH concentrations (Kalin et al., 2004). Pharmacological blockade of CRF transmission within the CeA produces a similar anxiolytic phenotype (e.g. (Rassnick et al., 1993; Bakshi et al., 2002; Asan et al., 2005) while infusion of CRF into the BLA, a CeA target (Roozendaal et al., 2002), increased the amplitude and duration of the GC response to restraint stress (Daniels et al., 2004) suggesting that *CRF is at least in part responsible for the effects of the amygdala on the HPA axis*.

The following experiments were designed to test the hypothesis that chronic overexpression of CRF produced within and released from neurons in the CeA results in HPA axis hyperactivity similar to that observed in human patients during a depressive episode.

MATERIALS and METHODS

A. Animal Subjects

Endocrine testing was performed in rats previously assessed for anxiety and depressive-like behavior; animal housing and lentiviral vector injection surgery are described in Chapter 3. The LVCRFp3.0CRF vector is shown in FIGURE 3-1; the timeline and design of Experiment 1 and Experiment 2 are displayed in FIGURE 4-1 and 4-2, respectively.

B. Experiment 1 Endocrine Analysis

From each cage, one rat was randomly assigned the Dex/CRF condition and the other the Sal/Sal condition. The Dex/CRF test was performed in three groups of ten rats per day. Injections and decapitations were timed to allow one minute between cage mates and three minutes between cages such that the time between injections and decapitation was consistent between subjects.

Beginning at 1200, rats received an IP injection of either 20μg/Kg dexamethasone (in a concentration of 40ug/ml) or an equivalent volume of sterile saline. 90min later an I.V. (tail vein) injection of saline or 0.5ug/Kg rat/human CRF (Sigma-Aldrich - United States) which had been diluted in saline to a concentration of 2μg/ml was administered. Approximately 25min after the I.V. injection, rats were sacrificed by live decapitation, brains were removed and fresh-frozen on dry ice and trunk blood was collected in cold 50ml Falcon tubes. Whole blood was spun at 2100rpm for 10min in a refrigerated centrifuge. Plasma was transferred to 2ml cryovials on ice and stored at -80^{o}C until assayed for ACTH and corticosterone concentrations.

C. Experiment 2 Endocrine Analysis

Animals were randomly assigned to the control, DST, CRF-stimulation test, or Dex/CRF test. Beginning at 1200, rats received an IP injection of either 20µg/Kg dexamethasone (in a concentration of 40ug/ml) or an equivalent volume of sterile saline. 90min later rats received an I.V. (tail vein) injection of saline or 0.5ug/Kg rat/human CRF (Sigma- USA) diluted in saline to a concentration of 2µg/ml. Drugs were assigned such that there were four groups:

1. Control: Saline + Saline
2. The dexamethasone-suppression test: Dexamethasone + Saline
3. The CRF-stimulation test: Saline + CRF
4. The Dex/CRF test: Dexamethasone + CRF

Approximately 25min after the I.V. injection, rats were sacrificed by live decapitation. Brains and pituitary glands were collected and fresh frozen on dry ice. Adrenal glands were also removed, cleaned of fat, and weighed. Trunk blood was collected in cold 50ml Falcon tubes for corticosterone and glass EDTA-coated tubes for ACTH (BD Vacutainer®). Whole blood was spun at 2100rpm for 10min in a refrigerated centrifuge. Supernatant was transferred to 2ml cryovials on ice. Samples were then stored at -80^{o}C until assayed for ACTH and corticosterone concentrations.

D. ACTH and Corticosterone Radioimmunoassay

ACTH was measured by immunoradiometric assay from a commercially available kit according to the manufacturer's instructions (Dia Sorin, Stillwater, MN). Corticosterone was measured using ImmuChemTM Double Antibody Radioimmunoassay (MP

Biomedicals, Orangeburg, NY). All samples were run together in a single assay run of each analyte (ACTH and corticosterone). Run along with both assays are two levels of a commercial quality control serum and two serum pools constructed in our lab. Both the commercial controls and the serum pools were within expected ranges. Additionally, the slopes, intercepts, and 20, 50, and 80% binding points were all within expected ranges. The assays were performed by the Ritchie Lab (Emory University, Atlanta GA) and run independently by two different technicians.

E. Statistical Analysis

For the baseline (saline/saline) and DEX/CRF conditions, one-tailed t-tests were used to assess the effects of virus on ACTH and corticosterone. For experiment two, two-factor ANOVA was performed for the dexamethasone-suppression test and for the CRF-stimulation test with *post-hoc* Bonferroni tests. Grubbs test for outliers were performed for all groups (TABLE 4-1C). Due to high degree of potential biological variability in these assays, outliers remain in the data set. Details of data analysis are presented in corresponding figure legends.* significant difference from LVCMVGFP Sal/Sal. † significant difference from LVCRFp3.0CRF Sal/Sal * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RESULTS

A. Experiment 1 Endocrine Analysis

Ten weeks of chronic CRF-OE within neurons of the CeA resulted in HPA-axis hyperactivity (FIGURE 4-3). In the control (saline/saline) condition, one-tailed t-tests

identified a significant increase in ACTH concentration in rats overexpressing CeA CRF ($p = 0.0188$) but no significant effect of virus on corticosterone concentration ($p = 0.1$). However, in the more sensitive DEX/CRF test, there was a significant increase in CORT ($p = 0.0301$) and ACTH ($p = 0.0367$) concentration in rats overexpressing CeA. Grubbs test identified one outlier in the corticosterone data from the LVCRFp3.0CRF Sal/Sal group (value=519.88ng/ml; z-score = 2.19; significant outlier $p < 0.01$).

B. Experiment 2 Endocrine Analysis

1. Dexamethasone-Suppression Test

Using two-factor ANOVA there was a significant main effect of injection on plasma corticosterone ($F(1, 18) = 16.49$, $p < 0.001$) and ACTH ($F(1,21) = 29.44$, $p < 0.0001$).

Post-hoc Bonferroni tests demonstrated that within the LVCMVGFP group, both plasma corticosterone ($p < 0.01$) and ACTH ($p < 0.001$) concentrations were significantly decreased by dexamethasone administration.

Based on data from human patients with MDD, we expected LVCRFp3.OCRF rats to exhibit DST non-suppression. In fact, dexamethasone administration failed to produce a significant suppression of corticosterone concentration ($p > 0.05$), although there was one significant outlier (594.60, z-score 1.7866; $p < 0.01$). However, ACTH concentration was significantly suppressed by dexamethasone ($p < 0.05$). (FIGURE 4-4 and TABLE 4-1)

2. CRF-Stimulation Test

Two-factor ANOVA identified a significant effect of injection, but not of virus, on plasma corticosterone ($F(1,21) = 14.61$, $p = 0.001$) and ACTH ($F(1,23) = 20.58$, $p < 0.001$) concentrations.

Post-hoc Bonferroni analysis revealed that within the LVCMVGFP group, exogenous administration of CRF significantly increased plasma corticosterone concentration ($p < 0.05$) but not ACTH concentration ($p > 0.05$). There was one significant outlier in the corticosterone data (812.4, z-score 1.9792, $p < 0.01$).

Based on data from human patients with MDD, we expected LVCRFp3.0CRF to have a blunted response in the CRF-stimulation test relative to the control virus. However, exogenous CRF administration stimulated corticosterone ($p < 0.05$) and ACTH ($p < 0.001$) concentrations. (FIGURE 4-5, TABLE 4-1).

3. Dex/CRF Test

It was expected that chronic CeA CRF-OE would result in HPA axis hyperactivity. However, t-tests identified no significant differences in plasma ACTH or corticosterone between the LVCMVGFP control group and rats overexpressing CeA CRF in either the Sal/Sal condition or in the more sensitive Dex/CRF test. (FIGURE 4-6, TABLE 4-1).

4. Adrenal Gland and Body Weight

Adrenal glands were collected but there were no differences in gland weight, body weight, or adrenal weight per body weight (TABLE 4-2).

DISCUSSION

Elevated plasma concentrations of ACTH and cortisol in MDD patients in the Dex/CRF test correlate with symptom severity and may predict response to treatment (Schule et al., 2006). This HPA axis hyperactivity has been attributed to CRF-OE in the hypothalamic PVN. Research in laboratory animals has shown that PVN CRF is regulated by extrahypothalamic sources of CRF such as the CeA (FIGURE 1-3, 1-4 and TABLE 1-1). Interestingly, among MDD patients, those who were DST non-suppressors exhibited higher CSF CRF concentrations than DST suppressors (Pitts et al., 1995).

While endocrine output is relatively simple to measure in human subjects, neurochemical alterations in the amygdala cannot be assessed; disruptions in PVN CRF could be secondary to overproduction and release of CRF from the CeA. The goal of this study was to demonstrate that increased CRF output from the CeA will elicit HPA axis disruptions similar to that seen in humans with MDD or in animal models of chronic stress.

A. Experiment 1

Ten weeks of chronic CeA CRF-OE increased plasma ACTH under baseline (Sal/Sal) conditions relative to LVCMVGFP control subjects, and increased both ACTH and corticosterone concentrations in the Dex/CRF test.

The lack of effect of CeA CRF-OE on corticosterone in the Sal/Sal group likely does not reflect a less-disrupted HPA axis in these CeA CRF-OE subjects compared to their cage-mates assigned to the Dex/CRF group. Rather, it supports previous research

showing that the Dex/CRF test provides a more accurate and sensitive measure of total HPA axis reactivity.

HPA axis hyperactivity in the CeA CRF-OE subjects from Experiment 1 supports the hypothesis that increased CRF drive from the CeA may be responsible for the HPA axis disturbances observed in human subjects. Via direct and indirect connections between the CeA and PVN, this elevation in extrahypothalamic CRF may overpower negative feedback, leading to chronic hyperactivity of the HPA axis. This result could have important clinical implications given that CRF regulation in the PVN is quite distinct from CRF regulation in the CeA. These implications will be discussed in more detail in Chapter 6.

B. Experiment 2

The purpose of Experiment 2 was to clarify potential confounding effects of behavioral testing on the HPA axis in Experiment 1. In Experiment 2, subjects were tested in the sucrose-preference test (SPT), but not the OF, EPM, DW, or FST. In the six-weeks of Experiment 2, rats were handled only for cage changes and were otherwise not manipulated. The endocrine analysis in Experiment 2 was also expanded to include the DST, a marker of HPA axis negative feedback, and the CRF-stimulation test, which reflects sensitivity of anterior pituitary corticotrophs to CRF.

Rats injected with LVCMVGFP exhibited significant suppression of plasma corticosterone and ACTH concentration in the DST (FIGURE 4-4) and significant

elevation of corticosterone (FIGURE 4-5) in the CRF-stimulation test in CeA CRF-OE subjects from Experiment 2.

Based on results in human patients in a depressive episode, chronic CeA CRF-OE was expected to decrease negative feedback, as measured by non-suppression in the DST, and to decrease anterior pituitary sensitivity to CRF administration, as measured by a blunted ACTH and corticosterone response to exogenous CRF in the CRF-stimulation test. Consistent with dexamethasone non-suppression, there was no significant decrease in plasma concentrations of corticosterone in the DST, although ACTH concentration was significantly suppressed by dexamethasone administration (FIGURE 4-4). However, there was also no significant difference in plasma corticosterone or ACTH compared to LVCMVGFP rats in the DST. Furthermore, rats overexpressing CeA CRF did not exhibit a blunted response in the CRF-stimulation test; rather plasma corticosterone and ACTH concentrations were significantly increased (FIGURE 4-5).

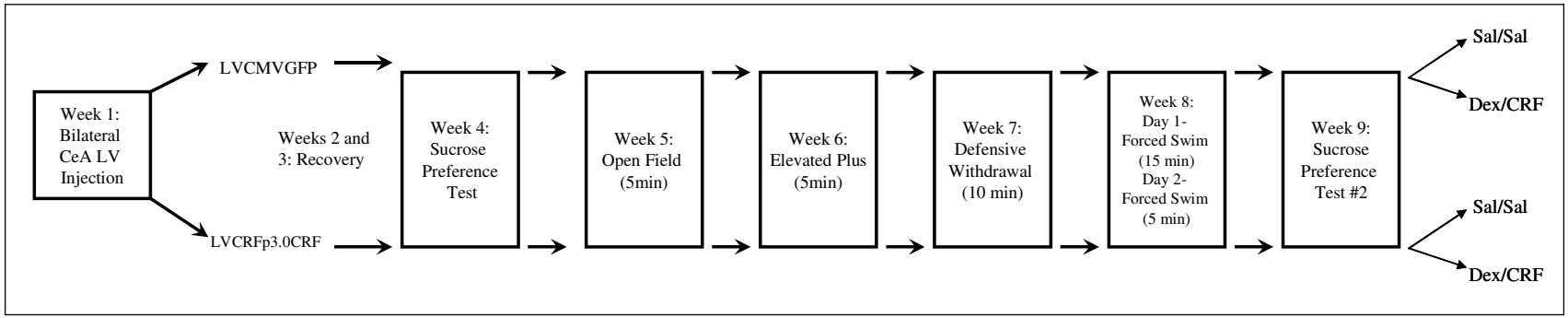
Chronic CeA CRF-OE in Experiment 2 was expected to increase plasma concentrations of corticosterone and ACTH in the Dex/CRF test. However, there were no group differences between rats injected with LVCMVGFP or LVCRFp3.0CRF. These results are inconsistent with the hypothesis that chronic CeA CRF-OE, in the absence of other behavioral stress, produces disruptions in HPA axis regulation. Overall the endocrine results from experiments 1 and 2 suggest that the influence of amygdalar CRF on the HPA axis is dependent on the environmental context and experiential history. This result contradicts previous research showing that electrical simulation of the CeA activates the HPA axis even in the absence of other behavioral stress (Feldman and Weidenfeld, 1998), and could suggest that activation of CeA neurons

other than and/or in addition to CRF-producing cells are involved in the HPA axis effects of electrical CeA stimulation.

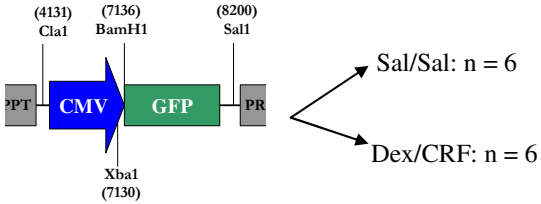
This interpretation is consistent with studies in human demonstrating a necessary interaction between genes which predispose one to psychopathology and environmental stress to precipitate psychopathology. It is also consistent with preclinical research in laboratory animals showing differential effects of particular stressors—physical vs. psychological, novel vs. familiar, acute vs. chronic, and controllable vs. uncontrollable—which correspond to differential activation of NT systems and pathways in the stress-response system (e.g. (Natelson et al., 1988; Korte et al., 1999; Singh et al., 1999; Keeney et al., 2006; Romeo et al., 2006; Christianson et al., 2008b; Christianson et al., 2008a).

.

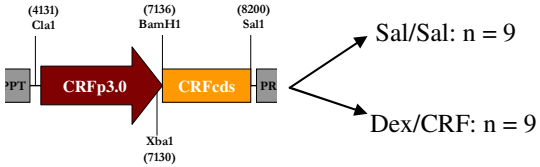
FIGURE 4-1: Experiment 1 Timeline and Experimental Design



LVCMVGFP (n = 12)



LVCRFp3.0CRF (n = 18)

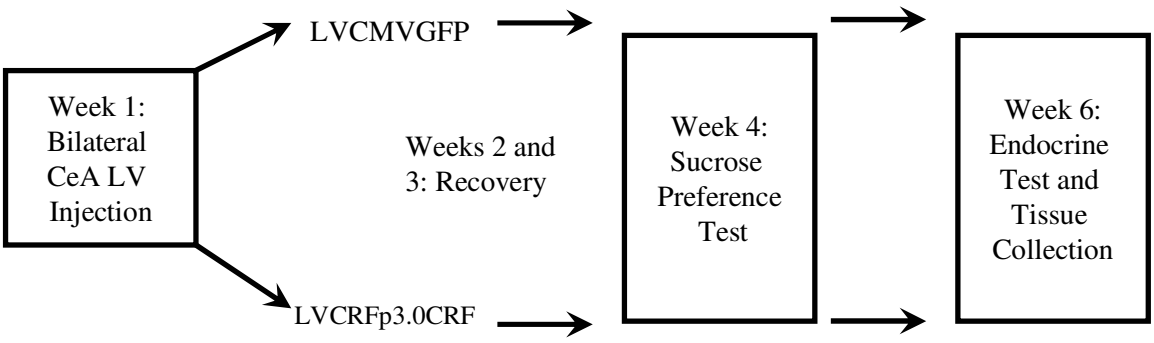


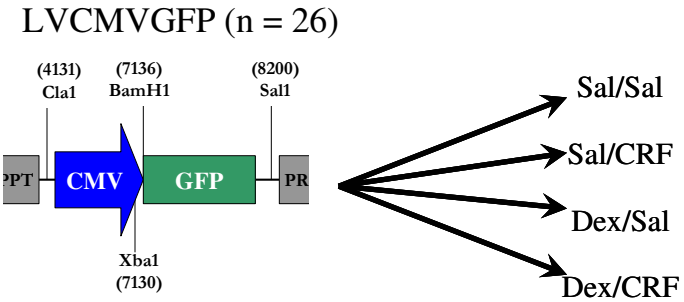
After histological verification of injection placement

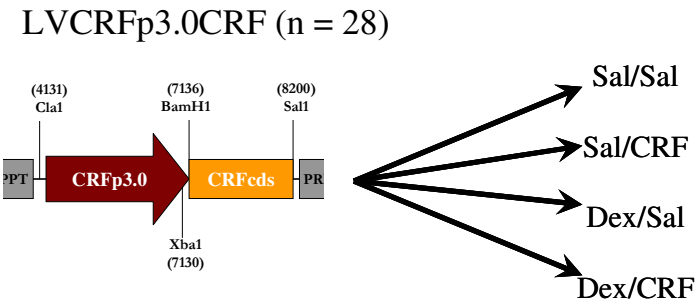
Sal/Sal: n = 7

Sal/Sal: n = 5

FIGURE 4-2: Experiment 2 Timeline and Experimental Design







1 rat in the LVCMVGFP group did not survive surgery; 3 rats from the LVCRFp3.0CRF group were eliminated after histological verification of injection placement

FIGURE 4-3: Dex/CRF Test in Experiment 1

Rats overexpressing CeA CRF exhibit HPA axis hyperactivity. In the control (saline/saline) condition, one-tailed t-tests identified a significant increase in ACTH concentration in rats overexpressing CeA CRF ($p = 0.0188$) but no significant effect of virus on corticosterone concentration ($p = 0.1$). However, in the more sensitive DEX/CRF test, there was a significant increase in CORT ($p = 0.0301$) and ACTH ($p = 0.0367$) concentration in rats overexpressing CeA. Grubbs test identified one outlier in the corticosterone data from the LVCRFp3.0CRF Sal/Sal group (value=519.88ng/ml; z-score = 2.19; significant outlier $p < 0.01$)

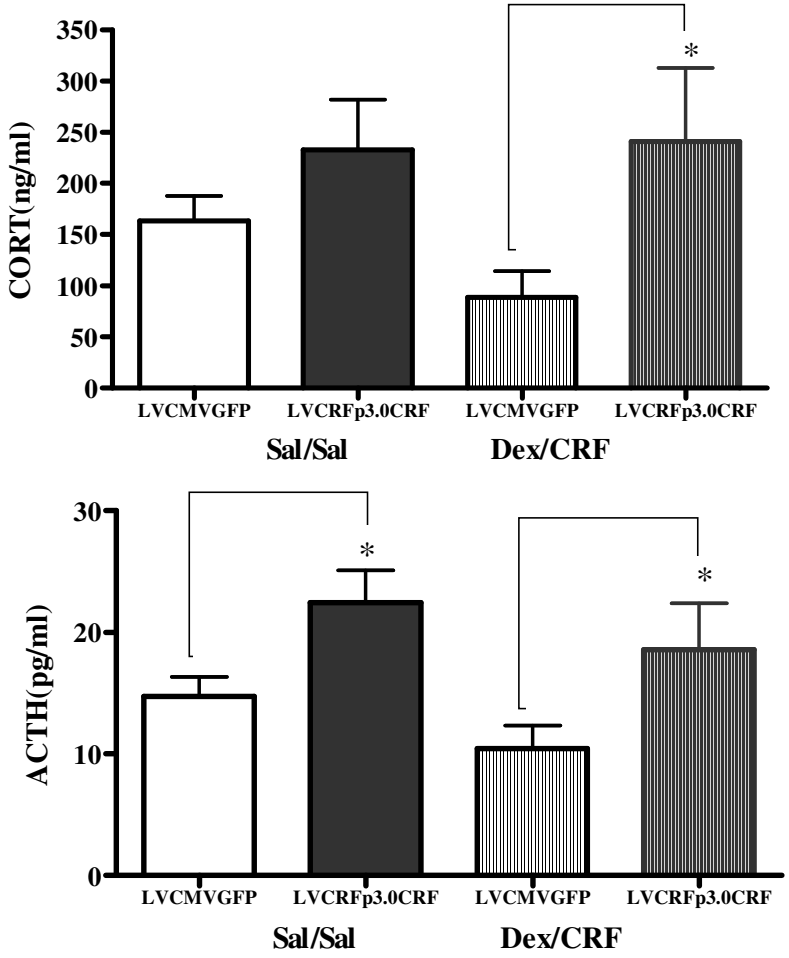
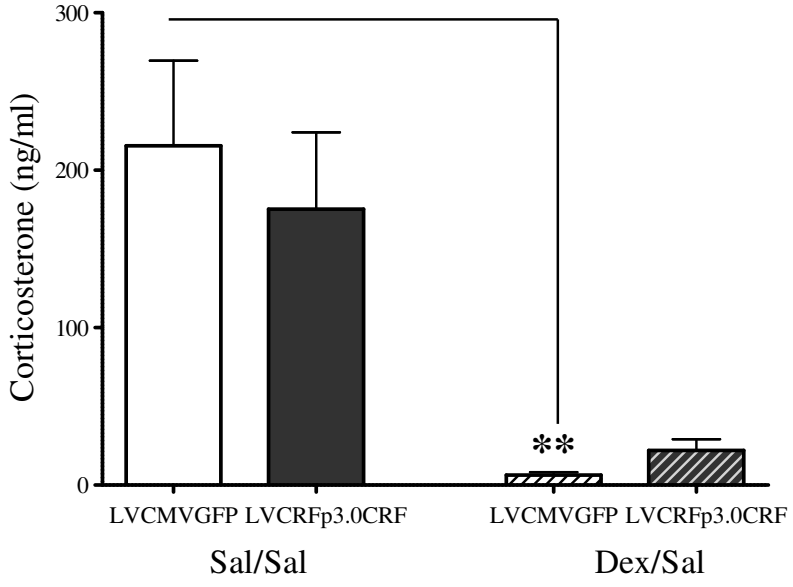


FIGURE 4-4: Dex-Suppression Test in Experiment 2

Using two-factor ANOVA there was a significant main effect of injection on plasma corticosterone ($F(1, 18) = 16.49$, $p < 0.001$) and ACTH ($F(1,21) = 29.44$, $p < 0.0001$). *Post-hoc* Bonferroni tests demonstrated that within the LVCMVGFP group, both plasma corticosterone ($p < 0.01$) and ACTH ($p < 0.001$) concentrations were significantly decreased. In rats overexpressing CeA CRF, dexamethasone administration failed significantly suppress corticosterone concentration ($p > 0.05$), although there was one significant outlier (594.60, z-score 1.7866; $p < 0.01$). ACTH concentration was significantly suppressed by dexamethasone ($p < 0.05$).

* = significant difference from LVCMVGFP Sal/Sal; $p < 0.05$; ** $p < 0.01$
† = significant difference from LVCRFp3.0CRF Sal/Sal; $p < 0.05$; ††, $p < 0.01$; ††† $p < 0.001$



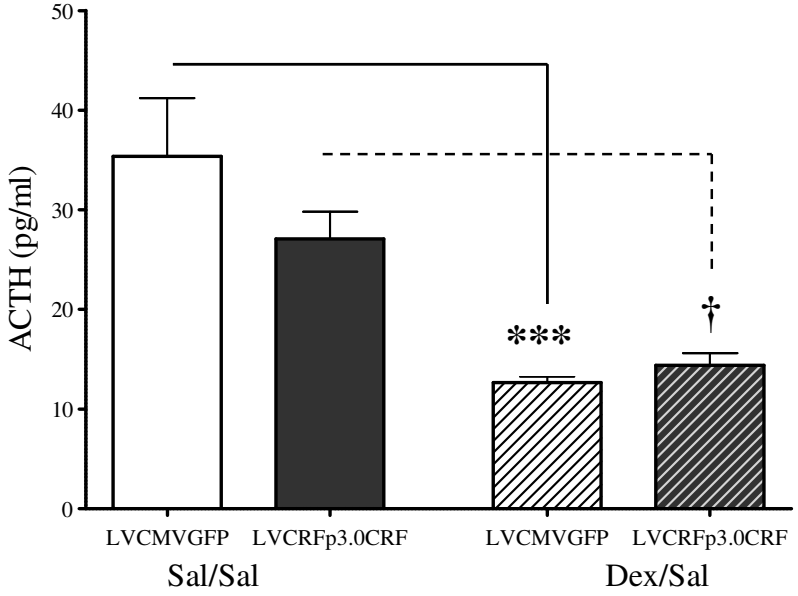


FIGURE 4-5: CRF-Stimulation Test in Experiment 2

Two-factor ANOVA identified a significant effect of injection, but not of virus, on plasma corticosterone ($F(1,21) = 14.61$, $p = 0.001$) and ACTH ($F(1,23) = 20.58$, $p < 0.001$) concentrations. *Post-hoc* Bonferroni analysis revealed that within the LVCMVGFP group, exogenous administration of CRF significantly increased plasma corticosterone concentration ($p < 0.05$) but not ACTH concentration ($p > 0.05$). There was one significant outlier in the corticosterone data (812.4, z-score 1.9792, $p < 0.01$). In rats chronically overexpressing CeA CRF, exogenous CRF administration stimulated corticosterone ($p < 0.05$) and ACTH ($p < 0.001$) concentrations.

* = significant difference from LVCMVGFP Sal/Sal; $p < 0.05$; ** $p < 0.01$
† = significant difference from LVCRFp3.0CRF Sal/Sal; $p < 0.05$; ††, $p < 0.01$; ††† $p < 0.001$

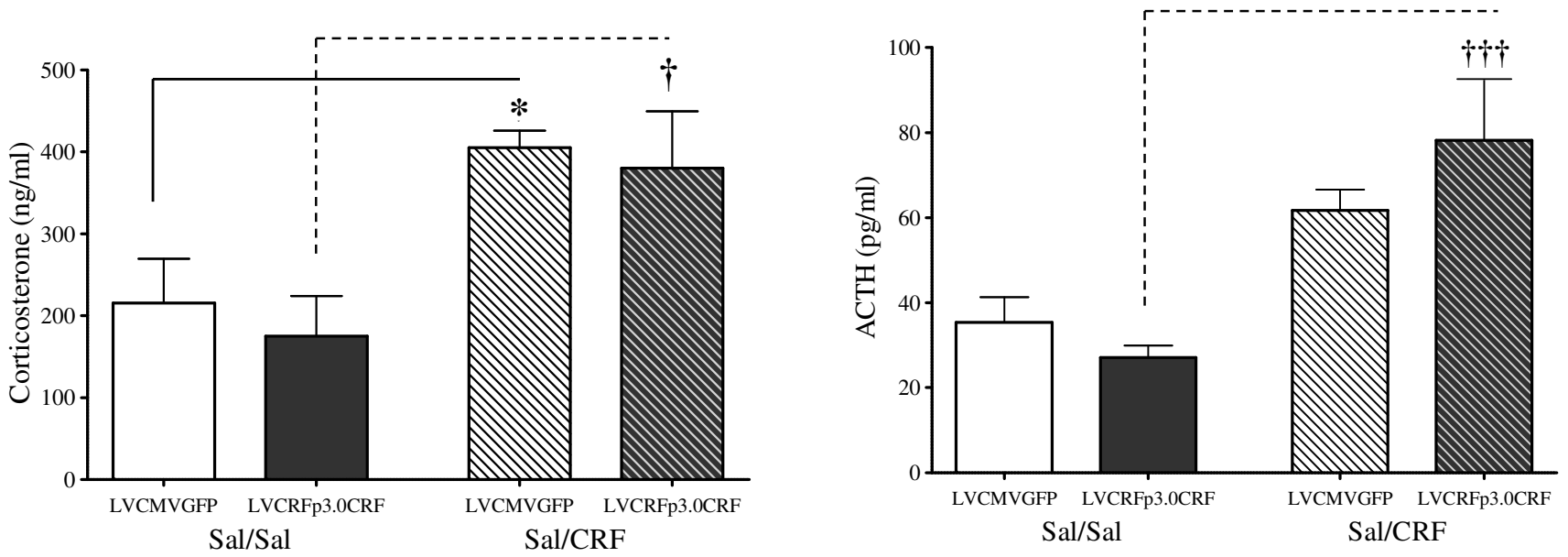


FIGURE 4-6: Dex/CRF Test in Experiment 2

T-tests identified no significant differences in plasma ACTH or corticosterone between the LVCMVGFP control group and rats overexpressing CeA CRF in either the Sal/Sal condition or in the more sensitive Dex/CRF test.

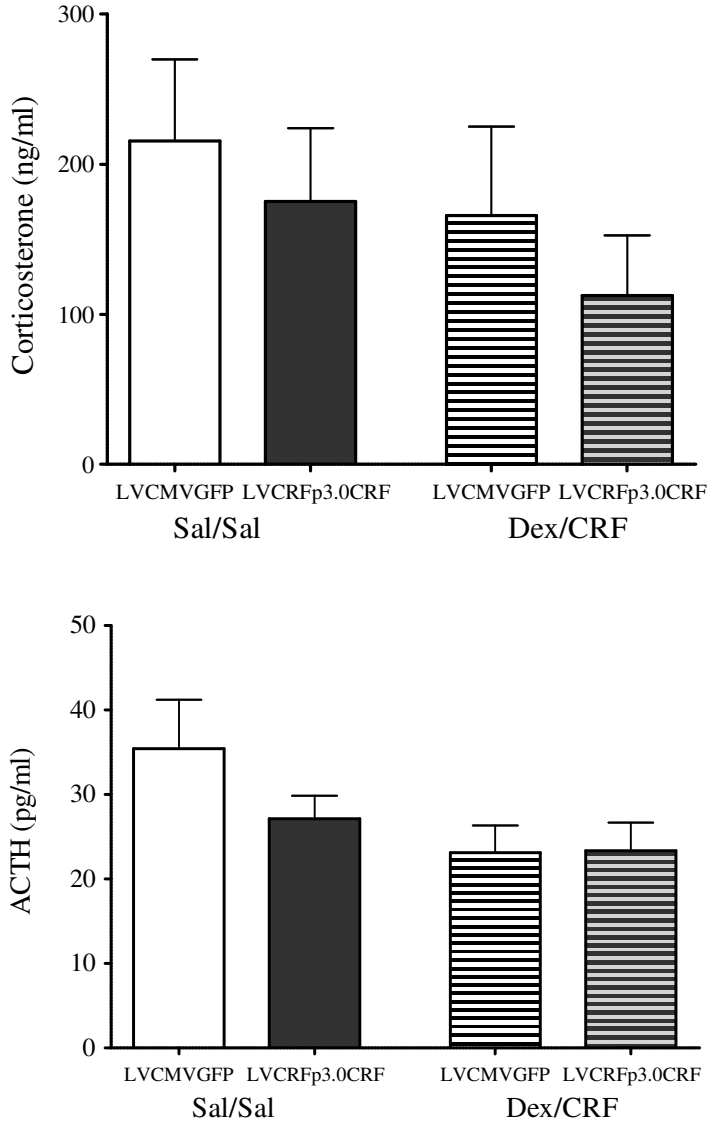


TABLE 4-1: Experiment 2 Additional Endocrine Results and Outliers

TABLE 4-1A CORT (ng/ml)

	LVCMVGFP	**LVCRFp3.0CRF**
Sal/Sal	215.43 +/- 54.26	175.17 +/- 48.75 p > 0.05
Dex/Sal	26.7 +/- 15.78	130.60 +/- 98.07 p > 0.05
Sal/CRF	472.92 +/- 64.83	530.65 +/- 174.21 p > 0.05
Dex/CRF	172.98 +/- 57.58	103.72 +/- 40.12 p > 0.05

TABLE 4-1B ACTH (pg/ml)

	LVCMVGFP	**LVCRFp3.0CRF**
Sal/Sal	35.4 +/- 5.82	27.09 +/- 2.76 p > 0.05
Dex/Sal	13.58 +/- 0.85	13.47 +/- 1.22 p > 0.05
Sal/CRF	61.67 +/- 4.89	78.16 +/- 14.55 p > 0.05
Dex/CRF	22.67 +/- 3.34	23.84 +/- 3.12 p > 0.05

TABLE 4-1C Outliers (Grubb's test for outliers)

Corticosterone (ng/ml)	**LVCMVGFP**	**LVCRFp3.0CRF**
Dex/Sal	Value = 99.80 * z-score = 2.0313 p < 0.01	Value = 594.60 * z-score = 1.7866 p < 0.01
Sal/CRF	Value = 812.40 z-score = 1.9792 p < 0.01	1432.70 z-score = 2.1139 p < 0.05

TABLE 4-2: Experiment 2 Adrenal Gland and Body Weight

	LVCMVGFP	**LVCRFp3.0CRF**
Adrenal Weight (g)	0.0484 +/- 0.0026	0.0493 +/- 0.0024 $p > 0.05$
Body Weight (g)	427 +/- 0.0056	426 +/- 0.0067 $p > 0.05$
Adrenal (g) per Body Weight (Kg)	0.1131 +/- 0.0058	0.1166 +/- 0.0061 $p > 0.05$

CHAPTER 5:

Chronic CeA CRF-OE Alters Expression of Genes Involved in HPA Axis Regulation

INTRODUCTION

Disproportionate HPA axis reactivity in MDD patients may result from alterations in the expression of genes whose products are responsible for HPA axis activation and feedback. Long-term gene expression changes may take place primarily in the PVN or may take place primarily in limbic structures, which then modulate the PVN. This question is of particular clinical relevance because PVN and CeA CRF cells are differentially regulated. GR activation in CRF neurons in the PVNmp directly inhibits CRF expression via binding to partial GRE in the CRF promoter (Herman et al., 1992; Malkoski and Dorin, 1999). However, it appears that the PVNmp is the only brain region in which GR has this effect on CRF transcription; in the CeA and BNST, GR activation actually increases CRF transcript, and in other CRF-producing regions, it has no effect (Swanson and Simmons, 1989; Makino et al., 1994b, a; Schulkin et al., 1998; Shepard et al., 2000; King et al., 2002; Shepard et al., 2003; Shepard et al., 2006).

GR activation in the hippocampus indirectly leads to HPA axis inhibition by simulating hippocampal glutamatergic projections to the PVN that activate inhibitory interneurons surrounding the PVNmp or by stimulating hippocampal GABAergic projections to the PVN that inhibit glutamatergic neurons in contact with CRF-producing

cells in the PVN. The hippocampus also provides tonic inhibition to regulate the overall tone of the HPA axis under baseline conditions; this tonic inhibition is mediated by MR, which are densely expressed in the hippocampus and up to 80% occupied at baseline (reviewed in (Reul et al., 2000b; Nicholson et al., 2004; King and Nicholson, 2007; Yao and Denver, 2007).

The effectiveness of the inhibitory transmission from the hippocampus to the PVN may rely on BDNF. Stress or GC-toxicity decrease hippocampal BDNF, thereby also diminishing the ability of the hippocampus to regulate the HPA axis, resulting in still increased GC-mediated toxicity (Barbany and Persson, 1992; Chao and McEwen, 1994; Schaaf et al., 1998). Antidepressant drugs increase BDNF in the hippocampus, ensuring reliable tonic inhibition of the PVN (Givalois et al., 2004).

There has been much debate as to whether HPA axis disruption is instigated by GR-insensitivity (the corticosteroid hypothesis of depression), decreased hippocampal integrity secondary to deficient BDNF (the neuroproliferation hypothesis), increased CRF in the PVNmp, or increased CRF in extrahypothalamic regions. It is my hypothesis that increased activity in extrahypothalamic CRF is responsible for initiating the sequence of events resulting in hippocampal and hypothalamic gene expression changes, followed by the HPA axis hyperactivity.

We have previously shown that a lentiviral vector using a 3.0Kb portion of the CRF promoter is able to target transgene expression to CRFergic cells (Chapter 2) and that a virus using this promoter to drive expression of CRF (LVCRFp3.0CRF), when injected into the CeA, increases anxiety-like behavior (Experiment 1, Chapter 3) and induces HPA-axis hyperactivity measured in the Dex-CRF test (Experiment 1, Chapter

4). Assessing regional changes in gene expression will help to clarify the sequence of events through which chronic CeA CRF-OE influences positive- and negative-feedback mechanisms of the HPA axis.

MATERIALS and METHODS

A. Animal Subjects

Gene expression analysis was performed in the rats previously tested for anxiety and depressive-like behavior (Experiments 1 and 2 in Chapters 3 and 4). The LVCRFp3.0CRF vector is shown in FIGURE 3-1 and the timeline and study design is shown in FIGURE 5-1.

B. Histological Processing

Brains were fresh frozen on dry ice, stored at -80^{o}C and sectioned at 20μm thickness on a Cryostat at -20ºC onto SuperFrost plus slides.

1. Riboprobe In situ Hybridization

In situ hybridization for CRF, BDNF, MR, and GR were performed using ^{35}S-UTP labeled riboprobes as described in Chapter 2. Plasmids were linearized and transcribed with the appropriate RNA polymerase to generate ^{35}S-UTP labeled riboprobes. For antisense probes, CRF was linearized with *PvuII* and transcribed with SP6, BDNF was linearized with *Not1* and transcribed with T7, MR was linearized with *EcoRI* and transcribed with SP6, and GR was linearized with *BamHI* and transcribed with T7. To optimize the riboprobe labeling procedures, antisense hybridization was compared with

sense-strand hybridization (data not shown). For MR, sense strand RNA was created by digesting with *HindIII* and transcribing with T7. For GR, sense strand RNA was created by linearization with *Xba1* and transcription with SP6. Sense strand labeling was comparable to non hybridized tissue; for the remaining assays, background regions within a section were used as the control comparison. Preparation, hybridization and washing of slides were performed as described in Chapter 2.

2. Oligo Probe In Situ *Hybridization*

The protocol for AVP oligo *in situ* hybridization was adapted from (Nishimori et al., 1996). The sections were fixed in 4% paraformaldehyde (pH 7.4) and rinsed for 5min in 1x PBS. Next, slides were dipped in H_2O, and then immersed for 10min in 0.1 mM triethanolamine containing 0.25% acetic anhydride, and incubated in 2x SSC for 3min followed by dehydration with increasing concentrations of EtOH. After the 100% EtOH incubation, slides were immersed for 5min in chloroform, immediately returned to 100% EtOH for 3min and then allowed to dry. Once dry, slides were coated with prehybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1x Denhardt's solution, 10mM DTT, and 1.0 mg/ml tRNA), coverslipped with parafilm, and incubated for 1hr at 37^{o}C. After prehybridization, slides were rinsed twice in 2x SSC for 5min each and again dehydrated in increasing concentrations of ethanol.

The AVP oligo probe sequence (CCTAAGCAGCAGCTCCCGGGCTGGCCCG-TCCAGC-TGCTGGGCGTTGCT) corresponds to 48bp complementary to the rat mRNA encoding amino acids 129-144 of the AVP precursor peptide. AVP oligo probes were labeled with ^{35}S-dATP using terminal deoxynucleotide transferase (TdT- Promega,

Madison, WI) to a specific activity of 1x 10^6/pmol and applied to the prepared tissue at a concentration of 4.3 pmol/ml in hybridization solution containing 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 1x Denhardt's solution, 10mM DTT, and 0.5 mg/ml tRNA.

Slides were hybridized in humidified chambers overnight at 37^{o}C. The following day, unhybridized probe was removed by washing slides three times in 1x SSC for 15min each at 60^{o}C followed by a fourth wash in 1x SSC at room temperature with rotation. Slides were dehydrated in increasing concentrations of EtOH and allowed to dry completely before exposure to Kodak Biomax MR film for at least 24 hours to obtain images for quantification (Nishimori et al., 1996).

3. Image analysis

Images from the *in situ* hybridization and receptor autoradiography films were digitized with a Dage-MTI CCD-72 (Michigan City, IN) image analysis system equipped with a Nikon camera as previously described (Skelton et al., 2000). Semiquantitative analysis was performed using Scion Image (version 3.0b) software. Optical densities were calibrated against ^{14}C-standards and expressed in terms of nCi/g of tissue equivalent. For the purpose of quantifying mRNA levels, specific signal density was determined relative to neutral background density present in the same brain section. In all cases, two to four sections per region were matched for rostrocaudal level according to the atlas of Paxinos and Watson (Paxinos et al., 1980) and used to produce a single value for each animal.

4. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). The unpaired one- or two-tailed student T-test or analysis of variance (ANOVA) was used where appropriate. Specific methods are addressed in figure captions. The Grubbs test for outliers was run on all data sets. Due to the potential for a high degree in biological variability in gene expression, outliers were not removed from the data sets but are shown in (TABLE 5-3). Significance is indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

RESULTS

FIGURE 5-1 compares the design of Experiment 1 and Experiment 2. For both Experiment 1 and Experiment 2, LVCRFp3.0CRF-mediated CeA CRF-OE was verified with *in situ* hybridization. Six animals were eliminated from the LVCRFp3.0CRF group in Experiment 1 and 3 were eliminated from Experiment 2. One LVCMVGFP subject was also eliminated in Experiment 2. The final n per virus was 12 for Experiment 1 and 25 for Experiment 2.

A. Experiment 1 Gene Expression

LVCRFp3.0CRF increases CeA CRF expression (180.15 +/-16.01 nCi/g vs. 255.75 +/-13.59 nCi/g; $p < 0.001$ in a one-tailed T-test). Histological analysis verified injection placement in 12 of the 18 rats injected with LVCRFp3.0CRF. (FIGURE 5-2). As hypothesized, CRF transcript in the PVN was significantly elevated in CeA CRF-OE

subjects (476.15 +/- 55.46 nCi/g in LVCMVGFP vs. 809.27 +/- 79.01 nCi/g in LVCRFp3.0CRF subjects; $p < 0.01$ in a one-tailed T-test.) (FIGURE 5-3). AVP transcript was also increased in the PVN of CeA CRF-OE rats (18.89 +/- 2.62 nCi/g in control rats vs. 24.34 +/- 1.6 in CeA CRF-OE subjects; $p < 0.05$ in a one-tailed T-test) (FIGURE 5-4).

In contrast to expected results, GR transcript was not altered in the hippocampal CA fields or DG of CeA CRF-OE rats (FIGURE 5-5; TABLE 5-1). However, results of two-tailed T-tests demonstrated a significant decrease in MR transcript in hippocampal CA1/2 (86.13 +/- 3.81 nCi/g in control subjects vs. 66.33 +/- 5.6 in LVCRFp3.0CRF rats; $p < 0.001$), CA3 (79.95 +/-3.44 nCi/g in LVCMVGFP group vs. 66.08 +/- 5.95 in the LVCRFp3.0CRF group; $p < 0.01$) and the DG (94.37 +/- 4.63 nCi/g in control rats vs. 69.02 +/- 4.9 nCi/g in CeA CRF-OE subjects; $p < 0.001$) (FIGURE 5-6).

Expression of the neurotrophic factor BDNF was expected to be decreased in the hippocampus of CeA CRF-OE subjects and increased in the PVN of rats overexpressing CeA CRF. Results of one-tailed T-tests identified no significant differences in BDNF transcript in either region. However, there was a trend towards an increase in BDNF in the PVN (38.73 +/-6.0 nCi/g in control subjects vs. 51.22 +/- 12.92 in CRF-OE rats; $p = 0.07$) and a trend towards decreased BDNF expression in the hippocampal CA3 field (241.97 +/- 22.58 nCi/g in control rats vs. 183.97 +/- 24.4 in CRF-OE subjects; $p = 0.06$) (TABLE 5-1).

B. Experiment 2 Gene Expression

Six weeks after lentiviral vector injection surgery, LVCRFp3.0CRF produced a substantial increase in CRF transcript in the CeA (302.24 +/- 22.12 nCi/g in control LVCMVGFP subjects vs. 1109.36 +/- 65.06 nCi/g in CeA CRF-OE subjects; $p < 0.0001$) (FIGURE 5-7). CRF transcript was also increased in the PVN of CeA CRF-OE rats (422.74 +/- 41.75 in control subjects vs. 545.09 +/- 52.96 in CeA CRF rats, $p < 0.05$) (FIGURE 5-8).

Unlike the previous experiment, there were very few significant changes in gene-expression. There was a slight trend towards an increase in AVP expression (356.41 +/- 26.78 vs. 422.62 +/- 36.98 in LVCRFp3.0CRF subjects, $p = 0.08$; FIGURE 5-9). There were also no differences in hippocampal MR or GR expression (TABLE 5-2). BDNF transcript was not altered in the hippocampus.

DISCUSSION

Mood and anxiety disorders are characterized by a variety of neuroendocrine, NT, and neuroanatomical disruptions; identifying the most functionally relevant is no easy task, particularly because brain regions and NT systems implicated in mood and anxiety disorders have wide-ranging functions. A myriad of studies have scrutinized classical NT systems, NPs, and neuroproliferative factors in experimental animal models and in patients with psychiatric disorders. The combined results of these analyses reveal a complex interaction between neurochemistry and emotional and behavioral output. One

consensus is that CRF is overexpressed from hypothalamic and extrahypothalamic sources.

Final PVN output is determined by summation of signals from limbic and brainstem sources. Increased CRF from the CeA may activate multi-synaptic pathways which excite PVNmp CRF cells and also lead to decreased hippocampal BDNF and GR, indirectly disinhibiting the PVN. The present experiments examined gene expression of HPA axis-regulatory genes including GR, MR, BDNF, AVP, and CRF.

A. Experiment 1 Gene Expression

Chronic CeA CRF-OE in Experiment 1 resulted in increased expression of both CRF and AVP transcripts in the PVN (FIGURE 5-3 and 5-4). These two peptides synergistically activate the HPA axis and the increase in their expression is consistent with the HPA axis hyperactivity also seen in Experiment 1 CeA CRF-OE subjects. Although there are few direct connections between the CeA and PVN, there are numerous indirect connectionss. For example:

1. CRFergic cells in the lateral division of the CeA, localized to inhibitory interneurons, project to the medial subdivision of the CeA, which provides tonic inhibition to the PVN, thus disinhibiting the HPA axis (Crane et al., 2003)
2. CeA glutamatergic connections to the LS also contain CRF, which has a net inhibitory effect on glutamate release to the LS. Less glutamate to the lateral septum (LS), decreases activity in LS neurons which inhibit the PVNmp (Gallagher et al., 2008)

3. CRF is also colocalized with glutamate in CeA projections to the BNST which synapse on glutamatergic or GABAergic PVN-projecting BNST neurons.
4. Similarly, CRF/glutamate neurons in the CeA project to noradrenergic cells in the LC which then project to the PVN and activate the HPA axis (Reyes et al., 2005)

The hippocampus contributes to HPA axis negative feedback regulation via activation of GR. In the present study, GR expression following ten weeks of CeA CRF-OE did not differ from LVCMVGFP subjects (FIGURE 5-4, TABLE 5-1). This lack of effect was unexpected given that chronic elevations in GC are associated with GR insensitivity and chronic stress has been shown to decrease (Makino et al., 1994b; Herman et al., 1995; Sterlemann et al., 2008) or even increase hippocampal GR transcript (Murakami et al., 2005). However, other studies have also observed a lack of effect on GR mRNA (Herman and Spencer, 1998). A glucocorticoid receptor binding assay may reveal a non-genomic effect of chronic CeA CRF on GR expression and sensitivity.

The hippocampus also regulates the HPA axis with tonic inhibitory connections. MR signaling in the hippocampus has a stimulatory effect on glutamatergic projections to GABAergic neurons in the peri-PVN region or other hypothalamic nuclei which project directly to CRF cells in the PVNmp (reviewed in (Reul et al., 2000a; Reul et al., 2000b; Reul and Holsboer, 2002).

While previous work has demonstrated an anxiogenic role for hippocampal MR activation in laboratory rodents (e.g. (Smythe et al., 1997; Bitran et al., 1998) and a depressive effect of MR agonists in human MDD patients (Young et al., 2003), expression of MR transcript was significantly decreased in the current study (FIGURE 5-

5). Studies examining the effects of chronic stress or chronic increases in circulating glucocorticoids have also observed decreased MR transcript (Sterlemann et al., 2008).

Hippocampal MR mRNA is negatively correlated with circulating GC concentrations (Hugin-Flores et al., 2004) and is decreased by exogenous CRF administration (Hugin-Flores et al., 2003); two potential mechanisms through which CeA CRF-OE causes the observed downregulation of MR transcript.

Furthermore, decreased MR transcript, which presumably corresponds to a decrease in functional MR receptors, could result in HPA axis disinhibition, contributing to the hyperactivity observed in the Dex/CRF test.

Previous research has identified a region-specific effect of BDNF transcript on the HPA axis. Hippocampal BDNF contributes to the negative regulation of the HPA axis, but in the PVN, BDNF transcript is *elevated* in response to stress. This elevation occurs in advance of increases in CRF and AVP transcript, potentiating rather than inhibiting HPA axis activity (Smith et al., 1995a; Givalois et al., 2004). Based on these data, expression of BDNF was expected to be decreased in the hippocampus and increased in the hypothalamus of CeA CRF-OE subjects (Smith et al., 1995a; Schaaf et al., 1998; Givalois et al., 2004; Murakami et al., 2005). Although BDNF transcript was not significantly altered by CeA CRF-OE, there was a trend towards decreased hippocampal BDNF and increased hypothalamic BDNF. Despite the relatively large number of subjects per group, the power was less than 0.80 and the statistical insignificance may reflect type-2 error (false negative) rather than an actual lack of biological effect on BDNF transcript.

B. Experiment 2 Gene Expression

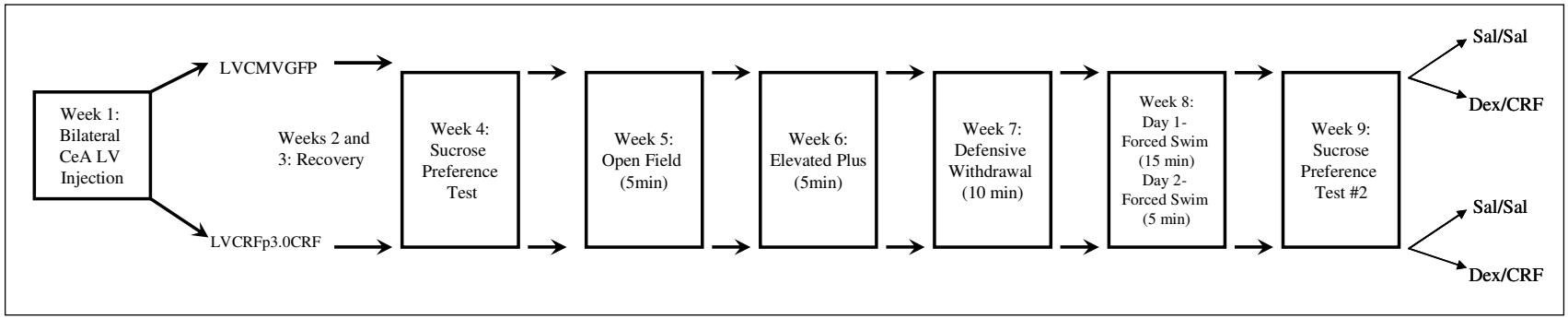
As mentioned previously, the goal of Experiment 2 was to provide a more comprehensive analysis of endocrine changes following chronic CeA CRF-OE in the absence of stressful behavioral testing. However, no significant HPA axis changes were observed in Experiment 2 CeA CRF-OE subjects as compared to subjects injected with the control (LVCMVGFP) virus.

In Experiment 2, overexpression of CRF was verified within the CeA and, as with Experiment 1, CeA CRF-OE did significantly increase CRF in the PVN, although to a lesser degree than that observed in Experiment 1. However, there was no significant difference in PVN AVP. There were also no differences in GR, MR, or BDNF expression in the hippocampus of CeA CRF-OE rats in Experiment 2. These negative data are consistent with the lack of HPA axis disruption observed in these subjects.

Results from Experiment 2 gene expression analyses could suggest that the exposure of Experiment 1 rats to behavioral testing was a necessary factor in downstream gene-expression and HPA-axis changes. Although PVN CRF transcript was elevated in CeA CRF-OE subjects, it is possible that this elevation was insufficient to overcome negative feedback mechanisms. In contrast, HPA axis negative feedback mechanisms are unable to overcome synergistic HPA axis activation elicited by elevated AVP and CRF in the PVN combined with decreased HPA axis inhibition due to decreased hippocampal MR receptors.

FIGURE 5-1: Timeline and Experimental Design for Experiment 1 and 2

(A) Experiment 1



(B) Experiment 2

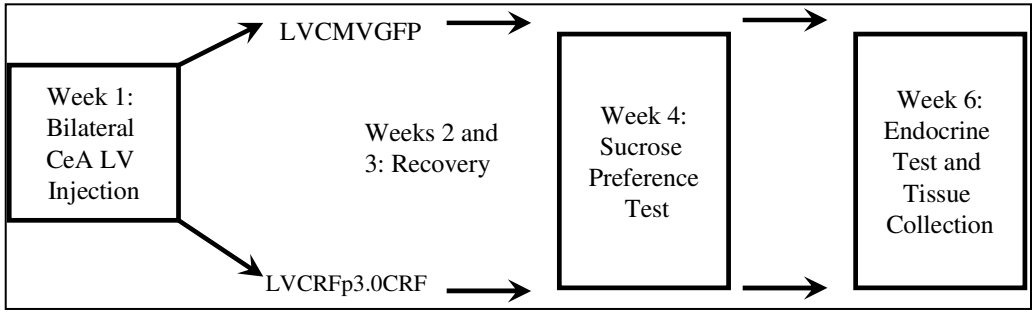


FIGURE 5-2: CeA CRF Transcript in Experiment 1 Subjects
Elevated CRF transcript (nCi/g) in rats overexpressing CeA CRF **(A)** Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **(B)** Representative example of lentiviral-vector mediated CRF-OE in the CeA in this study.

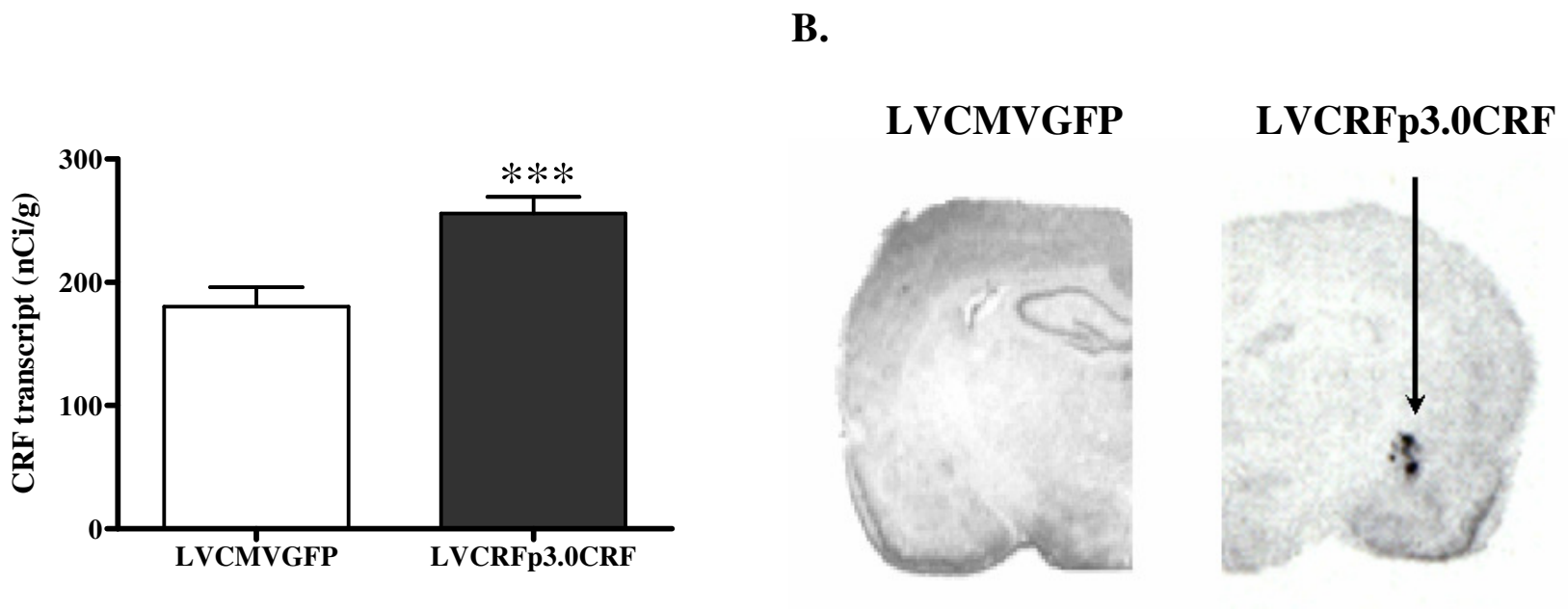


FIGURE 5-3: PVN CRF Transcript in Experiment 1 Subjects
(A) Elevated CRF transcript (nCi/g) in the PVN of rats overexpressing CeA CRF. Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis based on the a-priori hypothesis that increased CeA CRF expression would increase hypothalamic CRF expression (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **(B)** Representative *in situ* hybridization of CRF transcript in the PVN.

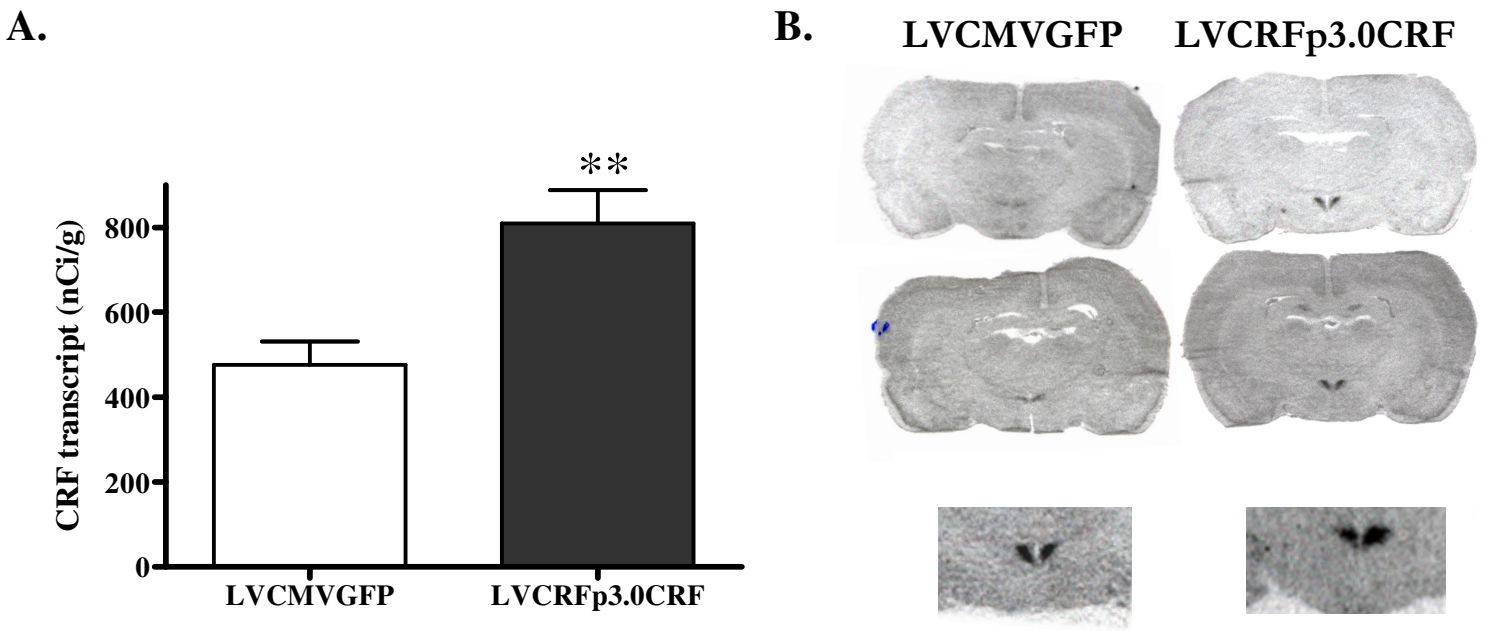


FIGURE 5-4: PVN AVP Transcript in Experiment 1 Subjects
Chronic overexpression of CeA CRF increases expression of AVP transcript (nCi/g) in the hypothalamic paraventricular nucleus. **(A)** Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis based on the a-priori hypothesis that increased CeA CRF expression would increase expression of AVP in the PVN (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **(B)** Representative example of oligo *in situ* hybridization for AVP.

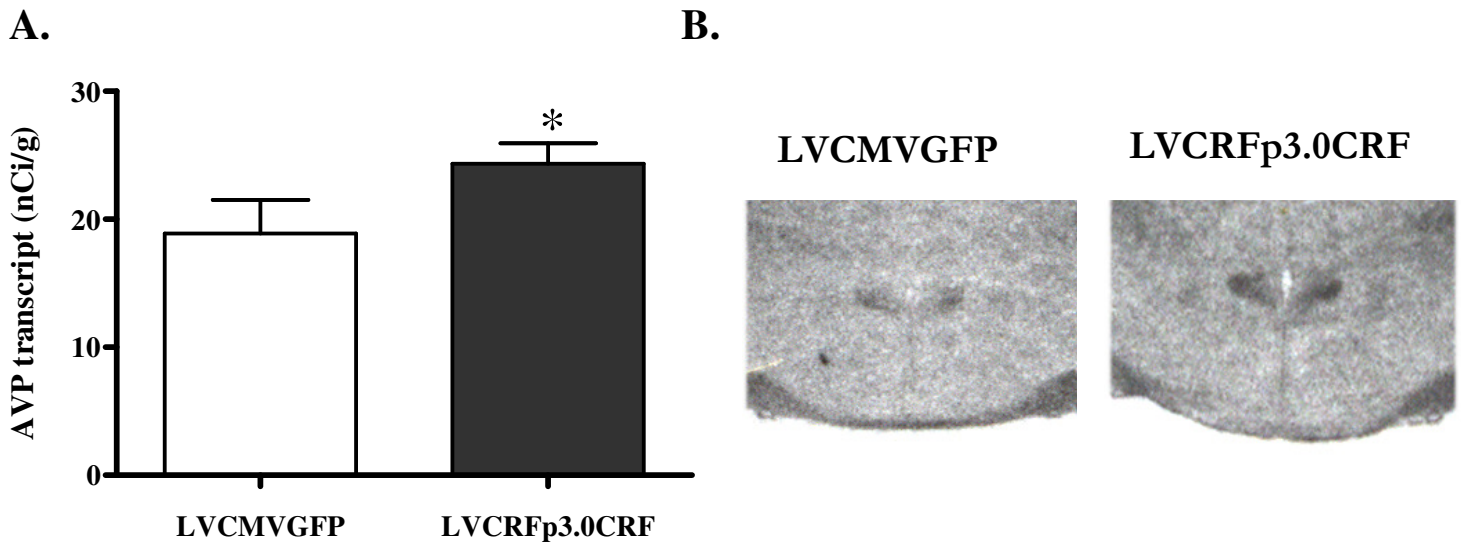
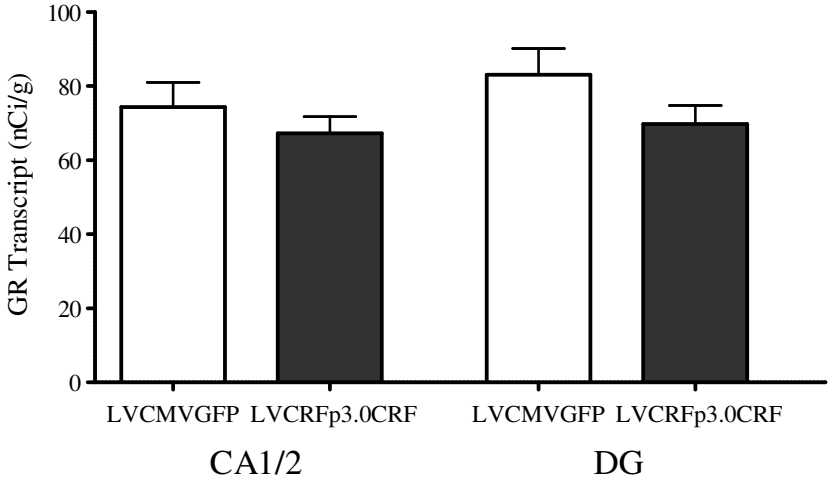


FIGURE 5-5: Hippocampal GR Transcript in Experiment 1 Subjects
Hippocampal glucocorticoid receptor mRNA is unaltered after chronic CeA CRF overexpression as shown with riboprobe *in situ* hybridization



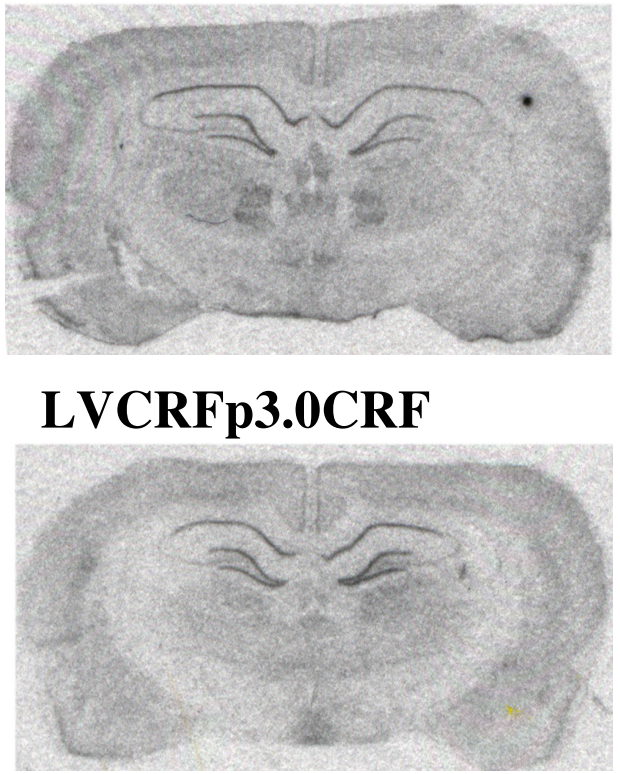


FIGURE 5-6: Hippocampal MR Transcript in Experiment 1 Subjects Mineralocorticoid transcript expression (nCi/g) is decreased in the hippocampus of rats overexpressing CeA CRF **(A)** Data are displayed as mean +/- SEM; p-values reflect results of two-tailed T-test analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **(B)** Representative example of riboprobe *in situ* hybridization for MR transcript.

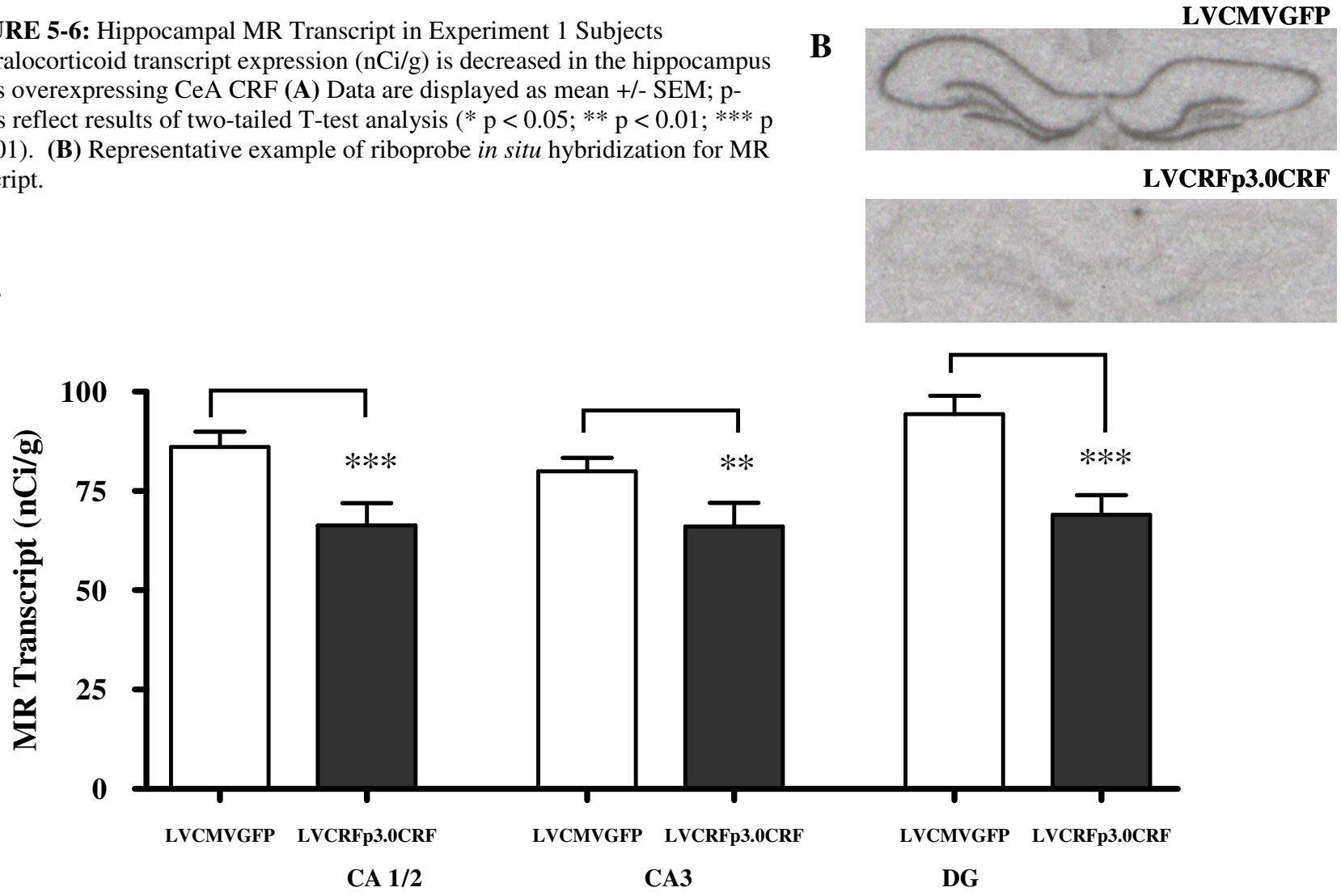


TABLE 5-1: Experiment 1 Additional *In Situ* Hybridization Data

TABLE 5-1A: CRF transcript in the hippocampus

	LVCMVGFP	LVCRFp3.0CRF
CA3	207.38 +/- 34.43	168.48 +/- 22.05 p > 0.05
DG	186.2 +/- 19.62	142.51 +/- 20.98 p > 0.05

TABLE 5-1B: GR transcript in the hippocampus

	LVCMVGFP	LVCRFp3.0CRF
CA 1/2	74.32 +/- 6.62	67.23 +/- 4.41 p > 0.05
DG	81.24 +/- 7.3	69.68 +/- 5.11 p > 0.05

TABLE 5-1C: BDNF transcript in the hypothalamus and hippocampus

	LVCMVGFP	LVCRFp3.0CRF
PVN	38.73 +/- 6.0	61.22 +/- 12.92 p = 0.07
Hippocampus		
CA1/2	80.56 +/- 10.73	75.17 +/- 11.95 p > 0.05
CA3	241.97 +/- 22.58	183.97 +/- 24.40 p = 0.06
DG	209.58 +/- 13.5	160.86 +/- 26.87 p = 0.08

FIGURE 5-7: CeA CRF Transcript in Experiment 2 Subjects
Elevated CRF transcript (nCi/g) in rats overexpressing CeA CRF **(A)** Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis (*** $p < 0.001$). **(B)** Representative example of lentiviral-vector mediated CRF-OE in the CeA in this study.

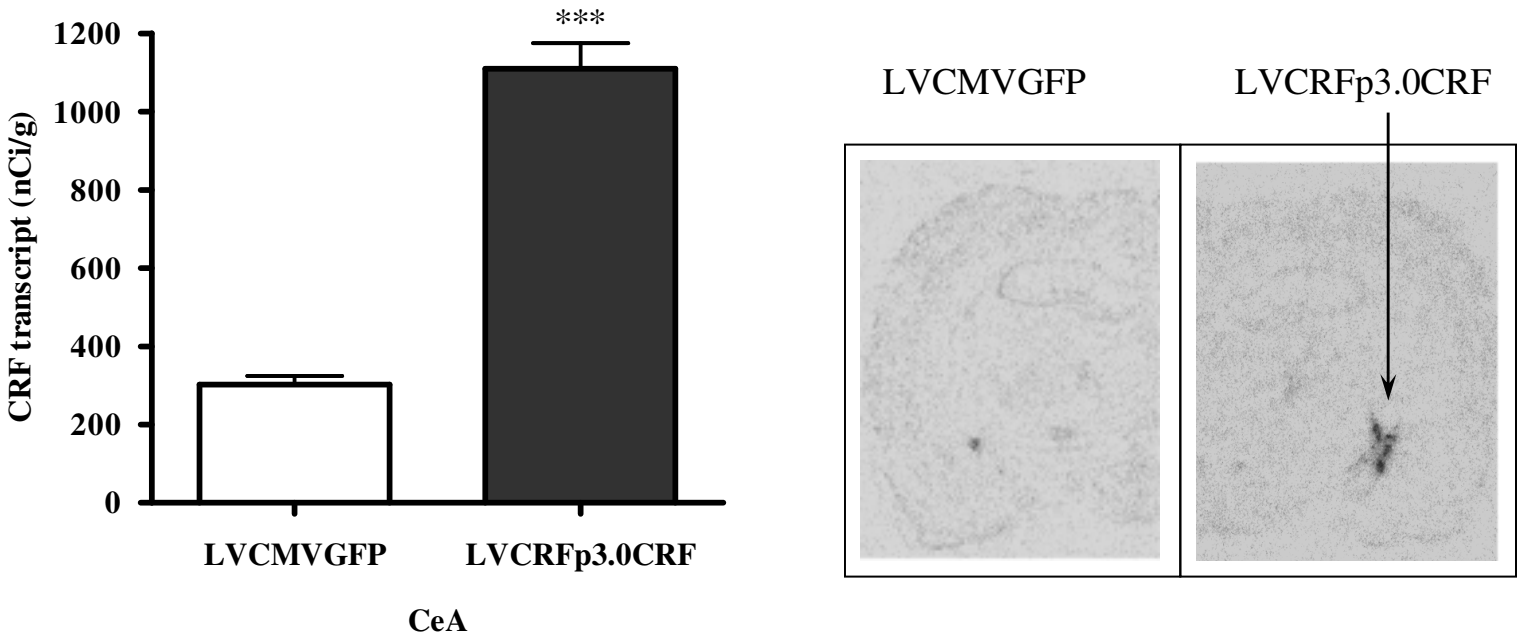
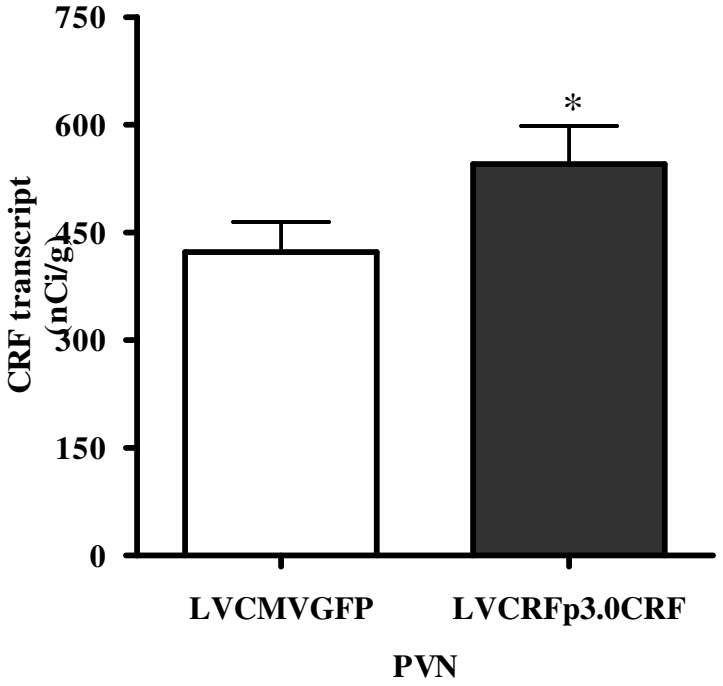


FIGURE 5-8: PVN CRF Expression in Experiment 2 Subjects
(A) Elevated CRF transcript (nCi/g) in the PVN of rats overexpressing CeA CRF. Data are displayed as mean +/- SEM; p-values reflect results of one-tailed T-test analysis based on the a-priori hypothesis that increased CeA CRF expression would increase hypothalamic CRF expression (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **(B)** Representative *in situ* hybridization of CRF transcript in the PVN.



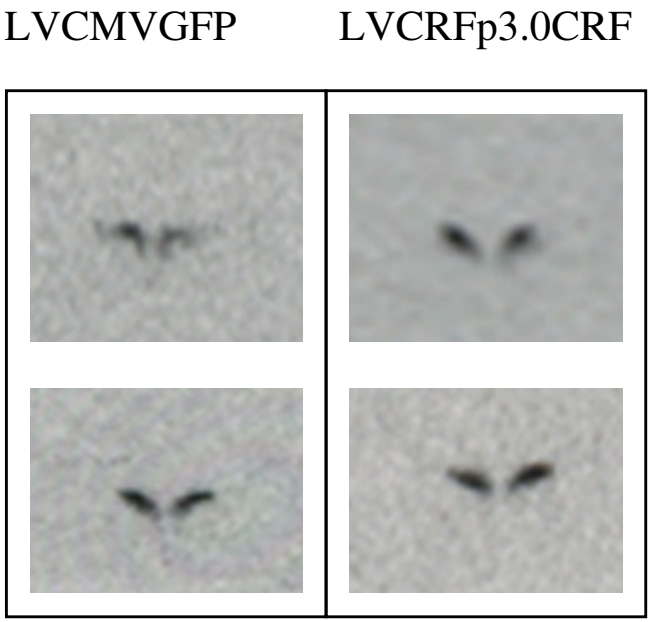
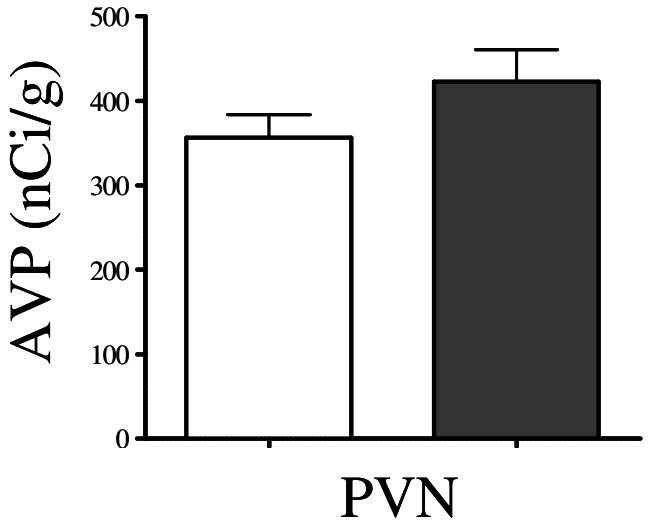


FIGURE 5-9: PVN AVP Transcript in Experiment 2 Subjects
PVN AVP transcript is unaltered after chronic CeA CRF-OE as shown with oligo *in situ* hybridization.



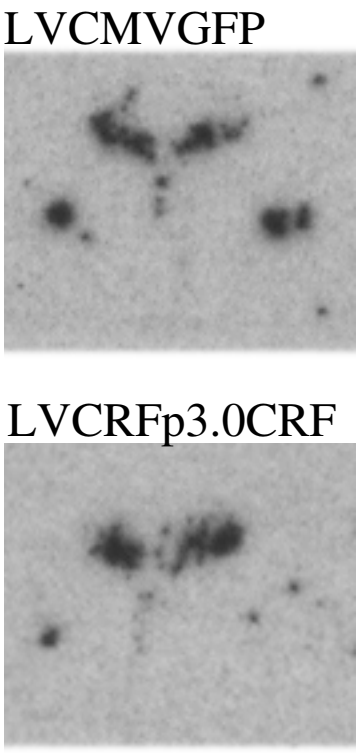


TABLE 5-2: Experiment 2 Additional *In Situ* Hybridization Data

TABLE 5-2A: AVP transcript in the hypothalamus

	LVCMVGFP	**LVCRFp3.0CRF**
PVN	387.44 +/- 40.59	461.02 +/- 52.78 p = 0.08

TABLE 5-2B: GR transcript in the hippocampus

	LVCMVGFP	**LVCRFp3.0CRF**
CA 1/2	46.04 +/- 4.49	49.48 +/- 5.33 p > 0.05
DG	64.07 +/- 5.85	63.49 +/- 6.32 p > 0.05

TABLE 5-2C: MR transcript in the hippocampus

	LVCMVGFP	**LVCRFp3.0CRF**
CA 1/2	341.45 +/- 18.04	312.40 +/- 18.42 p > 0.05
CA3	209.79 +/- 78	191.29 +/- 11.55 p > 0.05
DG	312.54 +/- 16.38	300.46 +/- 18.09 p > 0.05

TABLE 5-2D: BDNF transcript in the hippocampus

	LVCMVGFP	**LVCRFp3.0CRF**
CA 1/2	31.01 +/-7.06	34.34 +/- 6.69 p > 0.05
CA3	103.88 +/- 11.3	120.43 +/- 13.34 p > 0.05
DG	91.81 +/- 32.23	68.47 +/- 11.05 p > 0.05

TABLE 5-3: Experiment 2 Grubbs Test for Outliers

	LVCMVGFP	**LVCRFp3.0CRF**
PVN AVP	value = 1,070.12 z-score = 3.51 $p < 0.01$	value = 1382.75 z-score = 3.49 $p < 0.01$
BDNF CA1/2	value = 127.4 z-score = 2.98 $p < 0.05$	value = 112.25 z-score = 3.01 $p < 0.01$
BDNF DG	value = 664.75 z-score = 3.97 $p < 0.01$	valu e= 218.15 z-score = 3.03 $p < 0.01$

GENERAL DISCUSSION

A strong link between life stress and risk for depression and anxiety disorders has been well-founded (Mazure et al., 2002; Bradley RG, 2007; Anisman et al., 2008; Binder et al., 2008; Bradley et al., 2008). Risk for these disorders also has a heritable, genetic component. As the mediator of the stress-response, CRF is in a unique position to influence the interaction between genes and environment, and how those interactions translate to psychopathology. The goal of this research was to develop better tools to manipulate gene expression within CRF-producing cells, and to use those tools to address previously unanswerable questions regarding the effects of chronic CRF overexpression from within specific populations of CRFergic neurons.

CRFp3.0Cre Transgenic Mouse

The CRFp3.0Cre transgenic mouse expresses Cre-recombinase within CRF-producing cells. The utility of the CRFp3.0Cre strain is two-fold: **(1)** when crossed with the Cre-reporter strain mT/mG, CRF cells are easily identifiable for intracellular recording and fluorescence activated cell sorting (FACTS). **(2)** CRFp3.0Cre can be crossed with other extant mouse strains containing a floxed gene of interest. These crosses can extend our knowledge of interactions between CRF and other signaling molecules in the expression of stress-sensitive behavioral changes as well as the

molecular events which influence changes in CRF-expression patterns within specific sets of neurons.

A. Limitations and Methodological Considerations

The main limitation of the Cre-recombinase/LoxP system in conditional transgenic models is the permanence of the genetic change; once the LoxP site is excised, a cell will express Cre-recombinase and, in the case of the mT/mG cross, will fluoresce green even after it has ceased producing CRF. Furthermore, any daughter cells of this once-CRFergic neuron will also fluoresce green. Further characterization of the CRFp3.0Cre-mT/mG cross should include an analysis of the relative ratios of CRF-negative and CRF-positive green-fluorescing cells. This quality of the CRFp3.0Cre-mT/mG line can also be taken advantage of in identifying cell lineage (Muzumdar et al., 2007).

B. Continuing Progress and Future Directions

Several groups in our department have begun a diverse set of projects with the CRFp3.0Cre transgenic mouse. The Ressler group has crossed the CRFp3.0Cre mouse with other strains containing floxed genes of interest; the behavioral effects of these cell-type-specific manipulations will be rapidly forthcoming. The Rainnie lab has continued to record from putative CRF cells in the CRFp3.0Cre-mT/mG cross to develop a more comprehensive profile of the CRF-producing cell.

Currently available research has demonstrated CRF coexpression with numerous other NPs and NTs, however these studies have relied on tedious and expensive tract-tracing, double-labeling, and electron microscopy experiments. With the CRFp3.0Cre-

mT/mG cross, CRF cells can be identified and isolated using laser-capture or FACTS. Once separated, CRF and non-CRFergic neurons from a single region can be subjected to quantitative gene expression analysis. This project could rapidly compare the transcriptome of CRFergic and non-CRFergic cells in specific brain regions under baseline conditions and in response to stress (early life stress, physical vs. psychological, novel vs. familiar, acute vs. chronic, and controllable vs. uncontrollable) and pharmacological treatments at different ages (adolescent, advanced age). Such high-throughput analysis will inform the next stage of CRF research.

LVCRFp3.0CRF Lentiviral Vector

In human patients, research is necessarily limited to non-invasive assessments. The DST, CRF-stimulation, and Dex/CRF tests are useful measures of overall functioning in the HPA axis stress-response system. However, such peripheral analyses cannot assess the underlying neurochemistry and neuroanatomical connectivity. Lentiviral vectors are extremely useful for *in vivo* studies in the CNS because they have a large insert capacity, generate little or no immune response, maintain expression for the life of the animal, and can transduce non-dividing cells, preferentially infecting neurons when injected into the brain. Because these vectors are replication deficient, they do not leave the site of injection, making it possible to achieve site-specific manipulation. In contrast to previous research in this field, lentiviral-vector mediated CRF-OE is *region*, *time*, and *cell-type* specific. Such specificity elicits increased CRF overexpression within

endogenous CRF circuits, providing a model much more similar to the chronic CRF overexpression hypothesized to occur in human patients with MDD and PTSD.

A. Limitations and Methodological Considerations

The main limitation of Experiments 1 and 2 with LVCRFp3.0CRF is the inability to compare directly the results. After completing Experiment 1, Experiment 2 was designed to expand and clarifying the endocrine effects of chronic CeA-OE. However, results were less robust and less consistent than those from Experiment 1.

A logical interpretation is that the behavioral testing in Experiment 1 interacted with the chronic CRF overexpression to elicit the observed decreases in hippocampal MR and increases in hypothalamic CRF and AVP, leading to HPA axis hyperactivity, which was observed in the Dex/CRF test. In contrast, CeA CRF-OE in the absence of behavioral testing did not significantly increase PVN AVP expression, nor did it impact hippocampal MR. As such, despite increased PVN CRF, regulation of the HPA axis was largely left in tact in Experiment 2 and LVCRFp3.0CRF-injected subjects exhibited no significant deviation in plasma ACTH or corticosterone compared to the LVCMVGFP control subjects. However, it is possible that other subtle measures of HPA axis regulation were altered. For example, the circadian rhythm of HPA axis activation may be flattened as in other studies of chronic stress (Sterlemann et al., 2008).

Other between-test variabilities certainly contribute to, and could potentially account for the distinct results of each experiment. First, although I would have expected a ceiling effect of chronic CRF overexpression after just a few weeks, it is possible that the duration of Experiment 1 (10-weeks) compared to Experiment 2 (6-weeks) explains

the variance in gene expression. Ideally, Experiment 1 and 2 would have been carried out at the same time such that within the same batch of animals one group have been exposed to behavioral testing while another group remained unhandled. This design would have allowed direct comparison between handled and unhandled subjects to address specifically the role of behavioral testing.

Second, housing conditions were not identical between experiments. Surgeries for both experiments were performed in the same room, and for both experiments, animals were transferred to the ABSL-2 facility. Experiment 1 rats remained in the ABSL-2 cubicle for the duration of the 10-weeks. In accordance with Emory University biosafety requirements for the use of lentiviral vectors, Experiment 2 subjects were housed in the ABSL-2 for a mandatory 3-day quarantine but then returned to the psychiatry department housing facility. In both experiments, researchers rather than facility staff were responsible for cage changes, but other environmental factors such as the amount of human traffic and the size of the cubicle compared to the larger housing facility could have influenced the effect of CeA CRF-OE on gene expression and downstream HPA axis activity.

Last, although both experiments used the same viral construct, they were different batches of virus and the possibility exists that components of the virus solution could have influenced infectivity and incorporation (Torashima et al., 2006). That 6 out of 18 rats were eliminated from the LVCRFp3.0CRF group for Experiment 1 while only 3 out of 28 rats were eliminated from Experiment 2 speaks to some difference in the virus, surgery, or both.

B. Continuing Progress and Future Directions

A third LVCRFp3.0CRF experiment has already been carried out. Experiment 3 was designed to assess whether chronic administration of the SSRI escitalopram can reverse the behavioral effects of CeA CRF-OE and prevent CeA CRF-OE-induced HPA axis hyperactivity. The design for this CRF/SSRI project is shown in FIGURE 6-1. Data have already been collected and analysis of histology and behavior is currently in progress. While we have not yet been able to verify injection placement, data thus far have shown no differences in body or adrenal weight, or rate of weight gain over the course of the experiment. This result is consistent with both Experiment 1 and 2.

Histological analysis from LVCRFp3.0CRF Experiments 1, 2, and 3 is still ongoing. For example, binding assays for MR and GR will validate the *in situ* hybridization results. Expression patterns of CRF_1 and CRF_2 can also be assessed with *in situ* hybridization and binding assays. Expression of tyrosine hydroxylase (TH, the rate-limiting enzyme in the production of norepinephrine) can be assessed in the LC and inform to what degree CeA CRF-OE influenced noradrenergic circuitry.

As a novel tool, LVCRFp3.0CRF can be employed in a wide variety of future projects. For example, decreased hippocampal MR and increased hypothalamic CRF and AVP each have the potential to increase HPA axis activity; it would be interesting to develop a more detailed timeline of these changes. Does increased PVN CRF expression precede or follow decreased hippocampal MR and increased hypothalamic AVP? Would

administration of an agonist for MR prevent CeA CRF-OE-induced HPA axis hyperactivity?

Second, these experiments were designed based on the general understanding that both CRF and the CeA (and CRF in the CeA) are responsible for coordinating the endocrine, autonomic, and behavioral stress response and are implicated in symptoms of depression and anxiety disorders. Having completed this project, it would be interesting to compare these results to the effects of LVCRFp3.0CRF injection into other brain regions. Would PVN CRF-OE increase CRF in the CeA and/or BNST? Would BNST CRF-OE produce the same changes in MR and AVP gene expression as did CeA CRF-OE?

Most interesting to me, the CRFp3.0Cre transgenic project could be combined with the LVCRFp3.0CRF project. In fact, the behavioral, endocrine and gene-expression tests of chronic CeA CRF-OE was planned to be carried out in a transgenic mouse such as the CRFp3.0Cre-mT/mG cross in which CRFergic cells would be easily identified and gene expression analysis could initially be carried out on a much larger scale.

CONCLUSION

It is my hypothesis that the HPA axis disruptions serves as a valuable marker for overall dysregulation of central CRF circuits but does not in and of itself contribute to symptoms of depression and anxiety disorders. Rather, hyperactivity in the amygdala overwhelms inhibitory signals from higher cognitive centers, resulting in misinterpretations of social cues and disconnections between emotions and external events, increasing the risk for developing depression or anxiety disorders. Final PVN output is determined by summation of signals from limbic and brainstem sources and can be modulated in minute gradients (TABLE 6-1).

Different types of stress rely on distinct circuits to initiate the autonomic, endocrine, and behavioral stress systems (Hwang and Guntz, 1997; Palkovits et al., 1998; Palkovits, 2000). Exposure to an acute, life-threatening, physiological threat immediately and rapidly stimulates the brainstem noradrenergic nuclei which have direct connections with PVN CRF cells and facilitate activation of the HPA axis. Noradrenergic neurons in the LC also receive excitatory efferents from a population of CRFergic neurons in the PVN distinct from the HPA axis activating cells (reviewed in (Herman and Cullinan, 1997). These connections could provide a short-acting positive feedback loop.

In contrast to the fast, monosynaptic connections activated by systemic danger, the brain processes psychological stress by activating higher-order structures. These higher-order regions interpret the salience of perceived danger. Information about processive stress reaches the PVN through a complicated network of multi-synaptic and disinhibitory connections. CRFergic cells in the PVNmp are surrounded by local inhibitory GABA interneurons with cell bodies in the peri-PVN region or other

hypothalamic nuclei. These local GABAergic neurons constitutively inhibit CRFergic cells in the PVNmp. The strength of the inhibitory surround is modulated by remote input from other limbic system structures (Bali and Kovacs, 2003; Bartanusz et al., 2004). Limbic regions responsible for dampening the HPA axis include the PFC, LS, and hippocampus. The CeA has a primary facilitory role. These negative- and positive-regulators of HPA axis activity communicate with each other and with the inhibitory surround but have very few direct connections to the PVNmp.

The BNST may be a key player in gating the information to the PVN. Subregions of the BNST project either glutamatergic or GABAergic neurons to the PVN and to the inhibitory surround. Activity of these PVN-projecting neurons within the BNST is modified, in a stressor-specific manner, by input from the PFC, amygdala, and hippocampus. For example, processive stress activates the limbic forebrain. The limbic forebrain synapses on GABAergic neurons within the BNST that project to the PVNmp and inhibitory surround. In this way, the limbic system can augment or diminish the HPA response based on prior experience and ongoing activation (Crane et al., 2003).

The complex network of excitatory and inhibitory connections is fine-tuned by neuromodulators. CRF plays an important role in modulating numerous connections within the processive-stress-responsive limbic network, particularly in connections to and from the CeA. Reciprocal glutamatergic projections between the CeA and LS corelease CRF. In the LS, activation of CRF_1 receptors hinders the excitatory effects of glutamate; activation of CRF_2 on the pre- and post-synaptic cell facilitates the effects of glutamate at this synapse. This pattern is reversed at synapses in the CeA where activation of postsynaptic CRF_1 receptors facilitates the excitatory effect of the glutamatergic efferents

from the LS while activation of CRF_2 receptors hinders the effects of glutamate at this synapse (Gallagher et al., 2008). These data are in concordance with the hypothesis that CRF_1 initiates the stress-response while CRF_2 is responsible for return to homeostasis. This differential effect is made possible by the complex interaction between second messenger systems, which are influenced by other ongoing signals.

In addition to higher limbic connections, CRFergic neurons in the CeA also project to the DRN and LC. In the DRN, CRF_1 and CRF_2 are located on serotonergic and GABAergic neurons. The DRN and LC both provide facilitory projections to the CeA. Exogenous administration of CRF to the DRN results in fast increase in 5-HT release in the CeA to activate CeA projections that disinhibit the PAG, which increase freezing behavior. At the same time, CRF_2 receptor activation in the DRN initiates a slow, prolonged release of 5-HT into the mPFC, which inhibits the freezing response, to return to homeostasis (Forster et al., 2006). CRF is also coreleased with DA and glutamate in projections from the VTA to the NAc and LS and in projections from the mPFC to the CeA where it works with DA to modulate the tone of the synaptic connection (Gallagher et al., 2008).

CRF may even modulate its own biosynthesis; CRF neurons in the PVNmp are directly innervated by CRFergic projections from the perifornical and dorsal hypothalamic nuclei as well as the BNST and DRN (Champagne et al., 1998).

Among these complex circuits and signals, a primary stress response and a secondary corrective response to reestablish homeostasis are superimposed upon one another and utilize the same NTs and receptors to different effects. This high degree of interconnectivity is incredibly efficient in healthy subjects but also highly vulnerable to allostatic load.

This vulnerability may explain the ability of genetic alterations to increase the risk for developing mood and anxiety disorders upon stress-exposure, with increasing risk depending on the number and degree of environmental stress (Nemeroff, 2004; Binder et al., 2008; Bradley et al., 2008; Heim et al., 2008). Changes in the responsiveness or efficacy of just one component of the stress-response system may unbalance the entire network.

The "one component" has been the great source of debate. One family of thought implicates GC, others hippocampal volume or CRF in the PVN. The position of the CeA in the limbic processive-stress response system shows that its disruption can activate brain regions responsible for the endocrine, behavioral, and autonomic stress response via disinhibition as well as excitation. The anatomical positioning of the CeA along with previous research demonstrating an anxiogenic effect of its activation, and, in particular, research from human patients showing exaggerated amygdala activity at rest in MDD patients and in symptom-provocation paradigms in patients with anxiety disorders, has led me to the hypothesis that it is CRF in the CeA which is responsible for the host of downstream effects.

In experiment 1, increased CeA CRF causes HPA axis hyperactivity, which is observed in human patients; and decreases hippocampal MR; an effect of chronic

elevations in GC. Decreased MR diminishes the inhibitory tone to the PVN. Diminished inhibitory tone is permissive for increased secretory activity of CRF from the PVNmp (Bartanusz et al., 2004) such that, even in the presence of high circulating GC, GR-mediated negative feedback is insufficient to overcome the excitatory drive from the CeA (FIGURE 6-2).

The results from these studies could have important clinical implications; CRF is colocalized with numerous other NPs, particular CRFergic pathway activation is stressor-specific, and regulation of CRF is region-specific. Each of these variables must be considered when identifying potential targets for novel pharmaceuticals. If CRF in the CeA is a primary instigator of depression and anxiety symptoms, then an ideal treatment could directly target this CRF system. With a more precise target, it may be possible to develop drugs with a faster onset of action.

FIGURE 6-1: Timeline and Experimental Design for LVCRFp3.0CRF Experiment #3

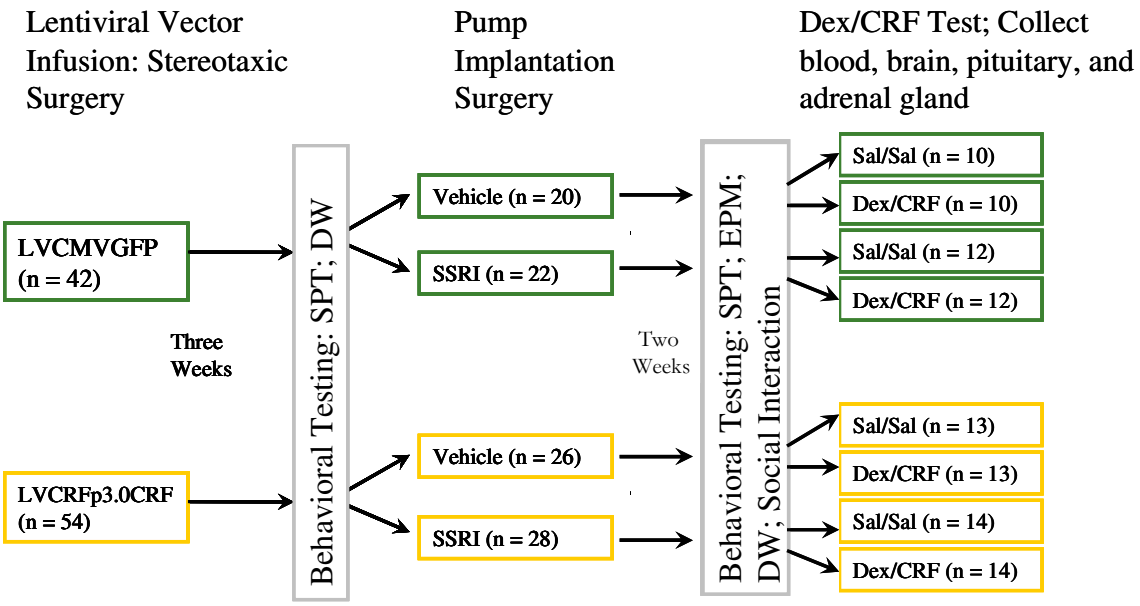


TABLE 6-1 Hypothalamic regulation by limbic sources in rat models of chronic stress (See (Ziegler, 2002) for a more detailed review)

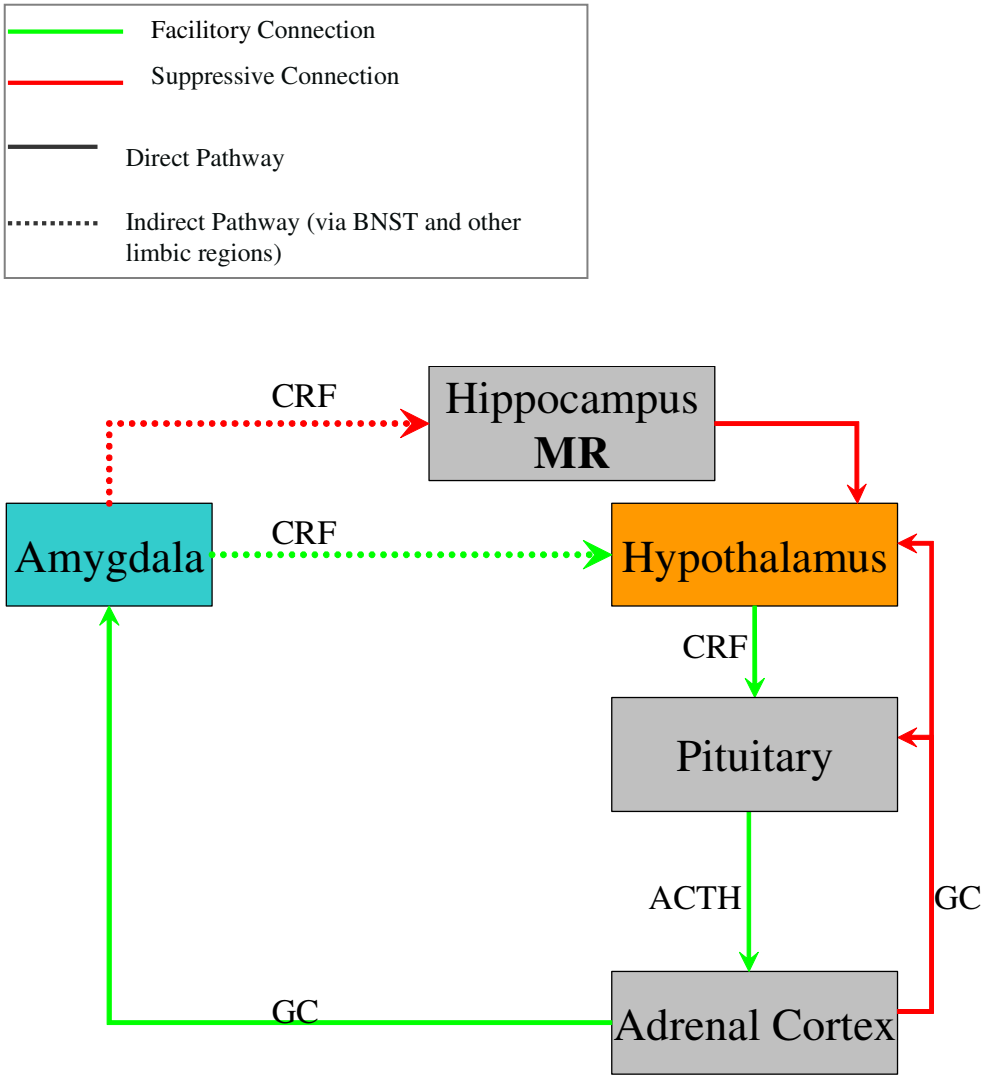
A. Excitatory Regulation of PVN CRF Neurons

Type of Stress	Brain Region	Neurotransmitters	Role in Stress-Response System
Physiological stress → Activation of…	LC	NE	Increase arousal, direct monosynaptic connections with the PVN and indirect connections via forebrain
	DRN	5-HT	
Complex Stressors (Social stress, restraint, novelty) → Activation of…	Sensory and Association Cortex	Glutamate-glutamate or GABA-GABA bisynaptic connections	Integrate stimuli
	Amygdala		Filter stimuli for significance
	Hypothalamus, BNST, and peri-PVN zone	Local regions surrounding PVN CRF cells	Integrate limbic signals to produce highly specific response in PVN CRF cells

B. Inhibitory Regulation of PVN CRF Neurons

Brain Region	Neurotransmitters	Role in Stress-Response System
PFC	Bi-synaptic glutamate-GABA connections	Evaluate significance of stress signal from sensory pathways.
Hippocampus		
BNST	GABAergic dendrites receive excitatory input from PFC and Hippocampus	Glucocorticoid-responsive negative feedback to the HPA axis
Other Hypothalamic Nuclei		
Peri-PVN Zone	GABAergic interneurons surrounding PVN CRF cells	

FIGURE 6-2: Limbic Networks Facilitate and Suppress HPA-Axis Activity



APPENDIX A: Continuous expression of Corticotropin Releasing Factor in the Central Nucleus of the Amygdala Emulates the Dysregulation of the Stress and Reproductive Axes

Erin Keen-Rhinehart[1, 3], Vasiliki Michopoulos[1, 3], Donna J. Toufexis[1, 2, 3], Elizabeth I. Martin[2], Hemu Nair[1, 3], Kerry J. Ressler[1, 2, 3], Michael Davis [1, ,2, 3], Michael J. Owens[2], Charles B. Nemeroff[2], Mark E. Wilson[1, 3].

Yerkes National Primate Research Center[1], 954 Gatewood Rd. and School of Medicine, Department of Psychiatry and Behavioral Sciences[2], 101 Woodruff Circle, Center for Behavioral Neuroscience[3], Emory University, Atlanta, GA 30322 USA

Abbreviated title: Continuous CRF synthesis in CeA simulates chronic stress

Keywords: CRF, CeA, emotionality, stress, sexual motivation, fertility

Corresponding Author:

Dr. Mark E. Wilson

Yerkes National Primate Research Center

954 Gatewood Road

Atlanta, GA 30329

Phone: 404-727-9058

Fax: 404-727-8088

e-mail: mark.wilson@emory.edu

Abstract

An increase in corticotropin releasing factor (CRF) is a putative factor in the pathophysiology of stress-related disorders. Because CRF expression in the central nucleus of the amygdala (CeA) is important in adaptation to chronic stress, we hypothesized that unrestrained synthesis of CRF in the CeA would mimic the consequences of chronic stress exposure and cause dysregulation of the hypothalamic – pituitary – adrenal (HPA) axis, increase emotionality, and disrupt reproduction. To test this hypothesis, we used a lentiviral vector to increase CRF expression site-specifically in the CeA of female rats. Increased synthesis of CRF in the CeA amplified CRF and arginine vasopressin (AVP) peptide concentration in the paraventricular nucleus of the hypothalamus (PVN) and decreased glucocorticoid negative feedback, both markers associated with the pathophysiology of depression. In addition, continuous expression of CRF in the CeA also increased the acoustic startle response and depressive-like behavior in the forced swim test. Protein levels of gonadotropin releasing hormone (GnRH) in the medial preoptic area (MPOA) were significantly reduced by continuous expression of CRF in the CeA and this was associated with a lengthening of estrous cycles. Finally, sexual motivation but not sexual receptivity was significantly attenuated by continuous CRF synthesis in ovariectomized estradiol - progesterone primed females. These data indicate that unrestrained CRF synthesis in the CeA produces a dysregulation of the HPA axis, as well as many of the behavioral, physiological, and reproductive consequences associated with stress-related disorders.

Introduction

Individuals adapt to stress exposure to restore homeostasis and maintain physical and emotional health. However, exposure to chronic stressors results in dysregulation of the HPA axis, mood-related disorders, and a disruption in reproduction (Weissman and Olfson, 1995). This is of particular importance to women because the occurrence of stress-related mood disorders is more prevalent in women (Berga and Loucks, 2005) and stress-induced infertility in women, or functional hypothalamic anovulation (Dallman et al., 2003), is associated with increased risks for cardiovascular disease, osteoporosis, and dementia (Jakobsson and Lundberg, 2006).

A factor common to both stress-related psychopathology and reproductive dysfunction is the over-activity of central CRF. Notably, levels of CRF in CSF are elevated in depression and posttraumatic stress disorder (PTSD) (Naldini et al., 1996a). In addition, CRF also disrupts GnRH production and suppresses reproductive behavior (Zufferey et al., 1999). Thus, the inability to restrain central CRF is a precipitating factor in the stress-induced dysregulation of both of these systems.

CRF is heterogeneously distributed throughout the brain, including the PVN, the CeA, and a portion of the extended amygdala, the bed nucleus of the stria terminalis (BNST) (Zufferey et al., 1999). These regions are candidate sites wherein unconstrained CRF expression may be responsible for the disruption of affect and reproduction. During chronic stress, CRF is up-regulated in the CeA and the BNST (Naldini et al., 1996b; Rattiner et al., 2004; Heldt et al., 2007). In fact, as little as 24 hrs of increased glucocorticoid secretion stimulates the production of CRF in the CeA (Long, 1922; Everett, 1989). Furthermore, concentrations of AVP are increased whereas CRF levels are decreased in the PVN in response to chronic stressors (Long, 1922; Everett, 1989). This increase in AVP maintains adrenocorticotropic hormone (ACTH) release from the pituitary in the face of reduced CRF release from the PVN (Porsolt et al., 1978b). Thus, the down-regulation of CRF in the PVN and the up-regulation of CRF in the CeA are

crucial for the adaptation to prolonged exposure to stressors (Porsolt et al., 1978b). A disruption of this control may be involved in the development of a maladaptive response to chronic stress and result in disturbances in emotional regulation and reproduction.

In this study we hypothesized that a continuous production of CRF in the CeA in female rats would dysregulate the HPA axis, increase anxiety behavior, and disrupt reproduction. To test this, we used a lentiviral vector to express site-specifically CRF constitutively in the CeA of female Sprague-Dawley rats and assessed changes in the regulation of the HPA axis, emotional and sexual behavior, and reproductive physiology.

Materials and Methods

Production and testing of recombinant lentiviral vectors. Lentiviral vectors are extremely useful for *in vivo* studies in the CNS because they have a large insert capacity, generate little or no immune response, maintain expression for the life of the animal, and can transduce non-dividing cells, preferentially infecting neurons when injected into the brain (Toufexis et al., 2004). Lentiviral vectors have proved to be useful vehicles for efficient, long-term, stable gene delivery into the CNS without generating an immune response (Uphouse et al., 2005). Because these vectors are replication deficient, they do not leave the site of injection (Patisaul et al., 2004), making it possible to do site-specific studies such as the ones described in this analysis.

Plasmid Construction. Viral vectors are derived from the HIV-based lentiviral backbones optimized by the laboratory of Dr. Didier Trono (Simmons DM, 1989). The Lenti-CMV-GFP viral plasmid is the "pCM02" vector, which was a generous gift from the lab of Dr. Joshy Jacob. PCM02 was created by inserting the 1.4kb BamHI/XhoI fragment containing GFP-WPRE from the pHR'-CMV-GFP-WPRE plasmid (Paxinos and Watson, 1986) into BamHI/XhoI sites of the pHR-GFP-SIN backbone in place of the GFP fragment (Pike et al.). The resulting pCM02 lentivirus-packaging vector contains a CMV promoter driving GFP expression followed by a woodchuck posttranscriptional regulatory element (WPRE).

The Lenti-CMV-CRF-IRES-GFP virus (hereafter referred to as "LENTI-CMV-CRF") was constructed as follows: The CRFcds plasmid, a generous gift from Wylie Vale (Salk Institute), was digested with EcoRI and cloned into pIRES2-EGFP (5.3Kb; Clontech Laboratories.) The CRF-IRES-GFP segment was then double digested with BglII and HpaI and the lentiviral vector backbone pCMO2 was digested with EcoRI and BamHI. Both of these plasmids were incubated with T4 DNA polymerase (New England Biolabs), following the manufacturer's protocol, to make blunt ends and then ligated together following the manufacturer's protocol using DNA ligase (New England Biolabs). The final viral vector clone was restriction digest verified and tested for expression efficiency and coexpression of GFP and CRF as in FIGURE 1.

Preparation of viral stocks. Virus was generated by transient co-transfection of the expression plasmid (20 □g), VSV-G pseudotyping construct (10□g), and the packaging construct pCMV□R8.91 (20□g) into a 150mm plate of 90% confluent 293T cells as previously described (Arborelius et al., 1999; de Kloet, 2003; Berga and Loucks, 2005; Swaab et al., 2005). Medium was collected 48 and 72 hrs post-transfection, cleared of debris by low-speed centrifugation, and filtered through 0.45-□m filters. High-titer stocks were prepared by an initial ultracentrifugation for 1 hr at 23,000 rpm (SW-28 rotor), and a secondary tabletop centrifugation at 13,000 RPM for 30min. Viral pellet was resuspended in 1% BSA/PBS, and stored at 80ºC. Viral titers were determined by infection of 293T cells. GFP positive cells were visualized by fluorescent microscopy. CRF positive cells were visualized by immunocytochemistry as described separately, with Anti-CRF dilutions of 1:1000-1:10,000.

Animals and Housing. Adult intact female Sprague-Dawley rats (age 40 days; n = 12; 125-150g) from Harlan Laboratories were single housed, on a 12:12 hour light/dark cycle (lights on at 0700 hr). These intact animals were used to monitor disruption of estrous cycles, glucocorticoid negative feedback, measures of emotionality, and provided tissue of immunohistochemical analysis. One female assigned to the group receiving the Lenti-CMV-CRF treatment died during

surgery, resulting in five animals in this group and six in the GFP-injected controls. A second set of adult OVX Sprague-Dawley rats (n = 12; 150-175g, Harlan Laboratories) were single housed, on a 12:12 hour reverse light/dark cycle (with lights out starting at 7am), and were used to assess sexual behavior. Both the GFP- and Lenti-CMV-CRF-injected groups had six animals each. All animals were provided with phytoestrogen-free diet (Harlan Diet #2016) and water *ad libitum*. The lentivirus will express CRF or GFP for the life of the animal. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Surgical Procedures. Animals were anesthetized with isoflurane and placed in a stereotaxic apparatus (Kopf Instruments, Model 900; Tujunga CA). A 10ul Hamilton microsyringe (22 gauge beveled-tip needle), previously coated with 1% BSA, was lowered to the target region. Injection coordinates relative to bregma were CeA: AP -2.3; ML 3.7; DV -8.0. Animals received 1 µl of virus per region at a rate of 0.2 µl/min (UltramicropumpII, World Precision Instruments, Sarasota, FL). The needle was left in place for 5min after the injection and slowly removed over a 5-minute period. The skin was closed using a 6-0 Vicryl suture (Ethicon, Johnson & Johnson, Piscataway, NJ). Animals were allowed two weeks for recovery and sufficient time for the virus to infect cells at the locus and induce them to start producing CRF or GFP. The lentivirus will express CRF or GFP for the life of the animal. Following completion of the tests and assessments described below, animals were sacrificed at ~7 mo of age.

Monitoring Estrous Cycles. Vaginal smears were taken daily between the hours of 10:00 and 13:00 for 6 weeks. Several drops of sterile water were inserted into the vagina via a glass medicine dropper and were withdrawn. The fluid was placed onto a microscope slide. Slides were examined while they were wet at 20X under a light microscope. The phase of estrous cycle was determined based on the predominant cell type present on each day according to standard criteria (Ma et al., 1999). Stages of the estrous cycle were: 1) large clumps of round, nucleated

epithelial cells, a few cornified cells and no leukocytes - proestrus, 2) clumps of cornified cells, little or no round nucleated epithelial cells, no leukocytes - estrus, 3) some round nucleated epithelial cells, some cornified cells and some leukocytes and mucus – metestrus and 4) mostly leukocytes, some round nucleated epithelial cells - diestrus (Bonaz and Rivest, 1998). At the conclusion of these 6 weeks, behavioral testing was initiated with one test paradigm a week.

Dexamethasone (DEX) suppression test. The DEX suppression test was conducted to assess the consequences of increased CRF release from the CeA on glucocorticoid negative feedback. DEX administration was timed to suppress the zenith of diurnal corticosterone rhythm. At 1100 hr or 4 hr after lights came on, a baseline plasma sample was obtained followed by a DEX injection (30 mg/kg given IP). Animals were returned to their home cages and remained undisturbed for the next 6 hours. Subsequent plasma samples were obtained at 1500 and 1700 hr. All plasma samples (0.2ml) were obtained by venipuncture of the saphenous vein while the animals were briefly anesthetized with isoflurane. All samples were obtained within 2min of removing the animal from its homeroom to minimize corticosterone release in response to environment change and handling (Heuser, 1998). Corticosterone levels were analyzed by radioimmunoassay using a commercially available kit (Diagnostic Products Corporation, Los Angeles, CA). The assay has a sensitivity of 5 ng/ml, assaying 50 µl of plasma. The inter- and intra-assay coefficients of variation were 5.88% and 1.31%, respectively.

Behavioral Tests. *Forced Swim Test.* The Porsolt methodology was followed to assess depressive-like behavior: time spent struggling versus time spent immobile (Groenink et al., 2002). Briefly, the rats were placed individually in a translucent container (40 x 24 x 60cm) during the light phase of their light cycle. This apparatus was filled with water (~24°C) to a depth of approximately 22cm so that the animals could not rest on the bottom nor reach the top of the container. A conditioning trial was given to animals the day before test day comprising of a

15min swim, after which animals were toweled off with paper towels and returned to their home cage. Twenty-four hours later, animals were given a 5-minute swim test that was video recorded for later scoring. An observer blind to experimental condition assessed time spent passively floating, barely moving so as to keep nose above the water, and time spent actively coping, struggling to get out of the water. Immobility time is a measure of depressive-like behavior that responds to the administration of anti-depressant drugs (Jacobson et al., 1988).

Baseline Acoustic Startle Test. Animals were tested in 8 X 15 X 15cm wire mesh cages where the floor consisted of four 6.0-mm-diameter stainless steel bars spaced 18 mm apart (Barbas, 2007). The cages are suspended between compression springs in a steel frame within a sound-attenuating, ventilated chamber (inside dimensions, 56 X 56 X 81cm; Industrial Acoustics, Bronx, NY). A General Radio (Concord, MA) type 1390-B noise generator provided background noise (60dB; wideband) that was delivered by high-frequency speakers (Supertweeter; Radio Shack, Tandy, Fort Worth, TX) that were positioned 5cm from the front of the cage. A Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) was used to measure sound levels with the microphone (type 4176) located 7cm from the center of the speaker. This distance approximated the distance between the rat's ear and the speaker during testing. Baseline startle responses were evoked by 50 msec white-noise bursts (5 msec rise-decay) generated by a Macintosh G3 computer sound file (0-22 kHz) that were run through a Radio Shack amplifier (100 watt; model MPA-2000) and played through the same speakers used for background noise. The amplitude of startle responses was measured using an Endevco (San Juan Capistrano, CA) 2217E accelerometer. The cage movements produced by the startle response of the individual rat results in the displacement of the accelerometer, the output of which was integrated to produce a voltage proportional to the velocity of the cage movement. An Endevco model 104 amplifier was used to amplify the output signal, which then was digitized by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced with a Macintosh G3 computer, on a 0-2500 unit scale. The startle amplitudes were defined as the

maximal peak-to-peak voltage that occurred within the first 200 msec after the onset of the startle-eliciting noise. Rats were given two baseline startle tests 24 hours apart, during the light period of their light/dark cycle. Startle measures from both tests were averaged together.

Sexual Behavior Tests. Following two weeks of recovery from neurosurgery, OVX females received standard hormonal priming (2.5 µg of estradiol benzoate in 100 □l of oil 72 hours before test, 10 □g of estradiol benzoate in 100 µl of oil 48 hrs before test, and 500 □g of progesterone in 100 µl of oil 4-6 hours before the test) and were tested for proceptivity and receptivity in a paced mating chamber for 10min (Jacobson and Sapolsky, 1991). All testing was done in the first several hours of the dark phase under red light. Lordosis frequency and lordosis quotients (number of lordoses/number of mounts; LQs) were calculated to assess sexual receptivity, and hops and darts were tabulated to assess proceptivity (Davis, 2006). Animals were tested on two separate occasions two weeks apart.

Immunohistochemistry. Animals were given an overdose of 4% chlorohydrate and perfused transcardially with 250ml of 0.9% sodium chloride containing 0.1% sodium nitrite, followed by 250ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein. Control females were sacrificed in diestrus. Because Lenti-CMV-CRF injected females were not cycling normally, these females were sacrifice on a day most closely resembling diestrus. Brains were removed and placed into 4% paraformaldehyde for post-fixation. Brains were serially sectioned at 30 microns on a microtome and processed for immunohistochemistry. Parallel series were processed for CRF, GFP, GnRH, and AVP immunoreactivity. Free-floating sections were rinsed in potassium PBS (KPBS; 0.1M, pH 7.4) and then washed for 30min in 0.5% hydrogen peroxide. Sections were washed again with KPBS and then incubated in primary antibody solution containing 0.4% Triton X at room temperature for an hour and transferred to 4°C. Incubation times with primary antibodies varied according to protein being targeted: CRF (a kind gift from Dr. Silverman at Columbia University) at a concentration of 1:100,000 for 48 hours, GFP

(Invitrogen, A11120) at a concentration of 1:10,000 for 48 hours, GnRH (Santa Cruz, HU11B) at a concentration of 1:10,000 for 96 hours, and AVP (Phoenix Pharmaceuticals, H-065-07) at a concentration of 1:100,000 for 48 hours. Sections were again thoroughly washed with KPBS and incubated at room temperature for 1 hour in biotinylated goat anti-rabbit IgG antibody (Vectastain Elite RTU ABC kit, Vector Labs). This was followed by more KPBS washing and a 1-hour incubation in avidin-biotin-peroxidase complex solution (Vectastain Elite RTU ABC kit, Vector Laboratories). Following this incubation, sections were washed in KPBS and then sodium acetate (0.175M) for 15min. Visualization of immunoreactivity was accomplished through a 3, 3'-diaminobenzedine (0.2 mg/ml) and 3% hydrogen peroxide (83 □l/ml) reaction in a sodium acetate solution. The reaction was terminated after 10-15min with thorough sodium acetate rinsing, followed by KPBS washes. Sections were mounted out of KPBS onto SuperFrost plus slides (Fisher Scientific), air dried overnight, and dehydrated through a series of graded ethanol, cleared in Histoclear (Fisher Scientific), and cover-slipped using permount. Immunopositive cells were quantified by eye by the same researcher. All sections for each protein stain were run in the same reaction as to minimize inter-assay variability.

***In situ* hybridization.** Lentiviral-vector induced CRF expression was examined using *in situ* hybridization. The rat pre-pro-CRF plasmid (K. Mayo, Northwestern University, Evanson, IL) was linearized with PvuII and transcribed with SP6 polymerase to generate a 593-base 35S-UTP labeled riboprobes. Prehybridization, slides were brought to room temperature, postfixed in 4% paraformaldehyde, pH 7.5, and rinsed in PBS. The remaining steps, included proteinase K treatment, acetylation, dehydration, overnight hybridization in a humidified chamber (50°C), RNase A digestion and washes to a final stringency of 0.1% standard saline citrate (Swerdlow et al., 1986), 0.1% DTT, 60°C for 30min, and were performed as previously described (Kitada et al., 1981). After being stringently washed, slides were dried and placed against Kodak (Rochester, NY) MR autoradiography film for at least 18 hours.

Results

Design of the Lenti-CMV-CRF vector and verification of CRF constitutive expression. A lenti-cytomegalovirus (CMV) vector coexpressing CRF and green fluorescent protein (GFP) was made as described in Methods and illustrated in FIGURE 1A. The virus was titered in 293 T cells by infecting equally confluent wells with serial dilutions of the virus and staining for CRF peptide expression using immunocytochemistry (ICC; Fig. 1B). We then confirmed that cells that expressed high levels of GFP also coexpressed high levels of CRF using double-fluorescence ICC (Fig. 1C). Finally, to test the infectivity of the virus *in vivo*, it was injected into the CeA of adult male Sprague Dawley rats weighing approximately 300g at the time of the surgery using a sham injection as the control. At least 10 days following surgery, rats were killed and lentiviral-vector induced CRF expression was examined using *in situ* hybridization (Fig. 1D-CRF) demonstrating enhanced CRF mRNA expression compared to control (Fig 1D-control).

Once we had demonstrated that the virus could successfully infect cells *in vivo* and cause cells to produce CRF, we proceeded to verify that we could get site-specific protein expression in the CeA in female rats using stereotaxic placement with coordinates from Paxinos and Watson (Wilson et al., 1978) and immunohistochemistry for CRF. Control animals in all these studies were injected with a control viral vector expressing only GFP and we verified that only animals with Lenti-CMV-CRF injected in the CeA showed increased CRF protein in the CeA (Fig. 2A-D; $t_9 = 4.82$, $p < 0.01$). Once we had demonstrated that Lenti-CMV-CRF injection yielded site-specific increases in CRF in the CeA in our female rats, we then proceeded to perform the physiological and behavioral experiments.

Dysregulation of HPA axis. CeA Lenti-CMV-CRF-injected females showed a significant increase in CRF (Fig. 3A, B) ($t_9 = 6.53$, $p < 0.01$) and in AVP (Fig. 3D, E) ($t_9 = 2.88$, $p = 0.02$) in the PVN. To examine the effect of increased CRF synthesis from Lenti-CMV-CRF in the CeA on HPA negative feedback, we performed a dexamethasone (DEX) suppression test (Fig. 4). The

response to dexamethasone varied significantly by treatment over time ($F_{2, 18} = 14.32$, $p < 0.01$). Although DEX suppressed plasma corticosterone similarly in control and Lenti-CMV-CRF-injected females at 4 hours post-injection, corticosterone levels were significantly elevated in Lenti-CMV-CRF-infected females compared to controls by 6 hours post-injection, ($t_9 = 5.67$, $p < 0.01$), indicating that the Lenti-CMV-CRF animals had escaped from glucocorticoid negative feedback.

Effects on emotionality. Locomotor activity, measured just prior to the first acoustic startle test, was not significantly different between Lenti-CMV-CRF-injected (0.214 ± 0.040) and GFP-injected females (0.192 ± 0.047; $p > 0.05$). The acoustic startle response for each individual was averaged across the three-decibel intensities and is shown in Fig. 5A. As can be seen, the baseline acoustic startle response was significantly greater in Lenti-CMV-CRF-injected females compared with GFP-injected females ($t_9 = 2.22$, $p = 0.05$), suggesting that basal levels of anxiety are increased in Lenti-CMV-CRF-injected females. In the forced swim test Lenti-CMV-CRF-injected females displayed increases in depression-related behavior in that they spent significantly less time attempting to escape ($t_9 = 2.38$, $p = 0.04$) and significantly more time floating ($t_9 = 3.39$, $p < 0.01$) than control females (Fig 5B and 5C).

Adverse consequences on reproductive parameters and sexual behavior. Lenti-CMV-CRF-injected females spent more days in diestrous (FIGURE 6A and B) and showed significantly fewer estrous cycles (FIGURE 6C; $t_9 = 3.83$, $p < 0.01$) with more days per cycle (FIGURE 6D; $t = 2.36$, $p = 0.04$), indicating that CRF continuously expressed in the CeA disrupts reproductive function. Comparison of the numbers of gonadotropin releasing hormone (GnRH) positive cells in the medial preoptic area (MPOA) between Lenti-CMV-CRF-injected and control animals (Fig. 7A-D) shows that CRF treated animals had significantly fewer GnRH positive neurons ($t_9 = 3.15$,

p = 0.01) than control animals, suggesting that CRF from the CeA is involved in the control of GnRH expression in this region.

Sexual behavior was assessed at two time points separated by two weeks following estradiol and progesterone priming to ovariectomized (OVX) females. As illustrated in FIGURE 8A, Lenti-CMV-CRF-injected females showed significantly fewer proceptive behaviors (hops and darts) compared to GFP-treated females ($F_{1, 10} = 32.59$, $p < 0.01$) and the differences in the frequency of these behaviors were significantly greater in the second test ($F_{1, 10} = 6.97$, $p = 0.03$). In contrast, the frequency of lordosis behavior (FIGURE 8B; $F_{1, 10} = 0.03$, $p = 0.87$) and mounts received from males (data not shown; $F_{1, 10} = 0.08$, $p = 0.88$) was not significantly different between Lenti-CMV-CRF injected and GFP females. Consequently, lordosis quotients (Adams et al., 1985) were not significantly different between Lenti-CMV-CRF and GFP treated females (FIGURE 8C; $F_{1, 10} = 1.00$, $p = 0.34$).

Discussion

This study demonstrates that lentiviral-induced continuous expression of CRF in the CeA of female rats causes an up-regulation of CRF and AVP in the PVN, an impairment of feedback inhibition of the HPA axis, increased acoustic startle and anxiety behavior, and an impairment of reproductive physiology and behavior. While a single report failed to find differences in CeA CRF concentrations between suicide victims and controls (Centeno et al., 2007), the data from the present study suggest that disrupted control over the up-regulation of CRF, which is known to take place in the CeA under conditions of chronic stress, produces many of the physiological and behavioral changes observed in stress-related pathologies (Baker et al., 2006).

Results here show increased concentrations of both CRF and AVP peptide in the PVN of Lenti-CMV-CRF-injected females. The increase in AVP in the PVN is a well-established consequence of chronic stress (Tsigos and Chrousos, 2002). However, CRF synthesis in the PVN following chronic stress has been shown to remain at basal levels (Rivest et al., 1993). Moreover, exposure to chronic stress down-regulates the production of CRF1 receptors in the PVN (Ortega

et al., 1994). Thus, the restriction of CRF synthesis and activity in the PVN after stress is believed to be of critical importance in limiting the stress response and preventing pathologies associated with excess CRF levels (Kalantaridou et al., 2004). However, the observation that increased CRF peptide in the PVN of CRF-injected females suggests that continuous expression of CRF from the CeA counteracts the reduction of hypothalamic CRF that usually follows stress. Indeed, I.C.V. administration of CRF increases the expression of CRF1 and increases CRF transcription within the PVN (Frohlich et al., 1999). Thus, continuous expression of CRF in the CeA may be constitutively up-regulating CRF1 receptors at the level of the PVN and result in the continuous transcription of CRF in this hypothalamic region.

A decrease in HPA inhibition is present in several psychiatric disorders, most notably major depression (Wallen, 1990), and is believed to reflect decreases in central glucocorticoid receptor (GR) number, binding affinity, or activation (Green, 2003). Transgenic mice over expressing CRF also exhibit HPA dysregulation (Shepard et al., 2000). However, this approach likely produces developmental compensation within the system that may not mimic the consequences of region specific increased CRF expression resulting from chronic stress (Weissman and Olfson, 1995). In this study, the increase in CeA CRF expression induced by the lentiviral vector significantly attenuated glucocorticoid negative feedback, suggesting mechanisms responsible for glucocorticoid negative feedback are compromised in these animals. While this could reflect a change in central GRs, other data suggest that the HPA axis is regulated by glucocorticoid–independent inhibition from a number of brain regions including the lateral and dorsomedial hypothalamus, the BNST, and the MPOA, all of which send gamma-amino-butyric acid (GABA) projections to the PVN (Gallager et al., 1983). The degree to which these pathways are stimulated directly or indirectly by GR activation is difficult to discern; however, adrenalectomized rats show inhibition of ACTH release, indicating that non-glucocorticoid mechanisms can curtail the stress response (Nemeroff and Owens, 2002). Therefore, continuous

expression of CRF in the CeA may be exerting a disruptive effect on one or more of the inhibitory neuronal pathways that regulate the HPA axis.

Of these aforementioned regions, the BNST is likely the intermediate nexus wherein limbic and cortical inputs involved in regulating the stress response are integrated and relayed to the PVN (Arborelius et al., 1999). The BNST receives afferents from cortical and limbic regions involved in the control of the HPA axis including the medial and central amygdala, the hippocampus, and the prefrontal cortex (Ising et al., 2005). Moreover, the BNST contains site-specific areas that activate (the anterior/lateral region) or attenuate (the posterior/medial region) the hormonal stress response (Pitts et al., 1995). Furthermore, these regions are preferentially innervated by either amygdaloid afferents, in the former case, or cortical/hippocampal afferents, in the latter (Mitchell, 1998). Thus, afferent input to the anterior/lateral BNST is from a site governing emotional activation (Ising et al., 2005), and inputs to the posterior/medial BNST are from brain regions involved in negative feedback of the HPA response (Binder et al., 2004).

The present data demonstrate that heightened CRF expression in the CeA produces a significant elevation in acoustic startle. The acoustic startle response is a short latency (8 ms) reflex mediated by a simple neural pathway that is modulated by emotion. Acoustic startle is elevated in fear and anxiety states and this is manifest in psychopathologies like PTSD (Liu et al., 2007). It is well-established that CRF infused into the ventricles of the brain or intra-BNST enhances acoustic startle (Bradley RG, 2007), an effect mediated by activation of the lateral region of the BNST, as reviewed recently (Gutman and Nemeroff, 2003). Anatomical studies show the existence of an efferent projection of CRF-expressing neurons from the CeA to the BNST (Zobel et al., 2000). Results here suggest a functional connection between CRF from the CeA and lateral BNST-dependent CRF-enhanced startle. Perhaps the persistent activation of the lateral BNST by CRF from the CeA that is producing enhanced acoustic startle is also responsible for the escape from glucocorticoid feedback inhibition in CRF-injected females.

Because acoustic startle is elevated by fear and anxiety and is reduced by anxiolytics (Yuuki et al., 2005), the elevated startle response observed in CRF-injected females implies that baseline anxiety is increased in these animals. This conclusion is substantiated by results from the forced swim test showing that CRF-injected females exhibit significantly less escape effort and significantly more immobility than control females, behaviors thought to represent depression-like states (Schule et al., 2006). These differences cannot be attributed to CRF-induced effects on locomotion; motor activity was not increased in CRF-injected animals prior to the initiation of the first day of acoustic startle testing.

In addition, results from this study indicate that CRF from the CeA negatively impacts reproductive physiology in female rats by lengthening the diestrus phase of the estrus cycle and significantly decreasing GnRH in the MPOA. Numerous studies have shown that activation of the stress axis inhibits reproduction. For example, macaques experiencing psychosocial stress associated with subordination stress have reduced fertility (Ising et al., 2007), secondary to an increased incidence of anovulation (Nikisch et al., 2005). Furthermore, macaque females that are stress sensitive have decreased GnRH positive neurons in the hypothalamus (Young et al., 2004). In rats, females subjected to chronic mild stress show a 40% lengthening in estrous cycle and decreased hypothalamic GnRH (Delbende et al., 1994), effects attributed to a stress-induced increase in CRF (Bugnon et al., 1983). For example, CRF decreases GnRH production in the hypothalamus (Tizabi et al., 1985) and LH and FSH production by the pituitary (Bugnon et al., 1983). Although it has been hypothesized that limbic circuitry, including the CeA and the BNST, is involved in the suppression of reproduction by stress (Tizabi et al., 1985), this is the first study tying CRF expression in the CeA directly to this inhibition. Hence, these data suggest that a constitutive increase of CRF in the CeA may be a causal factor in stress-related reproductive disorders like functional hypothalamic anovulation.

In addition to disrupting reproductive physiology, the continuous expression of CRF in the CeA also reduced the frequency of sexually motivated behavior. Ovariectomized, steroid-

primed Lenti-CMV-CRF-injected females showed significantly lower rates of proceptive behaviors compared to steroid primed controls. In contrast, lordosis behavior, even when expressed as the lordosis quotient, was not significantly different between GFP- and Lenti-CMV-CRF-treated females. It is not surprising that the lordosis behavior is not different, as male mounts were also similar between the two groups of females. We believe these data are highly significant because they indicate the more reflexive behavior, lordosis, that occurs in response to male stimulation (Nutt, 2001) is unaffected by continuous CRF from the CeA whereas sexually motivated proceptive behavior that serves to communicate a female's willingness to copulate with the male is attenuated. The neurobiology of these sexually motivated behaviors is complex (Ressler and Nemeroff, 2000), and likely involve limbic and reward pathways (Kent et al., 2002). The present results indicate that CRF expression in the CeA disrupts these circuits; a result consistent with observations of decreased libido or sexual desire characteristic of affective disorders (Tan et al., 2004).

A synthesis of all data garnered throughout the course of this study shows that uncontrolled CRF synthesis within the CeA produces dysregulation of the stress axis, increases emotional behavior, and disrupts reproduction. These results imply that the CeA mediates of all of these functions and suggest that increased expression of CRF within this particular limbic structure can lead to many of the maladaptive processes observed during psychopathology. Indeed, although there have been a substantial number of studies showing enhanced amygdala activity in both depressed people (Price and Lucki, 2001; Price et al., 2002) and those suffering from anxiety disorders (Grigoriadis et al., 1989; Owens et al., 1989), and central activation or infusion of CRF has been consistently associated with increased fear- and anxiety-related behaviors in animals (Grigoriadis et al., 1989), this study is, to the best of our knowledge, the first to tie endogenous synthesis of CRF in the amygdala with all of these outcomes.

In conclusion, findings in the present study demonstrate that continuous expression of CRF in the CeA of female rats results in a host of behavioral and neuroendocrine alterations that

resemble the changes observed in several stress-induced pathologies including PTSD, anxiety and depression. While the administration of glucocorticoids directly to the CeA increases amygdalar CRF expression in a fashion similar to that achieved with the lentiviral infection used in the present study (Veith et al., 1993), the results here show that the consequences of this increased expression of CRF affect multiple neurobiological targets and behavioral systems. Given the higher incidence of affective disorders in women compared with men (De Bellis et al., 1993), our model provides a valuable tool to assess sex differences on a number of behavioral and physiological endpoints in response to CRF over expression in the CeA. Finally, it could be argued that the results of the present study were due to seizure activity induced by high concentrations of CRF (Valentino and Curtis, 1991). However, this is unlikely as rats expressing CRF had an increase in startle and seizures are associated with a marked decrease in startle (Curtis and Valentino, 1991). Therefore, lentiviral vector-induced constitutive expression of CRF in the CeA constitutes a valuable new model for examining the neurobiological mechanisms underlying the dysregulation of the HPA and HPG axes, and may help to clarify a number of processes involved in the development of stress-related illness in women.

Acknowledgments and Author Contributions

The authors thank Ruth Connelly, Laura Canepa, Valencia Coston, and Teal Pelish for their expert technical assistance. We thank Drs. Gloria Hoffman and Andrea Gore for their immunohistochemistry expertise. This project was supported by a pilot grant from the Center for Behavioral Neuroscience as a part of the STC Program of the National Science Foundation under Agreement No. IBN-9876754 and by NIH grants HD46501, MH-42088, MH-52899, RR00165, and 5K12-GM00680.

E. Keen-Rhinehart oversaw the conduct of the studies, performed the ICC, and participated as first author. V. Michopoulos assisted Dr. Keen-Rhinehart with the conduct of the study and participated as first author. D. Toufexis conducted the startle tests and participated as first author. E.I. Martin worked on the development and characterization of the Lenti-CMV-CRF vector and contributed to the preparation of the manuscript. H. Nair contributed to the development of the Lenti-CMV-CRF vector and the preparation of the manuscript. K.J. Ressler supervised the development and characterization of the Lenti-CMV-CRF vector and contributed to the preparation of the manuscript. The startle studies were conducted in M. Davis' lab and he contributed to the preparation of the manuscript. The Lenti-CMV-CRF vector was developed in M.J. Owens' and C.B. Nemeroff's labs and both contributed to the preparation of the manuscript. With the exception of the startle tests, the in vivo studies were conducted in M.E. Wilson's lab and he contributed to the analysis of the data and the preparation of the manuscript.

FIGURE 1: Constitutive CRF Overexpression (A) Schematic diagram of the DNA plasmid encoding the lentiviral (LV) construct for constitutive corticotropin releasing hormone expression, CMV: cytomegalovirus, CRFcds: corticotropin releasing hormone cDNA, IRES: internal ribosomal entry site, eGFP: enhanced Green Fluorescent Protein, LTR: long terminal repeat. (B) *In vitro* functional assay. Immunocytochemistry with anti-CRF antibody demonstrates corticotropin releasing hormone (CRF) protein production in HEK 293 cells visualized with 3, 3 diaminobenzidine (DAB). (C) Coexpression of GFP and CRF in LENTI-CRF infected HEK293 cells is verified with double-immunofluorescent staining with both anti-GFP antibodies (green), anti-CRF antibody (red), and combined overlay. (D) *In vivo* functional assay. LVCMVCRF-induced increased CRF transcript expression in rat CeA (left) vs. control virus (right) demonstrated via *in situ* hybridization.

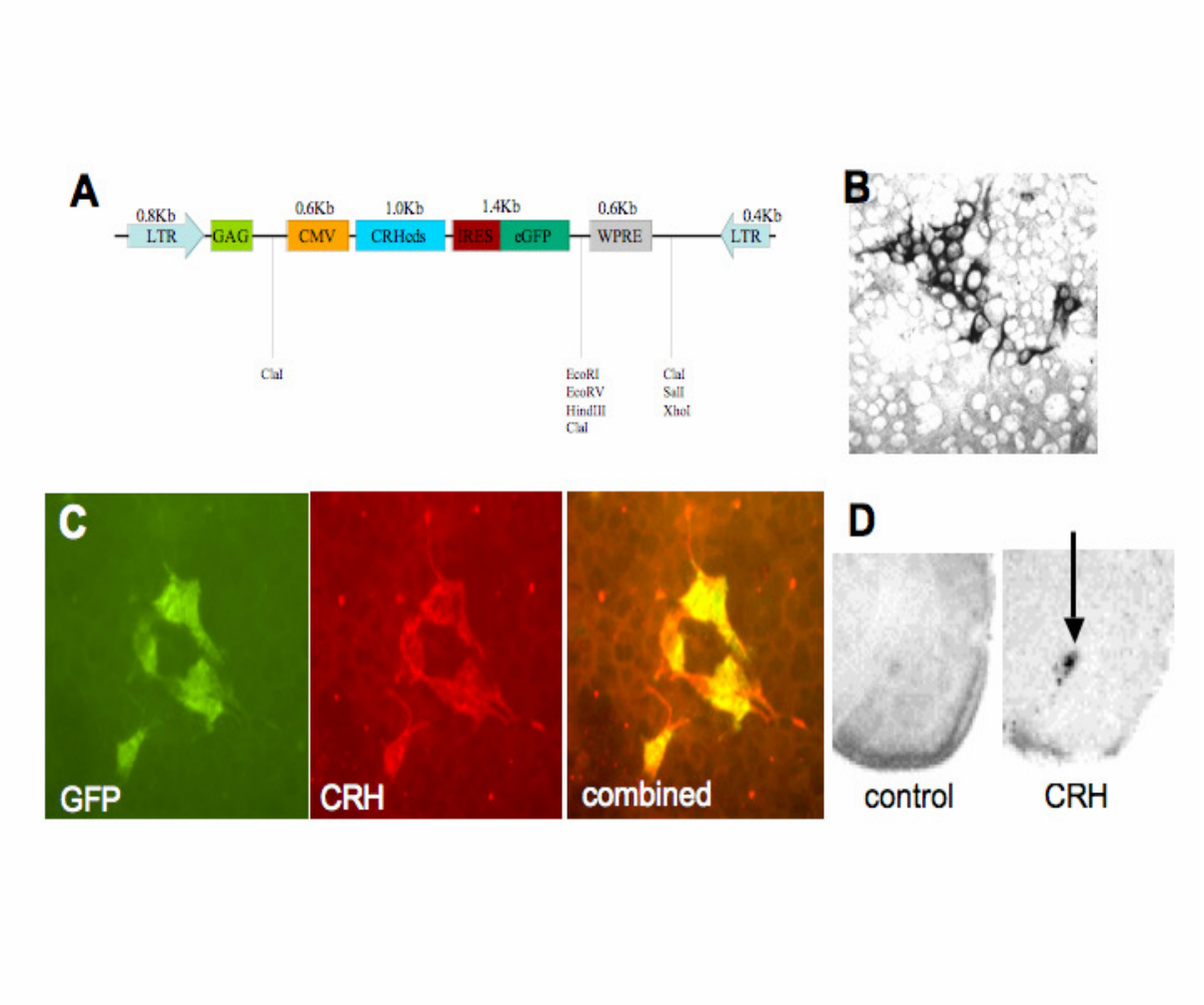


FIGURE 2: Lenti-CMV-CRF injection into the CeA significantly increased CRF protein production site-specifically. (A) Mean ± SEM number of positively labeled CRF cells in the CeA of Lenti-CMV-GFP (open bars) and Lenti-CMV-CRF treated female rats (closed bars) determined by immunohistochemistry. *=p<0.05. (B) A cresyl violet stained section representing the section used to quantify the number of CRF positive neurons in the CeA. (C) A representative section at 2X magnification with an additional 20X magnification inset showing the effects of Lenti-CMV-CRF injection into the CeA on the number of positively labeled CRF neurons. (D) A representative section at 2X magnification with an additional 20X magnification inset showing the amount of staining observed in the control, Lenti-CMV-GFP treated female rats.

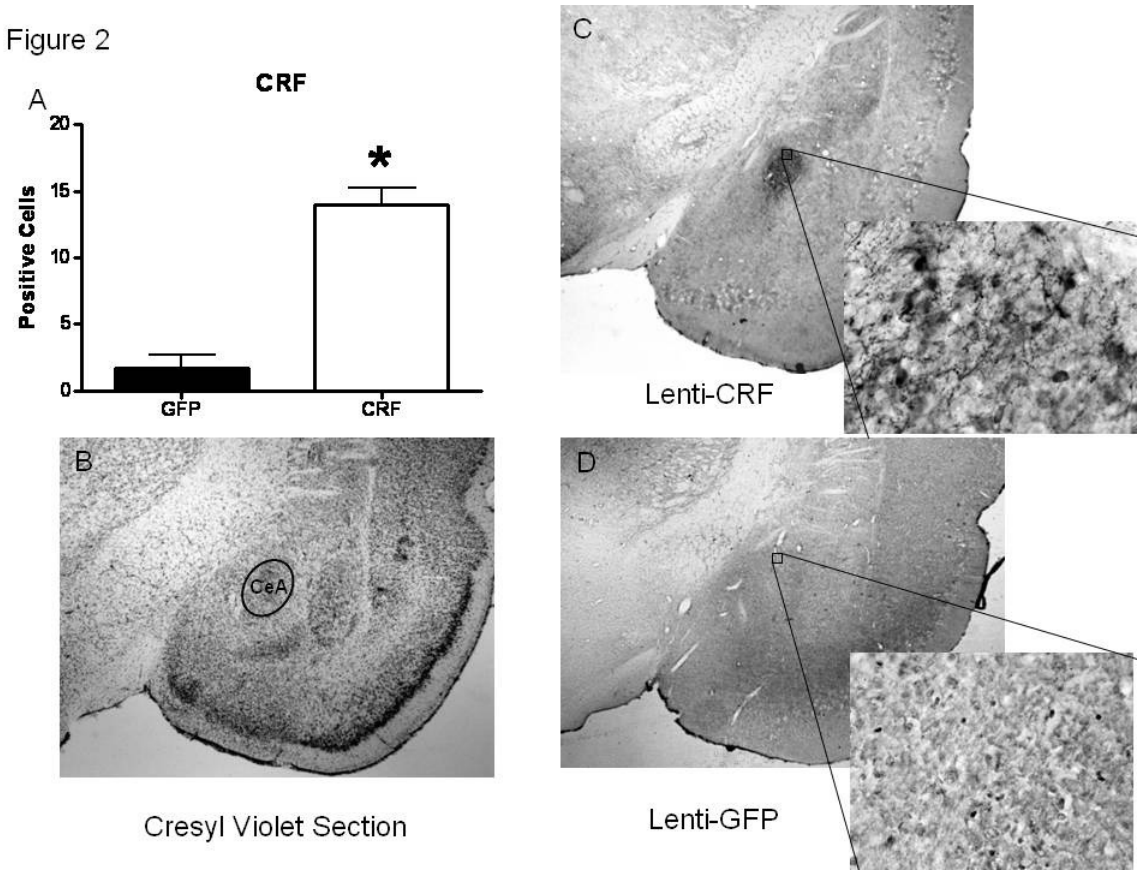


FIGURE 3: **Lenti-CMV-CRF injection increased CRF and AVP protein expression in the PVN.** (A) Mean ± SEM number of positively labeled CRF cells in the PVN of Lenti-CMV-GFP (open bars) and Lenti-CMV-CRF treated female rats (closed bars) determined by immunohistochemistry. *=p<0.05 (B) Representative coronal sections at 10X and 40X magnification showing the effects of Lenti-CMV-CRF injection into the CeA on the number of positively labeled CRF neurons in the PVN. (C) Cresyl violet stained coronal section representative of the section used to quantify and number of CRF and AVP positive cells in each animal. (D) Number of positively labeled AVP cells in the PVN of Lenti-CMV-GFP and Lenti-CMV-CRF treated female rats via immunohistochemistry. (E) Representative coronal sections at 10X and 20X magnification showing the effects of Lenti-CMV-CRF injection into the CeA on the number of positively labeled AVP neurons in the PVN.

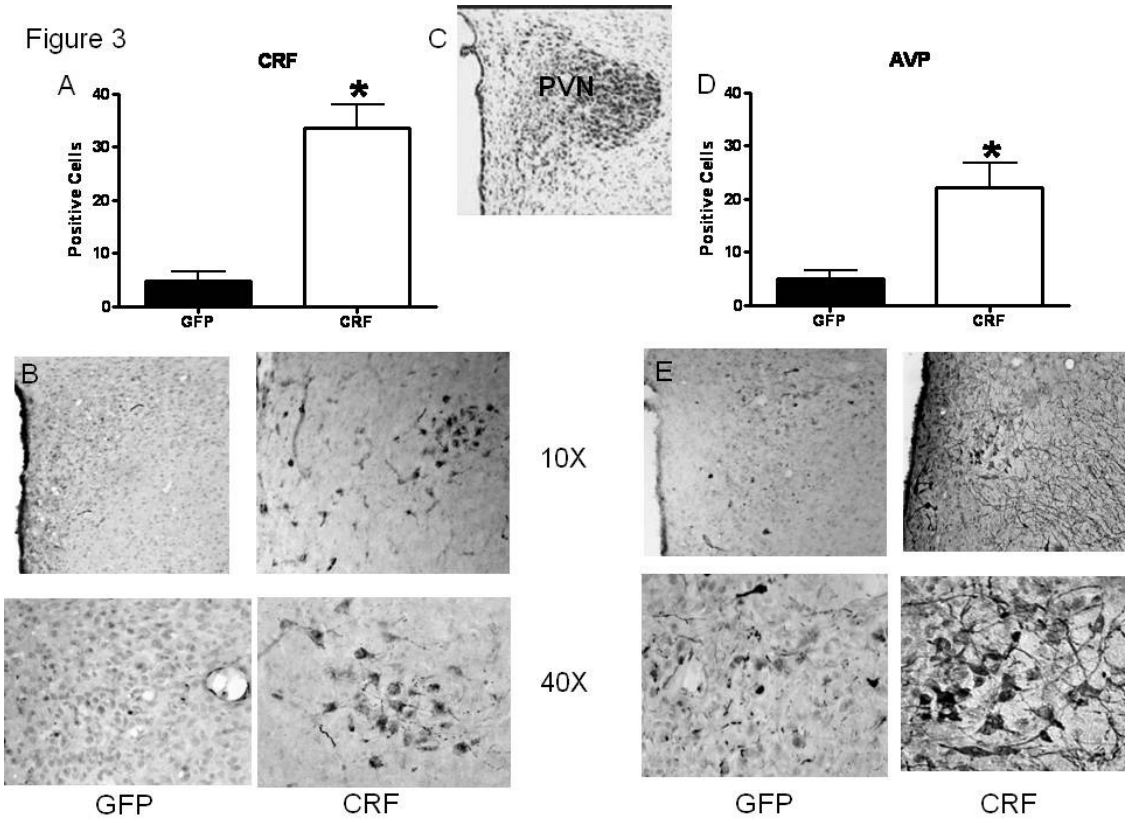


FIGURE 4: **Effect of Lenti-CMV-CRF injection on negative feedback regulation of the HPA axis as assessed by the dexamethasone suppression test.** Mean ± SEM corticosterone levels prior to and following a dexamethasone injection (shown by arrow) for GFP-injected control (open symbol) and Lenti-CMV-CRF-injected females (closed symbol). *p<0.05.

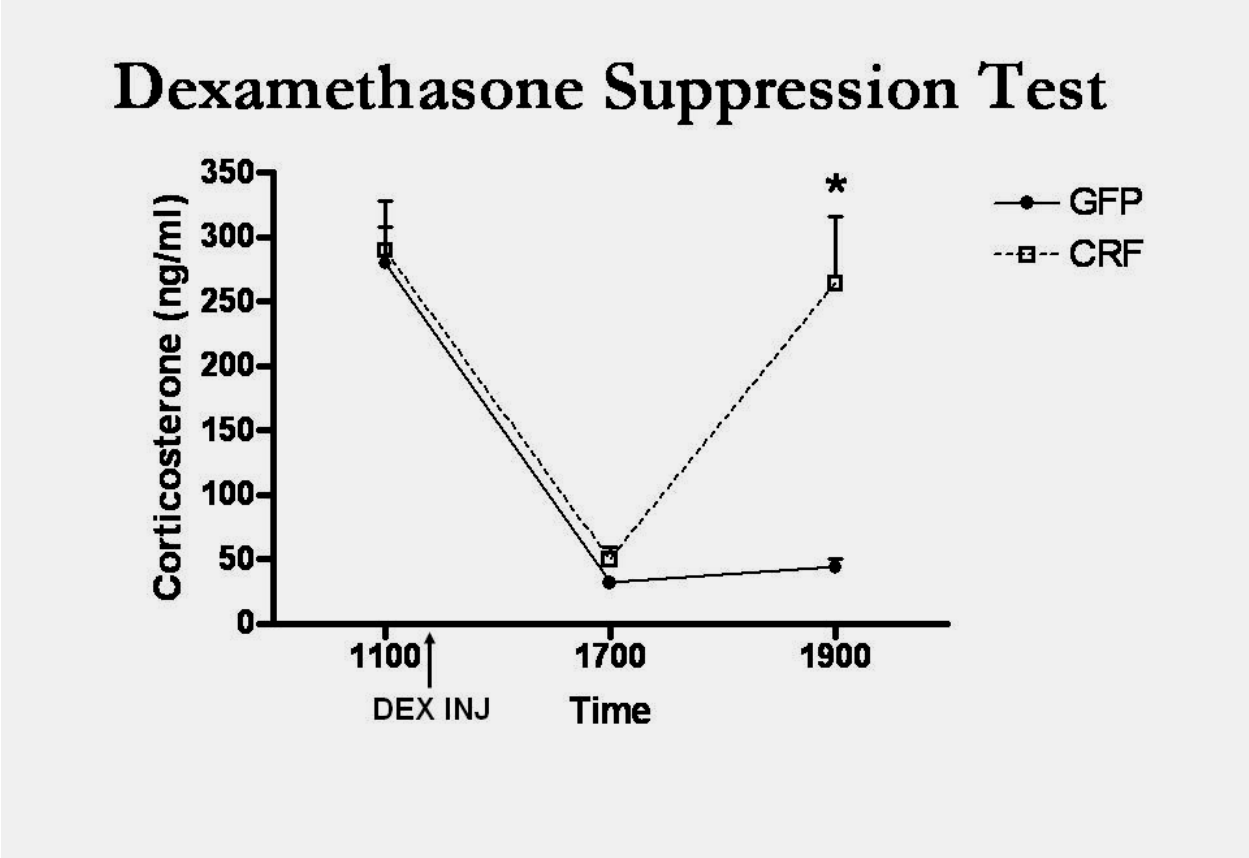


FIGURE 5: **Effect of increased CRF production in the CeA on anxiety- and depression-related behaviors.** Mean ± SEM measures of emotionality for GFP-injected control (open bars) and Lenti-CMV-CRF-injected females (closed bars). Shown is baseline acoustic startle response (A) and amount of time animals spent actively trying to escape (B) and time spent floating (C) in the forced swim test. *p<0.05.

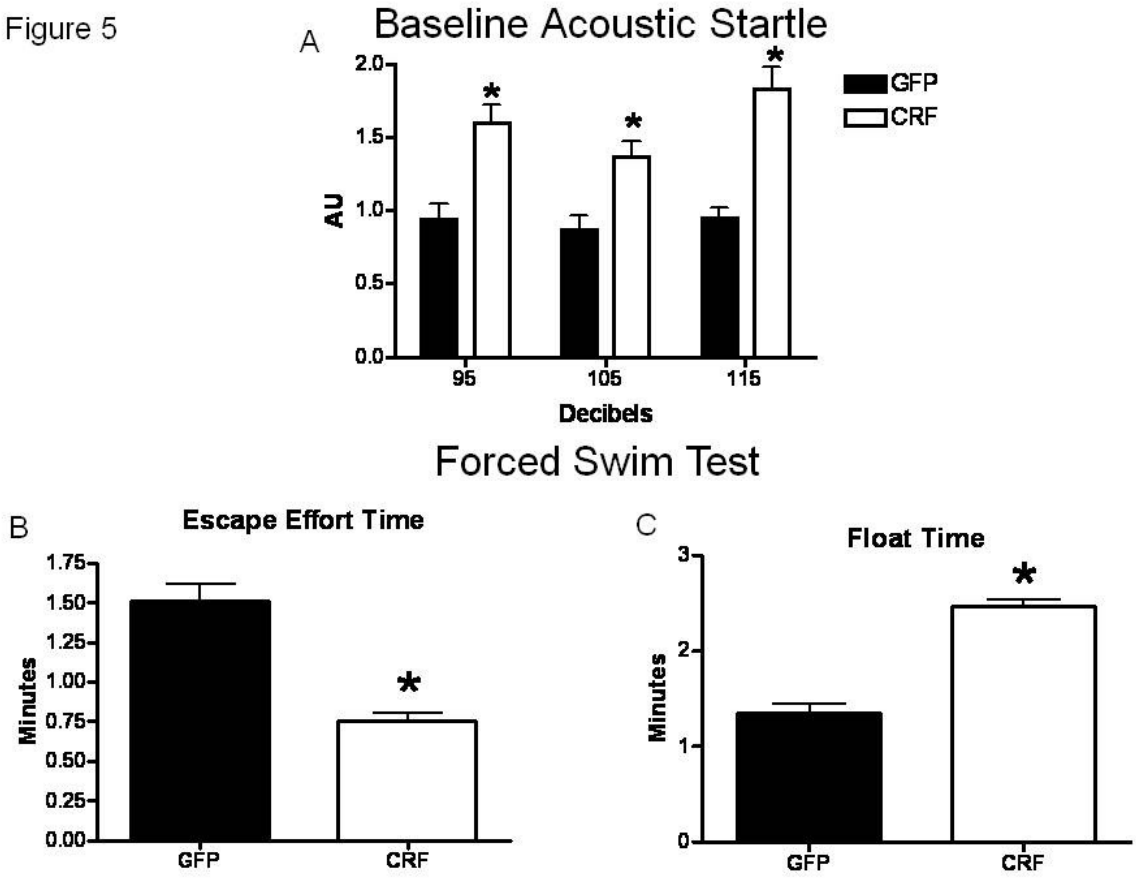


FIGURE 6: Increased CRF production in the CeA produces disturbances in the rat estrous cycle as determined by daily examination of vaginal cytology. Representative cycles of rats injected with (A) Lenti-CMV-GFP and (B) Lenti-CMV-CRF. Also shown are mean ± SEM number of cycles (C) and days per cycle in GFP-injected control (open bars) and Lenti-CMV-CRF injected females (closed bars). *=p<0.05.

Figure 6

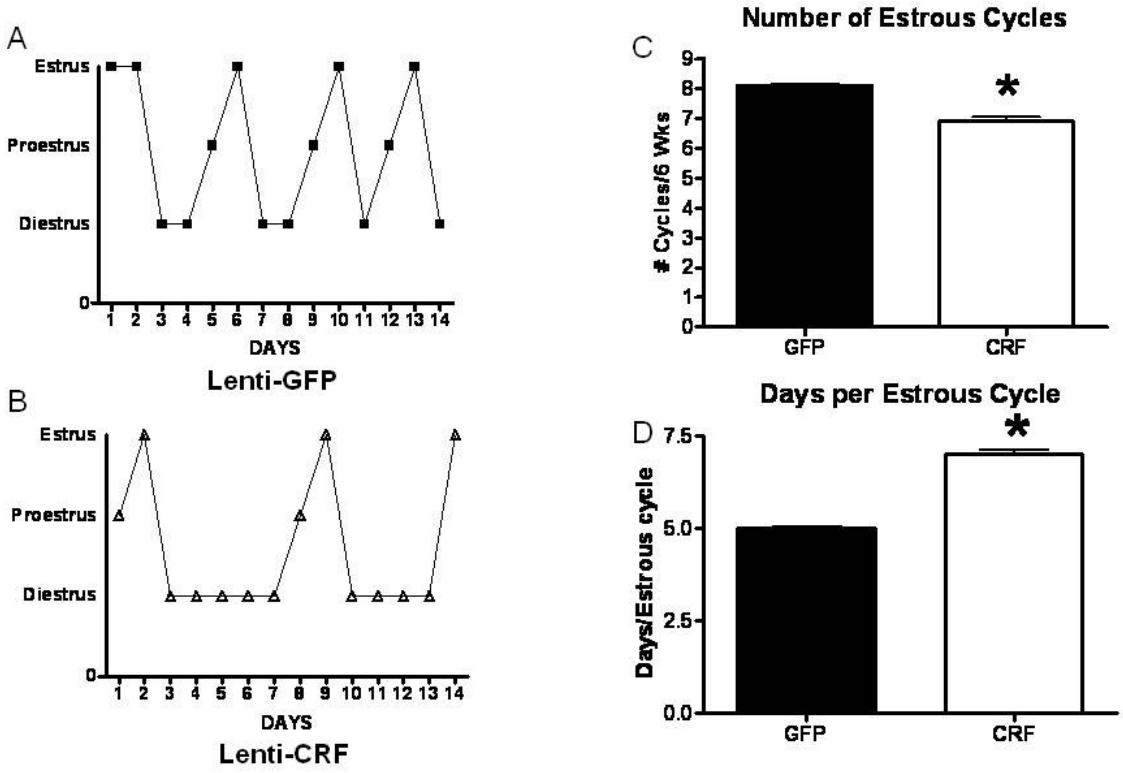


FIGURE 7: Increased CRF protein in the CeA decreases the number of positively stained GnRH cells. (A) Mean ± SEM number of positively labeled GnRH cells in the MPOA of Lenti-CMV-GFP (open bars) and Lenti-CMV-CRF treated female (closed bars) rats determined by immunohistochemistry. (B) Cresyl violet stained coronal section representative of the section used to quantify and number of GnRH positive cells in the MPOA in each animal. Representative coronal sections of (C) Lenti-CMV-GFP and (D) Lenti-CMV-CRF treated animals at 40X magnification. GnRH: gonadotropin releasing hormone, GFP: green fluorescent protein, CRF: corticotropin releasing hormone, MPOA: medial preoptic area. *p<0.05.

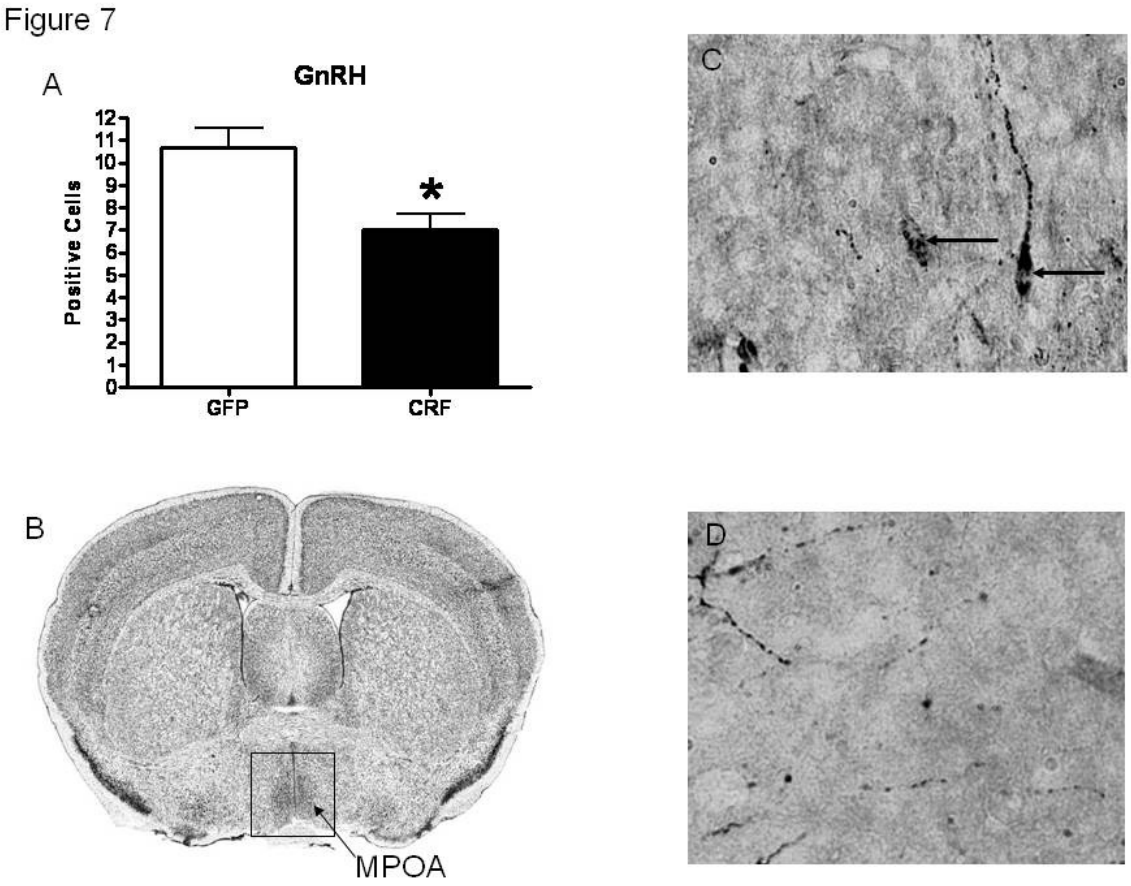
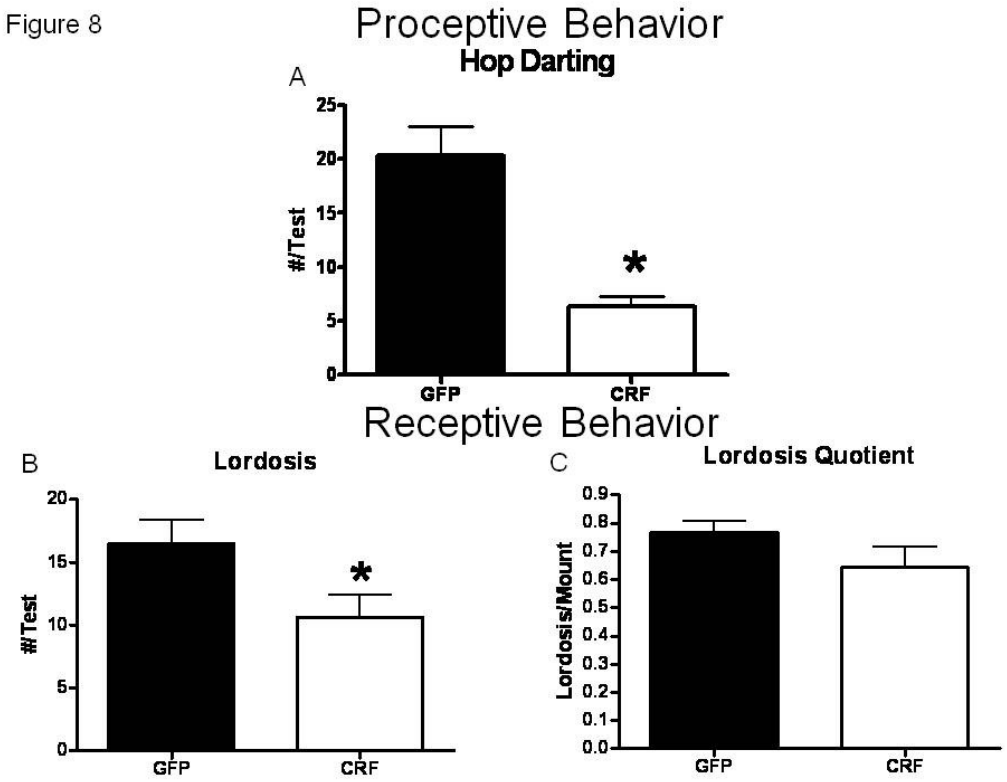


FIGURE 8: Effect of Lenti-CMV-CRF injection into the CeA on sexual behavior in ovariectomized rats. The consequences of continuous CRF production in the CeA of ovariectomized estradiol and progesterone primed GFP-injected (open bars) and Lenti-CMV-CRF-injected female rats (closed bars) at two time points one week apart on frequency (mean ± SEM) of (A) proceptive behavior measured by hop darting; (B) receptive behaviors measured by the lordosis; and (C) the lordosis quotient. * $p < 0.05$.



Appendix B:

The Role of Corticotropin-Releasing Factor in the Pathophysiology of Depression: Implications for Antidepressant Mechanisms of Action

Elizabeth I. Martin and Charles B. Nemeroff

Laboratory of Neuropsychopharmacology, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 101 Woodruff Circle, Suite 4000 WMRB, Atlanta, GA 30322, USA

Phone: 404-727-8382

Fax: 404-727-3233

For Publication in: PSYCHIATRIC ANNALS

Acknowledgements

Supported by NIH MH-77083, MH-69056, MH-58922, MH-39415, MH-42088, and RR-00039

Financial Disclosures

Dr. Nemeroff serves on the Scientific Advisory Board for Quintiles, Astra-Zeneca, Forest Laboratories, Janssen/Ortho-McNeil, American Foundation for Suicide Prevention (AFSP), NARSAD and PharmaNeuroboost. He holds equity, stock options or stock in Novadel Pharma, Corcept, CeNeRx, PharmaNeuroboost and Reevax. He is on the Board of Directors of Novadel Pharma, Mt. Cook Pharma, the AFSP, and the George West Mental Health Foundation. He is a grant recipient from NIH.

The Role of Corticotropin-Releasing Factor in the Pathophysiology of Depression: Implications for Antidepressant Mechanisms of Action

Current antidepressant drugs were developed largely based on two serendipitous findings; first, that monoamine depletion with the antihypertensive agent reserpine causes depression in some patients, and second, that the anti-tubercular agent isoniazid, which inhibits monoamine oxidase, the enzyme responsible for degrading monoamines extracellularly, was noted to improve patients' mood. Currently available antidepressants act by blocking presynaptic monoamine transporter proteins, inhibiting monoamine oxidase, and/or by differential actions at pre- and post-synaptic monoamine receptors. Despite relatively low remission rates and the lag time between treatment initiation and symptom resolution, new drug development has been slow. Much recent research has been devoted to identifying a common mechanism of action of antidepressant drugs that is more closely associated with the temporal sequence of symptom resolution. Moreover, identifying the basic underlying pathophysiology of major depressive disorder (MDD) may elucidate novel targets leading to development of more efficient and effective treatment (reviewed in (Valentino and Curtis, 1991)).

Corticotropin-releasing factor (CRF), a 41 amino acid-containing NP is the preeminent mediator of the mammalian stress response. CRF has long been the focus of research on developing novel antidepressant treatment. The present article will review recent data suggesting that CRF plays a major role in the pathogenesis of MDD and that the mechanism of action of current antidepressant treatments is, at least in part, an action on CRF-containing neural circuits which normalize the endocrine, autonomic, and behavioral stress response.

There is considerable evidence for hyperactivity of CRF-containing circuits in depression in both hypothalamic and extrahypothalamic brain regions including the amygdala and the bed nucleus of the stria terminalis. Chronically elevated activity of extrahypothalamic CRF systems and its associated increased synaptic availability of CRF is thought to be responsible for the decreased density of CRF_1 receptors and decreased mRNA expression of cortical CRF_1 receptors

in depressed suicide victims. Elevated cerebrospinal fluid (CSF) concentrations of CRF, observed in many MDD patients, is also thought to reflect the same hyperactivity in these extrahypothalamic and hypothalamic CRF-producing regions. In particular, CRF mRNA expression in the central nucleus of the amygdala (CeA) has been posited to be upregulated in MDD patients. As one main output of the amygdala, CRF projections from the CeA travel to cortical and brainstem regions including to noradrenergic cells in the locus coeruleus (LC). Overactivity in this CeA-LC projection could explain the observations of elevated CRF concentrations in the LC in MDD patients (reviewed in (Fadda et al., 1995)).

The endocrine response to stress is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, and ultimately CRF. HPA axis activation is initiated by CRF release from the parvocellular cells in the paraventricular nucleus of the hypothalamus (PVN) resulting in adrenocorticotropic hormone (ACTH) release from the anterior pituitary. ACTH acts on the adrenal cortex to provoke glucocorticoid release. Cortisol, the main glucocorticoid in primates, mobilizes energy stores to respond to a threat.

In many depressed patients, the HPA axis is hyperactive, as evidenced by elevated plasma ACTH and cortisol concentrations and by ACTH and cortisol responses in standardized endocrine challenge tests including the dexamethasone suppression test (DST) and the CRF stimulation test. In the DST, the synthetic glucocorticoid dexamethasone decreases plasma ACTH and cortisol concentrations via negative feedback at the level of the pituitary gland. It is well established, however, that many MDD patients are dexamethasone non-suppressors, suggesting that the HPA axis is hyperactive and/or that the negative feedback mechanism is disrupted in these patients (reviewed in (Stout et al., 2002)). Interestingly, among MDD patients, those who were DST non-suppressors exhibited higher CSF CRF concentrations than DST suppressors (Conti et al., 2004).

In the CRF stimulation test, intravenously administered CRF (which does not enter the CNS) elevates plasma ACTH and cortisol concentrations by stimulating CRF_1 receptors in the anterior pituitary. However, MDD patients as a group demonstrate a blunted ACTH response in

this test, likely due to chronic CRF hypersecretion. In fact, CRF concentrations and CRF mRNA expression is elevated in the hypothalamus of depressed patients as measured in postmortem tissue (reviewed in (Owens et al., 1989)).

A combination of the DST and the CRF stimulation test, the Dex/CRF test, developed by Holsboer and colleagues, is generally considered to be the most sensitive measure of HPA axis activity. In this test, many MDD patients exhibit elevated plasma ACTH and cortisol concentrations relative to healthy control subjects, suggesting that both glucocorticoid insensitivity and CRF overexpression contribute to HPA axis hyperactivity in depression (reviewed in (Skelton et al., 2000)).

Individual differences in genes involved in the CRF system and genes whose products are involved in HPA axis activation and feedback have been hypothesized to contribute to disturbed CRF signaling and HPA axis activity in MDD response to antidepressant treatment. Thus a single nucleotide polymorphism (SNP) in FKBP5, a glucocorticoid receptor-regulating (chaperone) protein, results in elevated expression of this gene. Increased FKBP5 expression is thought to trigger adaptive changes in the glucocorticoid receptor and HPA axis, explaining the observation that patients with this SNP had a greater number of depressive episodes but with less severe HPA axis disturbances during a depressive episode and, most importantly, had a more rapid antidepressant treatment response than other MDD patients (Owens et al., 1989). A SNP in the CRF_1 receptor gene has been associated with high anxiety in MDD patients and has also been correlated with response to fluoxetine treatment (Owens et al., 1989). Most recently our group (Grigoriadis et al., 1989) has demonstrated that SNPs of the CRF_1 receptor gene predict vulnerability to depression in adult victims of child abuse. A burgeoning database from preclinical (e.g. (Skelton et al., 2000)) and clinical (e.g. (Skelton et al., 2000)) research approaches have revealed that CRF_1 receptor antagonists possess antidepressant and anxiolytic properties and likely represent a novel class of antidepressants. Moreover, many such agents have been developed and are in various stages of testing from the laboratory to the clinic.

Currently available antidepressants, despite exerting their primary pharmacological effects on monoaminergic systems, all reduce the overall responsiveness of the HPA axis, and the activity of hypothalamic and extrahypothalamic CRF neurons. Importantly, somatic, non pharmacological antidepressant treatments such as electroconvulsive therapy also normalize HPA axis reactivity as measured by the Dex/CRF test (e.g. (Stout et al., 2001)), and reduce the elevated CSF CRF concentrations observed in depressed patients. These data are concordant with the hypothesis that CRF/HPA axis normalization is associated with symptom resolution.

The Dex/CRF test has been posited to be a potential biomarker to predict antidepressant response. Patients who exhibit improvement in depression symptoms without concurrent normalization of the Dex/CRF response are more likely to relapse (Stout et al., 2001; Gilmor et al., 2003). Improvement in the Dex/CRF test in MDD patients after two weeks of hospitalization was associated with improvement and remission three weeks later. In contrast, patients who exhibit persistent Dex/CRF test hyperactivity in the first two weeks of treatment are less likely to exhibit symptom reduction after five weeks of treatment (FIGURE 1) (Ising et al., 2007). Furthermore, the magnitude of the decrease in plasma cortisol concentration with the Dex/CRF test from the depressed pretreatment state to four weeks post-treatment with the SSRI citalopram predicted the magnitude of symptom improvement at the end of a 16-week study (Veith et al., 1993). Others have reported that patients with elevated ACTH concentrations in the Dex/CRF test prior to treatment were more likely to later fail to respond to the SSRI fluoxetine (Liotti et al., 2000).

Antidepressant-induced HPA axis normalization may reflect normalization of CRFergic signaling. This hypothesis is supported by the fact that changes in CRF mRNA expression and CRF concentrations as well as CRF_1 mRNA expression and binding have been demonstrated following chronic antidepressant administration in laboratory animals. These changes also roughly follow the time course of symptom resolution, supporting the hypothesis that

normalization of CRF neurotransmission plays a causal role in the mechanism of action of antidepressant drugs.

In rats, fifteen days of treatment with the antidepressant tianeptine, approved in Europe to treat depression and anxiety, decreased CRF concentrations in the rat hypothalamus and also decreased ACTH concentrations in the anterior pituitary gland. Tianeptine also blocked restraint-stress induced elevations in PVN CRF concentrations and plasma concentrations of ACTH and corticosterone, the major glucocorticoid in rodents (Lacerda et al., 2004). FIGURE **2** shows endogenous CRF mRNA expression in the rat PVN and hippocampus from our laboratory.

Numerous neuroanatomical connections between CNS monoamine and NP circuits suggest that disturbances in CRFergic activity might alter monoaminergic signaling. This neuroanatomical proximity also provides numerous opportunities by which antidepressants, whose primary actions are on monoaminergic neurons, can influence CRFergic neurotransmission. Monoamine depletion by reserpine increases CRF release from the rat median eminence (Liotti et al., 2002; Gemar et al., 2006) and posterior pituitary gland (Kilts, 2003). The monoamine oxidase inhibitors (MAOIs) tranylcypromine and pargyline can decrease CRF release (Goldapple et al., 2004) and chronic treatment with the tricyclic antidepressant (TCA) desipramine (20 mg/kg IP; 14 days) restores CRF-like immunoreactivity in the posterior pituitary (Mayberg et al., 1999).

During a depressive episode, CRF is hypersecreted in the CNS, likely including the LC, potentially via the aforementioned CeA-LC connection. Reciprocal noradrenergic projections from the LC to the amygdala activate CRF-containing cells (reviewed in (Liotti et al., 2000)). Elevations in noradrenergic neurotransmission in MDD may indirectly contribute to symptoms secondary to increased activity in amygdalar CRF cells (reviewed in (Mayberg, 1997)). Noradrenergic LC neurons also project to the DRN to elevate serotonergic firing while serotonergic projections from the DRN to the LC decrease noradrenergic firing. One hypothesized mechanism of action of SSRIs is that by increasing serotonin availability, SSRIs

decrease activation of the LC noradrenergic pathways to the amygdala; this decrease in amygdalar activation may contribute to the reduction in symptoms (Liotti et al., 2002).

Serotoninergic neurons in the DRN also project to the frontal cortex where they modulate GABAergic signaling. CRF influences serotonergic and GABAergic activity by potentiating the effects of serotonin on forebrain GABA (Keedwell et al., 2005). This interaction has previously been shown to alter depressive-like behavior in experimental animals (Drevets, 2001).

At the level of the brain stem, chronic but not acute administration of the TCA imipramine has been shown to increase CRF binding in rats (Keedwell et al., 2005). Chronic imipramine and desipramine administration to rats also showed a trend toward increased CRF binding in other brain regions including the striatum, cerebellum and frontal cortex, but not in the parietal/temporal cortex, hippocampus, or anterior pituitary gland (Liotti et al., 2002). Such increases in the density of CRF_1 receptor binding sites are likely secondary to antidepressant-induced reductions in CRFergic neuronal activity. These data are concordant with the observations that both normal controls and depressed patients exhibit reductions in CSF CRF concentrations after treatment with desipramine (Lacerda et al., 2004) and fluoxetine (Monk et al., 2006), respectively (FIGURE 3).

Antidepressants of different classes reduce CRF responsiveness to stress. Administration of the SSRI sertraline or the MAOI phenelzine can enhance the signal-to-noise ratio of rat LC neuronal activity, which is decreased by exogenous CRF administration (Liotti et al., 2000). In contrast, acute administration of the atypical antidepressant mianserin, an antidepressant available outside the United States which shares many properties with mirtazepine, elevated spontaneous discharge in rat LC neurons but reduced stimulation-induced LC activation; mianserin also prevented CRF-induced and CRF-dependent stress from activating LC neurons (Liotti et al., 2000). Chronic mianserin or desipramine administration has also been shown to reduce stress-induced LC activation (Liotti et al., 2002). Although chronic, but not acute mianserin or

imipramine treatment decreased hypothalamic CRF concentrations only the former also decreased extrahypothalamic CRF concentrations (Mayberg et al., 1999; Liotti et al., 2002).

Both the MAOI tranylcypromine and the SNRI venlafaxine, when administered chronically, reduce hypothalamic CRF responsiveness to stress. Both antidepressants reduced chronic variable stress-induced increases in CRF heteronuclear (hn) RNA expression in the PVN when compared to vehicle-treated control rats. Venlafaxine blocked chronic variable stress-induced elevations in CRF mRNA expression in the PVN. Because there were no changes in baseline CRF or HPA axis measures in antidepressant-treated rats, these results suggested that chronic antidepressant treatment decreases CRF neuronal sensitivity to stress. Importantly, antidepressant doses in this study were validated by monoamine transporter occupancy measurements and serum drug concentrations, allowing greater confidence in the interpretability of the data (Liotti et al., 2002).

Numerous mechanisms have been proposed by which antidepressants effect PVN CRF expression including influencing signal transduction cascades. Inducible cAMP early repressor (ICER) is an alternative splice variant of the gene for cAMP response element-modulator (CREM). This repressor is expressed in the hypothalamus at relatively high levels and is upregulated by electroconvulsive seizure (ECS), naturally leading to examinations of the role of ICER in the mechanism of action of other antidepressant treatments. To examine this hypothesis, mice deficient in ICER and wild type (WT) mice were exposed to forced swim stress and treated with desipramine or vehicle. In WT animals, desipramine increased ICER expression in CRF-containing PVN neurons and was able to reduce swim-stress-induced CRF expression. Desipramine had no effect on hypothalamic CRF in $ICER^{-/-}$ mice, suggesting that ICER is required for desipramine to reduce PVN CRF and, therefore, reduce plasma corticosterone, in response to swim stress (Liotti et al., 2000).

In addition to antidepressants, modulation of CRFergic signaling may also play a role in the mechanism of action of other psychotropic drugs including anxiolytics and mood stabilizers.

That CRF systems would play a role in a broader spectrum of psychiatric disorders and their treatment is not surprising in view of the fact that stress-sensitivity is linked to numerous major psychiatric illnesses as well as the high rates of comorbidity among these disorders.

Acute administration of the triazolobenzodiazepines alprazolam or adinazolam, anxiolytics used in the treatment of various anxiety disorders, produced decreases in CRF concentrations in the rat amygdala (Mayberg et al., 1999), LC (Drevets, 2001), and cortex, but elevations in the hypothalamus (Anand and Shekhar, 2003; Rauch et al., 2003). This elevation likely reflects decreased CRF secretion because it was associated with a decrease in plasma ACTH (Rauch et al., 2003). In contrast, chronic treatment with alprazolam, adinazolam, or the benzodiazepine diazepam decreased CRF receptor binding in the frontal cortex and hippocampus of rats (Drevets, 2003). Later studies also showed that rats treated with chronic, but not acute, alprazolam decreased baseline HPA axis activity, CRF mRNA expression in the CeA, and CRF_1 mRNA and receptor binding in the BLA (Drevets, 2001; Anand and Shekhar, 2003; Drevets, 2003).

Importantly, the timeline for the effect of alprazolam on LC CRF concentrations and the fact that alprazolam-induced changes in LC CRF concentrations do not develop tolerance highlight the possibility that the anxiolytic properties of benzodiazepines such as alprazolam are mediated, at least in part, by normalizing CRF circuit activity at the level of the LC (Drevets, 2003).

Valproate, an anticonvulsant and mood stabilizer approved for the treatment of acute mania including the mixed states, decreases activity in the CRF system. Valproate is particularly useful in treating the 40% of manic patients with dysphoric or mixed mania and these patients often exhibit HPA axis disturbances. In rats, acute valproate administration failed to alter CRF concentrations or HPA axis activity 90min post drug administration (Anand and Shekhar, 2003). Chronic valproate treatment decreased CRF concentrations in the median eminence and DRN. A

less substantial decrease in CRF mRNA expression was observed in the PVN and CeA, and CRF concentrations in the frontal cortex were elevated. (Drevets, 2003).

Summary and Conclusions

An overwhelming and ever-growing literature support the hypothesis that the CRFergic neurons, including extrahypothalamic as well as HPA axis components, represent a seminal stress-response system, that plays a causal role in the etiology of affective and anxiety disorders. A wealth of data have also demonstrated a role for CRFergic circuitry in the mechanism of action of current antidepressant therapies. The temporal sequence of symptom improvement during treatment with these agents more closely matches normalization of CRFergic signaling and HPA axis functioning than their primary effects on monoaminergic systems. Moreover, CRF_1 antagonists are known to possess antidepressant and anxiolytic properties and likely represent a novel class of antidepressants/anxiolytics that may be used both in monotherapy as well as in combination with currently available treatments.

FIGURE 1: ACTH (A) and cortisol (B) response to the combined Dex/CRF test at study inclusion and 2 to 3 weeks later in patients with improved (circles, *n = 36*) and unimproved HPA system function (triangles, *n = 14*) and in healthy control subjects (dashed line *n = 50*). The *p* values of the interaction term change in AUC scores by group are given. ACTH, adrenocorticotropic hormone; DEX/CRH, dexamethasone/corticotropin-releasing hormone; HPA, hypothalamus-pituitary-adrenocortical; AUC, area under curve. (From (Anand and Shekhar, 2003)).

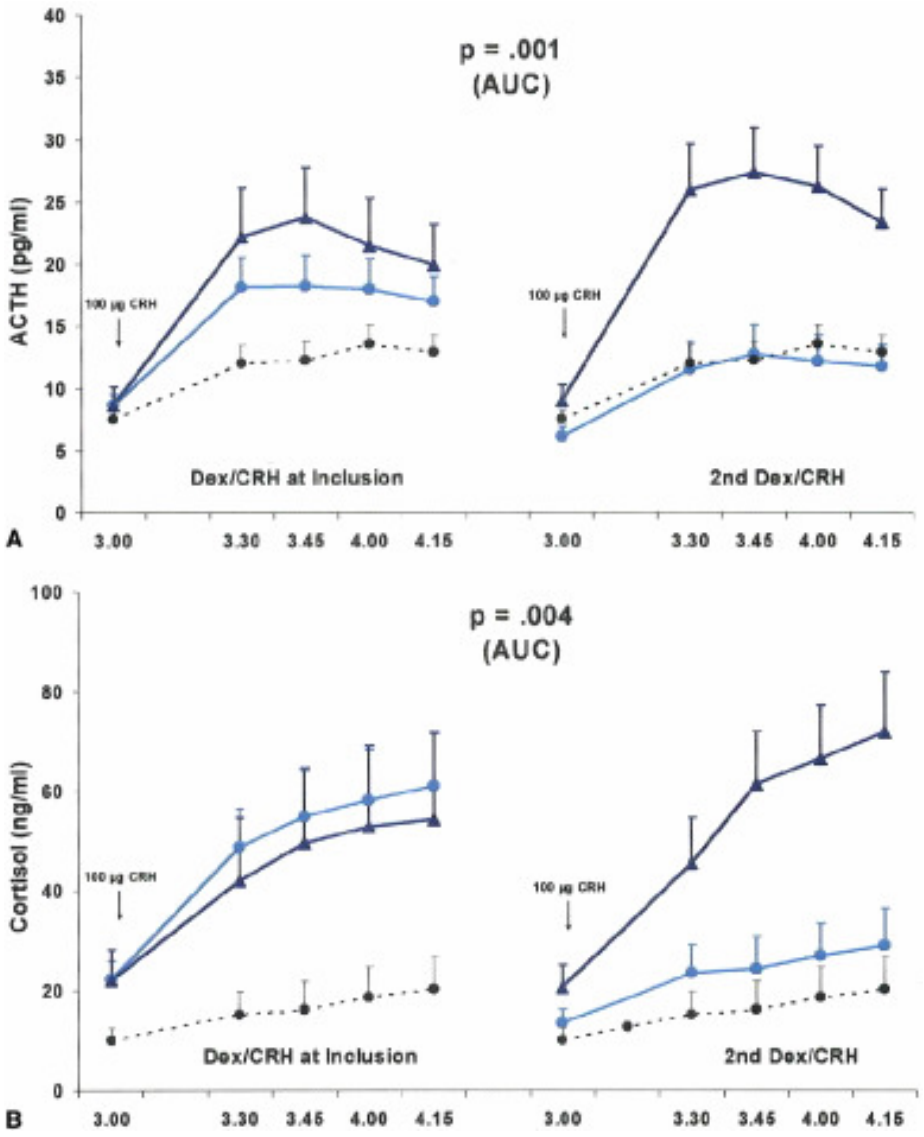


FIGURE 2: Rat endogenous CRF expression in (A) rostral hippocampus (B) caudal hippocampus and (C) paraventricular nucleus of the hypothalamus. FITC probe, Anti FITC POD, FITC Tyramide Amplification; FITC Filter CRF message probe is labeled with FITC-UTP. To amplify the signal an anti-FITC antibody is used. The antibody is conjugated to a peroxidase (POD) which reacts with the tyramide signal amplification (TSA). In this case the TSA uses FITC as the fluorophore. The amplification steps are necessary to achieve visible signal.

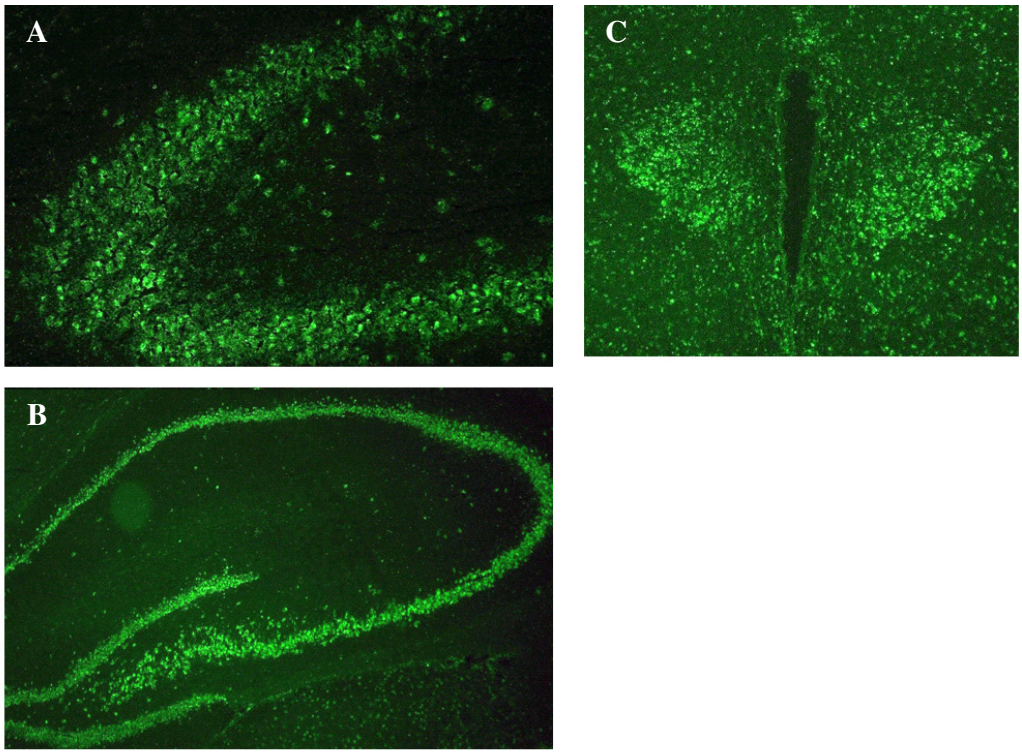
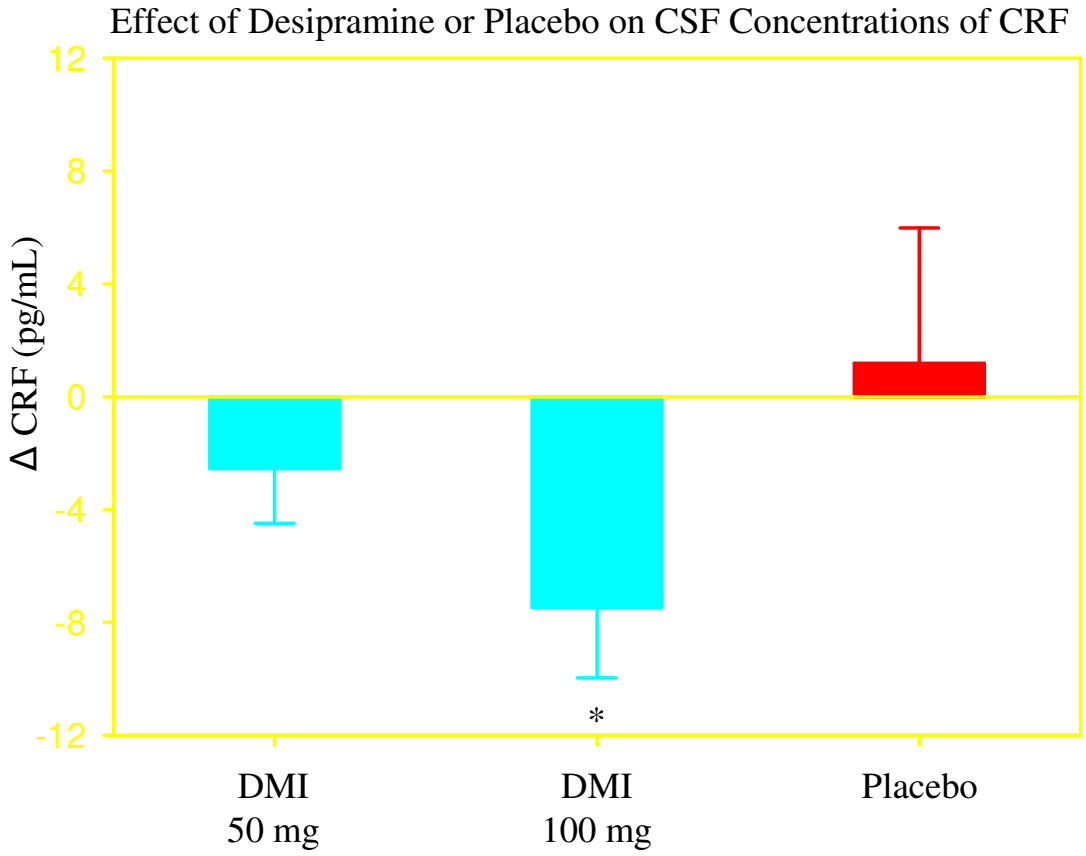


FIGURE 3: Dose- related change in cerebrospinal fluid levels of corticotropin releasing factor (CRF) in healthy volunteers after 50mg desipramine (DMI), 100mg DMI or placebo for 2 nights. Analysis of variance with repeated measures revealed a significant group x time interaction (*F = 5.9; df = 2,21: p<0.01*) indicating a differential effect of the two doses of DMI and placebo on CRF concentrations (* indicates a significant reduction from baseline by post hoc analysis: p = 0.02, basal vs. drug, *n = 8*) Modified from (Veith et al., 1993).



Appendix C: The Biology of Generalized Anxiety Disorder and Major Depressive Disorder: Commonalities and Distinguishing Features

Elizabeth I. Martin and Charles B. Nemeroff

Laboratory of Neuropsychopharmacology, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 101 Woodruff Circle, Suite 4000 WMRB, Atlanta, GA 30322, USA

For Publication in: The Future of Psychiatric Diagnosis: Refining the Research Agenda Depression and Generalized Anxiety Disorder

June 20-22, 2007

London, England

Generalized anxiety disorder (GAD) is commonly found comorbid with other anxiety disorders and with major depressive disorder (MDD). As syndromes, GAD and MDD share many symptoms and there are several treatments that are effective for both. However, despite this remarkable overlap, there exist many distinguishing features which are discordant with the hypothesis that GAD and MDD represent a single neurobehavioral disorder and should be described as such in the DSM-V. The goal of this review is to describe the key biological similarities and differences between MDD and GAD; these data should contribute towards the final determination as to whether MDD and GAD will remain distinct diagnoses in DSM V.

A. Anatomy of GAD and MDD

Symptoms of mood and anxiety disorders are thought to result in part from disruption in the balance of activity in emotional, limbic, centers of the brain relative to higher cognitive centers (Figures 1 and 2, TABLE 1). To paraphrase the late neuroanatomist Walle J.H. Nauta, in certain psychiatric disorders, the cerebral cortex is too loose a saddle on the limbic system. Recent advances in neuroimaging now permit elucidation of functional and anatomical alterations in patients with neuropsychiatric disorders. Unfortunately, remarkably few imaging studies have examined functional and structural CNS disruptions in GAD. Comparisons between different studies are made difficult due to variations in patient samples and diagnostic criteria, specific techniques employed, and methods of data analysis. Some discrepancies between earlier studies may have resulted from lack of resolution to identify differential activation of brain subregions.

1. Cortical activity is differentially disrupted in GAD and MDD in a subregion-specific manner.

The frontal cortex can be divided into numerous subregions, some of which exert unique effects upon normal (and pathological) mood and anxiety. The orbital frontal cortex (OFC) codes information, controls impulses, and regulates mood. In healthy control subjects, anxiety-inducing autobiographical memory scripts, but not sadness-inducing scripts, increase regional cerebral

blood flow (rCBF) in the left OFC. This result supports previous data demonstrating OFC activation in PTSD and panic disorder patients during symptom provocation (Liotti et al., 2000). In MDD patients, OFC volume has been reported to be decreased (Liotti et al., 2000). A role for the frontal cortex in MDD relapse vulnerability is suggested by data showing that acute mood challenge in non-medicated, acutely depressed and remitted MDD patients reduced rCBF in the OFC and medial prefrontal cortex (mPFC) compared to never-depressed control subjects (Keightley et al., 2003).

Successful treatment with antidepressant drugs influences frontal cortical activity in MDD and GAD patients. In GAD patients, successful treatment with paroxetine resulted in a diffuse set of metabolic changes; in MDD patients, paroxetine treatment demonstrated a more focused effect, decreasing OFC metabolism (Mayberg et al., 1999). Brain activity changes following cognitive behavioral therapy (CBT) has also been studied in MDD patients. Prior to treatment, the mPFC was hyperactive in depressed patients but successful CBT treatment decreased dorsal, ventral, and medial PFC activity. In contrast, paroxetine increased PFC metabolism (Mayberg et al., 1999). Antidepressant treatment has also been shown to increase right dorsal prefrontal cortical (dPFC) activity (Swanson and Simmons, 1989; Schulkin et al., 1998) and acute sadness induction in healthy subjects deactivated this region, suggesting dPFC hypoactivity may be involved in symptoms of depression (Holsboer, 2000). This hypothesis is supported by numerous studies showing hypometabolism and hypoperfusion in the dPFC (reviewed in (Arato et al., 1989; Bremner et al., 1997; Heim et al., 1997a, b; Holsboer, 2003)), though other studies reported no change in right PFC activity in euthymic or acutely depressed MDD patients during sadness induction (Plotsky et al., 1998).

The ventromedial (vm) subregion of the PFC is believed to be associated with MDD and the ventrolateral (vl) subregion with both GAD and MDD. The vmPFC is involved in reward processing (Holsboer and Barden, 1996; Owens and Nemeroff, 1999; Stout et al., 2002) and in the visceral response to emotions, which are enhanced by the right vmPFC and inhibited by the

left vmPFC (Koob and Bloom, 1985; Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Nestler et al., 2002; Claes, 2004). Disruption of the vmPFC in MDD patients is a potential neural correlate of anhedonia; in MDD patients, vmPFC activity is elevated in response to "happy" stimuli, while these stimuli had the opposite effect in healthy subjects (Nemeroff, 1988). In response to sadness induction, vlPFC activity is increased in euthymic and acutely depressed MDD patients compared to healthy controls (Stout et al., 2002) and vlPFC volume is decreased in MDD patients (Nutt, 2001). In adolescent GAD patients under resting conditions, vlPFC activity is also elevated relative to healthy control subjects. This elevation may represent a compensatory response rather than an underlying cause of GAD because within GAD patients, PFC activation correlates negatively with symptom severity (Fossey et al., 1996; Nutt, 2001; de Kloet et al., 2006; Risbrough and Stein, 2006).

2. Limbic and Paralimbic Regions are differentially disrupted in GAD and MDD

- **Insular Cortex and Cingulate Cortex**

The insular cortex integrates the sensory, affective, and cognitive components of pain and processes information regarding the internal bodily state. Like the frontal cortex, the insular cortex and cingulate cortex are divided into subregions distinguished by cytoarchitectonic, connectivity, and functional differences (Tsigos and Chrousos, 2002). The dorsal insula is granular, has major connections to the somatosensory cortex, and is activated by acute sadness. The ventral insula is agranular, involved in visceral sensation and autonomic responses via connections with the OFC and amygdala, and is activated by anxiety (Hendrick et al., 2000; Swaab et al., 2005).

The cingulate cortex also plays a role in the emotional components of pain perception. It can roughly be divided into anterior (ACC) and posterior (PCC) segments. The ACC can be further divided into pregenual and subgenual sections. The pregenual ACC is deactivated in euthymic, remitted MDD patients but activated in acutely depressed patients during provoked sadness (Seidman and Rabkin, 1998). Activity in the subgenual ACC is unchanged during a

depressive episode, but exhibits reduced activity during remission. Subgenual cingulate hypoactivity may result from treatment-induced compensatory changes, a reestablishment of homeostasis at a new set-point designed to better control intrusive thoughts and acute depression (Cameron and Nesse, 1988; Semeniuk et al., 2001). These and other data support the idea that ACC activity provides a marker for refractory MDD and is involved in emotion-attention interactions (Cooper and Ritchie, 2000). Subgenual ACC activity also increases following sadness-, but not anxiety-, induction in healthy subjects. In contrast, the PCC is deactivated by both sadness and anxiety compared to neutral memories (Kaneda and Fujii, 2001); in remitted MDD patients, PCC activity is increased (Le Melledo and Baker, 2004).

- **Amygdala**

The amygdala organizes the emotional response to stress; it is overactive in both GAD and MDD patients, potentially underlying the rumination on aversive or guilt-provoking memories that is common to both mood and anxiety disorders (Rubinow and Schmidt, 2006; Walf and Frye, 2006). Interestingly, some studies suggest that the left amygdala is most relevant to mood disorders while the right amygdala, which is more closely associated with fear and distress, plays a more prominent role in anxiety (reviewed in (Sichel et al., 1995; Gregoire et al., 1996)). The amygdala is highly interconnected with brain regions responsible for interpreting social behavior. As such, amygdalar hyperactivity may be associated with inaccurate interpretations of social behavior, a common symptom of GAD, via interactions with the superior temporal gyrus (STG), thalamus, and PFC (Schmidt et al., 2000).

Remarkably, an increase in resting amygdalar CBF may be specific to primary mood disorders; patients with OCD, phobias, or other neuropsychiatric conditions do not demonstrate increased resting amygdalar activity (Rubinow, 2005). The magnitude of increased rCBF and metabolism in the amygdala correlates with the severity of neurovegetative and emotional symptoms of a depressive episode (Musselman and Nemeroff, 1996). Amygdalar overactivity in MDD patients persists even in the absence of conscious processing as evidenced by sleep studies

(Rush et al., 2006), or in response to split-second presentation of fearful facial stimuli (Abraham et al., 2006; Lifschytz et al., 2006). This overactivity normalizes after successful antidepressant treatment (Cameron and Nesse, 1988). Some studies suggest that the left amygdala is functionally overactive but anatomically smaller in patients with MDD (Musselman and Nemeroff, 1996); others show that the amygdala is enlarged (e.g. (Nemeroff et al., 1985; Custro et al., 1994)). Unfortunately volumetric studies tend to be relatively inconsistent due to diverse patient samples and different methodologies used, rendering data interpretation difficult.

Although resting amygdala activation appears to be specific for mood disorders, symptom-provocation paradigms reveal anxiety-induced amygdalar activation, particularly in the right hemisphere (Cameron and Nesse, 1988; Munjack and Palmer, 1988). Unfortunately, of the few imaging studies in GAD patients, most were performed in the basal state and fail to identify GAD-related changes in brain activity. Additional mood-challenge studies specifically comparing GAD and MDD patients will likely be necessary to provide direct and much needed comparative data.

3. Distinct Cortical-Limbic Neural Networks mediate GAD and MDD.

Improved neuroimaging techniques and our greater understanding of the complicated interactions between brain regions highlight the importance of moving beyond simple examination of regional changes. To identify relevant distinctions between MDD and GAD, more research is needed to focus on identifying neural networks responsible for these disorders. Normal sadness and anxiety are thought to recruit completely separate cortical-limbic pathways; sadness is associated with a *dorsal* cortical deactivation while anxiety is associated with *ventral* cortical deactivation (Fossey et al., 1993). Future studies identifying neural network activity in GAD and MDD patients are expected to reveal similar distinctions between pathological sadness and anxiety.

Moreover, shifts in limbic-cortical networks may influence the transition between euthymic and episodic states in MDD patients. Both transient and chronic changes in negative mood may influence the direction of these limbic-cortical shifts. For example, connectivity

between subregions of the cingulate cortex, along with links to the brainstem, hypothalamus, and spinal cord, and ascending projections to the OFC, mPFC and dPFC provide a neural network through which primary autonomic information can influence learning, memory, reward, and reinforcement (Schatzberg, 2000). Sadness-induction studies support a role for this cingulate-PFC connection in depression; MDD patients, but not healthy control subjects, experience decreased activity in the dlPFC associated with sadness-induced elevations in the subgenual ACC (Schatzberg, 2000). Because these limbic-cortical connections are necessary for normal range and expression of emotion, disruption at any point in this network may result in symptoms of mood and anxiety disorders and, likewise, cognitive, pharmacological, and neurosurgical interventions may return homeostasis to this network via top-down or bottom-up effects (Karl and Herzog, 2006).

B. Neuroendocrinology

Neuroendocrine systems have been intensively studied in mood and anxiety disorders including the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadal (HPG), and hypothalamic-pituitary-thyroid (HPT) axes (TABLE 2).

1. HPA axis hyperactivity characterizes MDD but not GAD.

The HPA axis is comprised primarily of corticotropin-releasing factor (CRF) with cell bodies in the paraventricular nucleus of the hypothalamus (PVN), adrenocorticotropic hormone (ACTH) from the anterior pituitary and glucocorticoids (GC) from the adrenal cortex. This system is known to mediate the mammalian response to stress (e.g. (Hashimoto et al., 1996)). In many MDD patients, particularly those with severe or psychotic depression, the HPA axis exhibits marked hyperactivity as evidenced by the following:

1. At rest, plasma ACTH and cortisol concentrations are elevated compared to healthy volunteers,

2. Plasma ACTH and cortisol (and other GCs) are not suppressed by dexamethasone, a synthetic glucocorticoid, suggesting that HPA axis negative feedback is disrupted in MDD patients
3. When CRF is administered in a standard CRF stimulation test, plasma ACTH concentrations are blunted in MDD patients compared to healthy control subjects.
4. Administration of dexamethasone followed by CRF (the Dex/CRF test), generally considered the most sensitive measure of HPA axis activity, results in elevated plasma ACTH and GC concentrations in MDD patients compared to control subjects (reviewed in (Hou et al., 2006)).
5. Depressed patients exhibit elevated cerebrospinal fluid (CSF) CRF and cortisol concentrations.
6. Elevated concentrations of CRF and CRF mRNA expression in the PVN of depressed suicide victims.
7. Decreased CNS CRF_1 receptor mRNA expression in depressed suicide victims.

HPA axis hyperactivity in MDD has been hypothesized to result from decreased sensitivity to GC negative feedback and increased activity of hypothalamic CRF neurons. Evidence suggests that during a depressive episode, CRF is overexpressed in both hypothalamic and extrahypothalamic regions, the latter including the amygdala and bed nucleus of the stria terminalis (BNST). This is believed to result in elevated CSF CRF concentrations (e.g. (Griebel, 1999)). Elevated CSF CRF concentrations and HPA axis hyperactivity normalize upon recovery from depression, suggesting that these are state markers for a depressive episode rather than trait markers for MDD (Heilig, 2004). It has been hypothesized that return to normal HPA axis function is a shared property of all antidepressant treatments (e.g. (Sajdyk et al., 2004)).

Copious preclinical and clinical data support the hypothesis that CRF and stress play a causal role in symptoms of anxiety and depression (e.g.(Sajdyk et al., 2004)). Either chronic stress or direct CNS CRF administration in experimental animals lead to symptoms remarkably

similar to those observed in MDD patients (for review see (Yehuda et al., 2006)). Chronic antidepressant treatment prevents CRF activation *and* stress-induced behavioral responses but produces no such effect in unstressed controls, supporting the hypothesis that antidepressant efficacy depends on altered CRF signaling (Schatzberg, 2000).

Importantly, in the few studies available, GAD patients exhibit normal HPA axis activity and CRF secretion (Schatzberg, 2000). Thus GAD patients neither exhibit hypercortisolism nor DST non-suppression. HPA axis disruption and elevated CSF CRF concentrations have, however, been observed in other anxiety disorders, most notably, PTSD (reviewed in (Brawman-Mintzer et al., 1997; Koszycki et al., 2004)). That CRF and the HPA axis appear to play a less prominent role in GAD than in other anxiety disorders and MDD is somewhat surprising given the plethora of studies describing a role for CRF in anxiety-like behavior in experimental animals. Several explanations could explain this discrepancy: (1) more extensive GAD studies are required before concluding that CRF hyperactivity does not occur in GAD or (2) animal models based on stress-reactivity cannot adequately model GAD. Despite the lack of evidence of a role for CRF circuits in the pathophysiology of GAD, CRF antagonists have been demonstrated to possess anxiolytic as well as antidepressant activity.

2. The hypothalamic pituitary-gonadal axis in MDD and GAD.

The HPA and HPG axes are interlinked; the HPG axis is inhibited by CRF and GCs during the response to stress, and promoter elements in the CRF gene are regulated by sex-steroids (reviewed in (Pande et al., 1999)). Evidence suggests that the HPG axis is underactive in MDD as evidenced by decreased plasma concentrations of sex steroids (reviewed in (Schatzberg, 2000)). Furthermore, gonadal hormone replacement can reduce depression symptom severity in hypogonadal men who are unresponsive to SSRIs (Ogren et al., 2006).

Some evidence suggests that HPG axis activity is also decreased in GAD patients (Barrera et al., 2005; Karlsson and Holmes, 2006). In men, anxiety associated with hypogonadism is reportedly able to be treated with testosterone supplementation (e.g. (Nemeroff,

2003)). However, HPG axis alterations neither predicted treatment outcome nor correlated with anxiety symptoms in patients with GAD, suggesting a lack of a causal relationship between GAD and HPG axis alterations (Nutt, 2001).

Preclinical data suggest that female sex hormones influence anxiety, but very few clinical studies have specifically examined this relationship (reviewed in (Nemeroff, 2003)). Estrogen directly interacts with NP and NT systems involved in the regulation of mood and anxiety in humans and experimental animals, and estrogen receptors are found in brain regions relevant to mood and anxiety (see (Kalueff and Nutt, 2006) for review). In women, there is considerable evidence that estrogen exerts antidepressant effects particularly in postpartum depression (e.g. (Taylor et al., 2005)) and in the perimenopausal period (e.g. (Simon and Gorman, 2006)). These data do not, of course, necessarily support a direct causal role for estrogen in mood or anxiety disorders. The cyclicity of female sex hormone secretion may contribute to the susceptibility for mood and anxiety disorders by compounding genetic and environmentally-induced risk factors in other neural systems such as CRF neurons and the HPA axis. The individual response to this enhanced sensitivity is highly context dependent and likely depends on individual-specific alterations in other systems associated with anxiety and depression (discussed in (Zarate et al., 2006)).

3. HPT axis alterations are common in MDD but not in GAD.

Hypothyroidism is relatively common in MDD patients and may represent a trait marker in such MDD patients; unlike HPA axis disturbances which identify the state of a depressive episode, HPT axis alterations often have been demonstrated to be present in euthymic MDD patients (Chaw, 1967). There is considerable evidence that HPT axis disturbances play a causal role in MDD. Higher baseline plasma thyroxine (T_4) concentrations predict better outcome in depressed patients and T_4 concentrations normalize with effective treatment. Recent evidence from the large STAR-D study (Nutt, 2001) that augmenting antidepressants with triiodothyronine (T_3) in non-responders improves treatment response, confirm several previous findings ((Nutt, 2001)),

though it neither increases the rapidity or magnitude of the response in unselected patients treated with SSRIs [Garlow, Ninan, Dunlop and Nemeroff (in preparation)].

HPT axis function is best assessed using the thyrotropin-releasing hormone (TRH) stimulation test. One of the most reliable findings in MDD patients is the blunted thyroid-stimulating hormone (TSH) response to TRH (Porter et al., 2003). CSF concentrations of TRH have been reported to be elevated in depressed patients, which may in part explain this finding (reviewed in (Riedel et al., 2002)). There is also a very high prevalence rate of symptomless autoimmune thyroiditis (grade IV hypothyroidism) in MDD patients, as well as other types of hypothyroidism. Indeed primary hypothyroidism remains the leading medical cause of refractory depression (Malison et al., 1998; Maron et al., 2004). There is virtually no evidence of HPT axis alterations in GAD (Maron et al., 2004). CSF TRH concentrations are unaltered in patients with GAD, panic disorder, and OCD (Kent et al., 2002). However, the TSH response to TRH is blunted in OCD and panic disorder, but enhanced in PTSD patients (reviewed in (Kent et al., 2002)).

C. Neuropeptides

In addition to CRF, other neuropeptides such as neuropeptide Y (NPY), cholecystokinin (CCK), and galanin influence mood and anxiety (TABLE 2).

1. Neuropeptide Y is decreased in MDD and may be a neural correlate of resiliency to mood and anxiety disorders.

NPY is involved in the physiology of feeding behavior and is abundantly expressed in the CNS where it is colocalized with norepinephrine (NE) in several brain regions known to modulate emotion including the hypothalamus, hippocampus, and amygdala (Ressler and Nemeroff, 2000). NPY exerts an antidepressant-like effect in animal models of depression and may be involved in the pathophysiology of depression (see (Ressler and Nemeroff, 2000) for a review). Evidence supporting such a role includes reports that depressed patients have low plasma concentrations of

NPY, which are normalized by antidepressants (Ressler and Nemeroff, 2000). Interestingly, NPY concentrations in CSF were significantly lower in first episode MDD patients than in MDD patients in a recurrent episode, suggesting that NPY may be a valuable marker, and perhaps predictor, of a first depressive episode (Ressler and Nemeroff, 2000).

No clear role for NPY in the etiology of anxiety disorders has been established (Ansseau et al., 1988), but there are no published reports examining NPY in GAD. In experimental animals, NPY is known to exert anxiolytic effects (Coupland et al., 1992), potentially due to interactions with the CRF system (Pitchot et al., 1996). NPY and CRF are colocalized in, and have opposing effects on, the amygdala, locus coeruleus (LC) and periaqueductal gray (PAG). CRF receptor antagonists can prevent the anxiogenic effects of NPY receptor antagonists, and NPY can block CRF-induced anxiety (Meltzer et al., 1984). In humans, combat-exposed men without PTSD tended to have higher concentrations of plasma NPY than combat-exposed men with PTSD, suggesting that NPY could be a neural correlate of resiliency (Schittecatte et al., 1995).

2. Cholecystokinin provokes panic and anxiety but not depression.

CCK is found in the gastrointestinal system and vagus nerve and is located centrally in the amygdala, hippocampus, PAG, substantia nigra, and dorsal raphe nucleus (DRN) (reviewed in (Krishnan et al., 1988)). Remarkably, CCK agonists administered to healthy human subjects evoke severe anxiety symptoms similar to a short-lived panic attack that can be reduced with benzodiazepine treatment. Chronic administration of the antidepressant imipramine, also an effective treatment for panic disorder, decreases the acute anxiogenic effects of CCK (reviewed in (Tan et al., 2004)).

CCK has been hypothesized to play a role in GAD but not MDD; GAD patients are hypersensitive to CCK agonists, whereas MDD patients are not (Price and Lucki, 2001). These data suggest that a CCK receptor selective antagonist could represent a novel class of anxiolytics.

Such drugs have been developed and have not been demonstrated to possess anxiolytic efficacy (Price et al., 2002). They would also be unlikely to possess antidepressant properties.

3. Galanin has depressogenic effects and may modulate anxiety.

Galanin influences learning and memory, nociception, feeding, neuroendocrine and cardiovascular regulation (Nutt, 2001). Galanin is colocalized with monoamines in brainstem nuclei and inhibits firing in NE, 5-HT, and dopamine (DA) neurons. Galanin overexpression or administration in experimental animals has been reported to increase depression-like behavior. Importantly, i.c.v. administration of the nonselective galanin antagonist M35 produces antidepressant effects. Orally active non-peptidergic galanin antagonists are being developed and also appear to possess antidepressant properties (Kent et al., 2002). The role of galanin in anxiety, if any, is context-dependent and requires additional study (e.g. (Hoehn-Saric and McLeod, 2000).

D. Neurotransmitters

A myriad of studies have scrutinized classical NT systems in experimental animal models and patients with psychiatric disorders, revealing a complex interaction between neurochemistry and emotional and behavioral output (TABLE 3).

The aforementioned observed increases in CNS activity in GAD patients could result from decreased inhibitory or increased excitatory neurotransmission. Dysregulation of γ-aminobutyric acid (GABA) inhibitory neurotransmission has been documented in several anxiety disorders (reviewed in (Liotti et al., 2000)). The observed $GABA_A$ receptor downregulation in GAD patients is thought to play a role in the etiology of this illness (reviewed in (Papadimitriou et al., 1988)), and symptoms of GAD including excessive worry, hypervigilance, and psychomotor agitation are effectively treated with GABA receptor ($GABA_A$) agonists such as benzodiazepines and barbiturates (reviewed in (Dantzer, 2006; Raison et al., 2006; Pace et al., 2007).

Data supporting a role for GABAergic disruption in MDD are minimal and less consistent (see (Nutt, 2001; Vaswani et al., 2003) for review). Neuroimaging studies have identified reduced GABAergic activity and a reduced number of GABA neurons in the OFC of MDD patients (reviewed in (Nutt, 2001), and some studies suggested that GABAergic agonists may be effective in the treatment of depression. However, the US FDA rejected the application for adinazolam, a trialobenzodiazepine, as an antidepressant. Thus, unlike SSRIs (see below), benzodiazepines appear to be effective treatments for anxiety disorders but not major depression.

Evidence suggests that chronic antidepressant treatment downregulates the excitatory amino acid N-methyl-D-aspartate (NMDA) receptor which, in MDD patients, may be overexpressed. NMDA receptor downregulation could reduce brain excitability in anxiety patients. In depressed patients, antidepressant-induced elevations in neurogenesis could result from decreased glutamate-induced excitotoxicity secondary to NMDA receptor down regulation (reviewed in (Goldstein et al., 1996)). Recently, the NMDA antagonist ketamine has been reported to possess antidepressant properties (Anand and Shekhar, 2003).

Monoamine circuit disruption in the pathophysiology of mood disorders has been suggested since the serendipitous finding that monoamine depletion after reserpine treatment for hypertension causes depression in some patients. SSRIs and TCAs are effective antidepressants and anxiolytics, leading to the original hypothesis that 5-HT is deficient in MDD and GAD patients. Early studies found that brain concentrations of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) are decreased in depressed suicide victims (e.g. (Anand and Shekhar, 2003)). More recent data show that CSF 5-HT and 5-HIAA concentrations are normal in non-suicidal MDD patients and decreased in GAD patients (reviewed in (Mayberg et al., 1999)).

Further evidence supporting a segregated role for 5-HT circuitry in MDD and GAD etiology comes from challenge tests with 5-HT agonists. The 5-HT_{2c}/5-HT_3 agonist *m*-chlorophenylpiperazine (*m*CPP) elicits anxiety and anger symptoms in GAD patients but has no

such behavioral effects in MDD patients. In healthy subjects, administration of *m*CPP, the serotonin precursor L-tryptophan, or the 5-HT_{1A} agonist ipsapirone increased cortisol, ACTH and prolactin concentrations. Compared to control subjects, MDD patients exhibit a blunted prolactin response in the *m*CPP and L-tryptophan tests and a blunted cortisol response to ipsapirone challenge. Patients with primary anxiety disorders did not exhibit the blunted response to ipsapirone, even in those anxiety disorder patients with sub-syndromal depressive symptoms.

Midbrain serotonin transporter (SERT) density has been measured in MDD and GAD and was found to be decreased in the former and to negatively correlate with anxiety symptoms in both disorders. More recent studies replicated the negative correlation between SERT and anxiety symptoms in GAD, but also found no significant difference in SERT between GAD patients and control subjects. Additional studies measuring SERT in GAD patients before and after treatment would be of interest.

Some of the confusion regarding the role of 5-HT in anxiety may be explained by variability in target regions and receptor subtypes among 5-HT pathways. For example, presynaptic 5-HT_{1A} autoreceptor activation in the DRN is believed to be anxiolytic but postsynaptic 5-HT_{1A} receptor activation in the hippocampus is believed to be anxiogenic. 5-HT_{2A} receptor activation increases stress hormone release and 5-HT_{2A} antagonists are anxiolytic. Similarly, 5-HT_{2C} antagonists are anxiolytic, 5-HT_{2C} receptor agonists are anxiogenic, and some of the anxiolytic properties of antidepressants may result from 5-HT_2 receptor desensitization.

Although serotonergic transmission in general is thought to be decreased in MDD and GAD, NE neurotransmission is commonly thought to be elevated. Although increases and decreases in NE metabolites have been identified in MDD, anxiety symptoms have been associated with increases in NE metabolites. It has been hypothesized that NE signaling is elevated due to increased noradrenergic LC neuron firing and/or receptor "supersensitivity." Elevations in NE neurotransmission may indirectly contribute to MDD and GAD symptoms via amygdala overactivity and CRF overexpression.

In response to chronic antidepressant treatment, NE and NE metabolite concentrations are decreased in CSF and β-adrenergic receptors are downregulated. The effect on the NE system is the same whether the antidepressant has acute effects on 5-HT (SSRIs) or NE (SNRI) receptors. The ability of SSRIs and SNRIs to elevate 5-HT and decrease NE neurotransmission supports the hypothesis that both of these classes of drugs act to "reset" NT systems that are dysregulated in MDD and GAD.

As with 5-HT agonists, MDD and GAD patients respond differently to challenge tests with adrenergic and dopaminergic agonists. Clonidine, the presynaptic α2 partial agonist, and apomorphine, a dopaminergic agonist, elicit growth hormone (GH) release in healthy subjects. The response to both of these drugs is blunted in MDD patients, potentially due to a defect in catecholamines. Some studies have also suggested a blunted GH response to clonidine in anxiety disorders and a blunted GH response to apomorphine in OCD, but overall the data do not support the hypothesis of dopaminergic alterations in anxiety disorders. In MDD patients, the GH response to apomorphine correlated negatively with total duration of illness. Clonidine, administered during sleep, increases the interval between rapid eye movement (REM) stages of sleep. This response is blunted in MDD patients compared to healthy control subjects, GAD patients, and subjects with sub-syndromal depressive symptoms. These results are thought to reflect a deficit in central α2-adrenergic receptor signaling in MDD.

Neuroanatomical connections between NT and NP circuits render complex any interpretation of the role of individual NTs and NPs in the etiology of MDD and GAD. For example, 5-HT modulates GABAergic signaling within the PFC, but preclinical studies reveal that CRF enhances the effect of 5-HT on PFC GABA neurons, providing a pathway by which stress-induced CRF activation influences both 5-HT and GABA. This hypothesis is supported by previous data that CRF modulates brainstem serotonergic projections to forebrain regions, resulting in functional alterations in depressive-like behavior.

5-HT and CRF also influence noradrenergic signaling. Brainstem monoamine nuclei are reciprocally connected such that serotonergic projections from the DRN decrease NE cell firing in the LC whereas noradrenergic projections from the LC increase 5-HT cell firing in the DRN. Noradrenergic LC neurons interact with CRF systems coordinating the mammalian autonomic, endocrine and behavioral response to stress. It has been proposed that SSRIs, by increasing 5-HT availability, decrease activation of amygdala CRF neurons by LC NE projections. This decrease in amygdala activation may reduce anxiety and depressive symptoms (FIGURE 3).

These data suggest that, (1) although 5-HT and NE abnormalities exist in both MDD and GAD, the specific disturbances are quite different, (2) 5-HT and NE systems can influence mood through numerous pathways, and (3) the therapeutic effects of antidepressants result from complex effects on these systems.

E. GAD and MDD are characterized by distinct somatic symptoms.

Somatic symptoms of GAD and MDD may result from neuroanatomical, neuroendocrine, and NT disturbances in these disorders and these symptoms may also influence neural activity. One symptom common to all anxiety disorders is increased muscle tension. In contrast, psychomotor retardation and physical pain are common symptoms in depression, though psychomotor agitation is not infrequent. GAD patients also report excessive sweating, heart rate, and blood pressure when objective measures of these variables reveal no such alterations. After treatment, GAD patients report decreases in heart rate and muscle tension, despite the fact that no change in heart rate and muscle tension have occurred, suggesting that successful treatments "repair" the brain's ability to interpret internal bodily states.

Further support for somatic differences between GAD and MDD is derived from neuroimaging data. Invoking sadness increases motor and premotor cortex activity while acute anxiety activates the supplementary motor cortex and bilateral primary somatosensory cortex,

thought to result from intense feedback from somatic sensations, and likely contribute to incorrect interpretation of the body's internal state.

Sleep polysomnography studies show that, compared to healthy controls and GAD patients, MDD patients exhibit severe sleep disturbances including increased awakenings and increased shifts in sleep stage. Compared to healthy control subjects and GAD patients, MDD patients also exhibited longer duration of REM and shorter REM latency. In contrast, GAD patients exhibited longer sleep onset latency, shorter total sleep time, and shorter stage 2 sleep compared to healthy controls.

Additional biological distinctions between MDD and GAD include evidence that MDD is an inflammatory state, as for example illustrated by the increases in inflammatory cytokines. No such data exist for GAD.

Summary and Implications for Research

MDD and GAD are characterized by a variety of neuroendocrine, NT, and neuroanatomical disruptions; identifying the most functionally relevant differences is no easy task. NT system disruption in MDD and GAD is complicated by the high degree of interconnectivity between NT and NP-containing circuits in limbic, brain stem, and higher cortical brain areas. The well-documented effectiveness of SSRIs, in the treatment of depression and anxiety disorders likely results from the diverse role of 5-HT in the CNS and the manifold effects of SSRIs rather than a common underlying pathophysiology of serotonergic circuits in MDD and GAD.

Similarly, common neuroendocrine and NP systems are disrupted in MDD and GAD, but the magnitude and nature of those disruptions in symptom etiology is quite distinct. MDD patients often exhibit a hyperactive HPA axis, as measured by elevations in ACTH, cortisol and CRF. MDD patients also commonly exhibit a hypoactive HPG axis and numerous HPT axis alterations. In contrast, the HPA, HPG and HPT axes are largely unchanged in GAD patients. MDD patients appear to exhibit galanin hyperactivity. Its role in anxiety, if any, remains obscure.

NPY activity may influence resiliency to stress-sensitivity disorders including MDD and GAD, and CCK hypersensitivity is strongly implicated in anxiety disorders, but appears to play no role in depression.

Neuroanatomical studies also identify some similarities but clear differences between GAD and MDD patients. A 2001 article reviewing neuroanatomical findings in MDD and GAD suggested that GAD in general is associated with overactive neural circuitry whereas neural circuitry tends to be underactive in MDD patients. Modern neuroimaging research in psychiatric patients must examine not only regions of interest, but also interactions between brainstem, limbic, and higher centers and the NTs and NPs involved in those interactions. The amygdala in particular is a key structure in which CRF, monoamines, and psychological stress interact to potentially initiate symptoms of depression or anxiety. Some have suggested that amygdalar hyperactivity in MDD patients is secondary to decreased PFC activity. Decreased information processing in higher cognitive centers and increases in limbic centers may cause inaccurate perception of environmental and internal conditions, a symptom common to MDD and GAD. Reciprocal limbic-cortical networks clearly play an important role in emotional processing and additional research must dissect the network changes responsible for normal anxiety and sadness, as well as pathological mood and anxiety disorders.

Additional studies must also employ highly selective inclusion criteria to avoid confounds caused by comorbidity between both syndromal GAD and MDD, as well as taking into account patients with GAD with prominent depressive symptoms and the converse. It is not unlikely that conflicting data in much of the previous neuroimaging research results from overlapping subject populations in MDD and GAD studies, rather than a real similarity in the etiology of anxiety and depression. Importantly, despite the varied methodological differences between neuroimaging studies, provoked anxiety across diagnoses involves neural circuitry quite segregated from depression and normal sadness and continued research in this area is likely to

identify additional distinguishing features of normal and pathological anxiety compared to normal and pathological sadness.

The decision to classify MDD and GAD as distinct disorders must be based not only on clinical phenomenology but also on pathophysiology, genetics, course of illness, and treatment response data. Neuroendocrine, NT, and neuroanatomical differences between MDD and GAD patients, and healthy control subjects must be interpreted with care. Brain regions and NT systems implicated in mood and anxiety disorders have wide-ranging functions, many of which may be unrelated to the etiology of psychiatric disorders. Finally both of these disorders clearly represent complex gene-environment interactions. The clinical phenotype, GAD or MDD, may well be largely determined by individual differences in multiple genes that exhibit functional polymorphisms.

Acknowledgements

Supported by NIH MH-77083, MH-69056, MH-58922, MH-39415, MH-42088, and RR-00039

Financial Disclosures

In the past three years, Dr. Nemeroff consulted to, served on the Speakers' Bureau and/or Board of Directors, has been a grant recipient, and/or owned equity in one or more of the following: Abbott Laboratories, Acadia Pharmaceuticals, American Foundation for Suicide Prevention(AFSP), American Psychiatric Institute for Research and Educations(APIRE), AstraZeneca, BMC-JR LLC, Bristol-Myers-Squibb, CeNeRx, Corcept, Cypress Biosciences, Cyberonics, Eli Lilly, Entrepreneur's Fund, Forest Laboratories, George West Mental Health Foundation, GlaxoSmithKline, i3 DLN, Janssen Pharmaceutica, Lundbeck, National Alliance for Research on Schizophrenia and Depression(NARSAD), Neuronetics, NIMH, NFMH, NovaDel Pharma, Otsuka, Pfizer Pharmaceuticals, Quintiles, Reevax, UCB Pharma, Wyeth-Ayerst.

Currently, Dr. Nemeroff serves on the Scientific Advisory Boards of Astra-Zeneca, Johnson & Johnson, Pharma Neuroboost, Forest Laboratories, Quintiles and NARSAD. He is a grant recipient from NIH, NARSAD and AFSP. He serves on the Board of Directors of AFSP, APIRE, NovaDel Pharmaceuticals and the George West Mental Health Foundation. He owns equity in CeNeRx and Reevax. Dr. Nemeroff owns stock or stock options in Corcept, and NovaDel.

TABLE 1: Summary of select neuroanatomical and neuroimaging studies in MDD, GAD, and normal sadness and anxiety. "Treatment" refers to successful treatment.

	MDD and Normal Sadness	GAD, other Anxiety Disorders and Normal Anxiety
OFC	• Decreased volume in MDD • Decreased rCBF in euthymic and acute MDD • Decreased metabolism after SSRI treatment • Acute sadness had no effect	• Acute anxiety increase rCBF in left OFC • Overactive during symptom provocation in PTSD and panic disorder
mPFC	• Decreased rCBF in euthymic and acute MDD • Hyperactive in MDD • Decreased metabolism after CBT • Increased metabolism after SSRI	
dPFC	• Increased after antidepressant treatment • Hypometabolic in MDD	
vmPFC	• Happy stimuli increase activity in MDD but decrease in controls	
vlPFC	• Acute sadness increase activity in euthymic and acute MDD • Decreased volume in MDD	• Overactive in adolescent GAD patients as compensatory mechanism • Activation correlates negatively with severity.
Insular Cortex	• Acute sadness activates dorsal insula	• Acute anxiety activates ventral insula
Cingulate Cortex	• Pregenual ACC deactivated in euthymic MDD • Pregenual ACC activated in acute MDD • Subgenual ACC normal in acute MDD but hypoactive in remitted MDD patients • ACC and PCC activated by acute sadness	• Acute anxiety has no effect on ACC but deactivates the PCC
Amygdala	• Overactive at rest in primary mood disorders • Magnitude of activity correlates to severity • Overactivity without conscious perception • Normal activity after treatment • Smaller volume of left amygdala vs. controls	• Not overactive at rest • Overactive during symptom provocation • Right amygdala most relevant to anxiety

TABLE 2: Summary of select endocrine and neuropeptide disruptions in MDD, GAD, and normal sadness and anxiety

	MDD and Sadness	GAD, other anxiety disorders, and normal anxiety
HPA axis	• Hyperactive • Dexamethasone Non- Suppression • Blunted Response to CRF Stimulation	• Normal or slightly elevated in GAD • Associated with anxiety-like behavior in animals
CRF	• Overexpressed in hypothalamic and extrahypothalamic regions • Increased CSF concentrations	• Normal CSF concentrations in GAD • Overexpressed in PTSD and OCD
HPG axis	• Hypoactive • Increase susceptibility, not direct causality	• Hypoactive • Increase susceptibility, not direct causality
HPT axis	• Hypoactive in some patients • Blunted TSH response to TRH • Increased CSF TRH concentrations	• Unchanged • Normal CSF TRH concentrations • Blunted TSH response to TRH in OCD • Elevated TSH response to TRH in PTSD • Hyperthyroidism in panic disorder
NPY	• Decreased plasma concentrations • Lower CSF NPY in first episode MDD patients vs. recurrent MDD patients • Decreased in PFC of bipolar pts. • CSF NPY concentration is inversely proportional to anxiety scores in MDD	• Unchanged • Elevated NPY may confer resiliency to PTSD after combat exposure in men
CCK	• Unchanged	• CCK Hypersensitivity • Overexpressed in panic disorder Causes panic in healthy controls
Galanin	• Elevated in MDD	• Unclear, highly context dependent. • Modulatory, not causal

TABLE 3: Summary of select neurotransmitter abnormalities in MDD, GAD, and normal sadness and anxiety

	MDD and Sadness	GAD, other anxiety disorders, and normal anxiety
GABA	• Inconsistent • GABA-A agonists not approved for MDD by FDA	• Decreased GABA-A receptor density in GAD • GABA-A agonists are anxiolytic • Affinity for GABA-A predicts efficacy of benzodiazepines
Serotonin	• Decreased 5HIAA CSF concentrations in suicide victims • Normal in non-suicidal MDD patients • Blunted prolactin response to 5-HT agonists	• Decreased 5HIAA CSF concentrations in some studies
SERT	• Decreased density in midbrain • Density correlates negatively with anxiety symptoms in MDD	• Density correlates negatively with anxiety symptoms in GAD
$5HT_{1A}$		• Anxiolytic as DRN autoreceptors • Anxiogenic as hippocampus postsynaptic receptors
$5HT_2$	• Desensitized by antidepressants	• Anxiogenic • Antagonists are anxiolytic
Norepinephrine	• Elevated in CSF and plasma of severe melancholic MDD patients • Unchanged in nonmelancholic MDD patients • Blunted GH response to clonidine • Blunted REM response to clonidine	• Unchanged in GAD

FIGURE 1: Cortical Regions involved in GAD and MDD

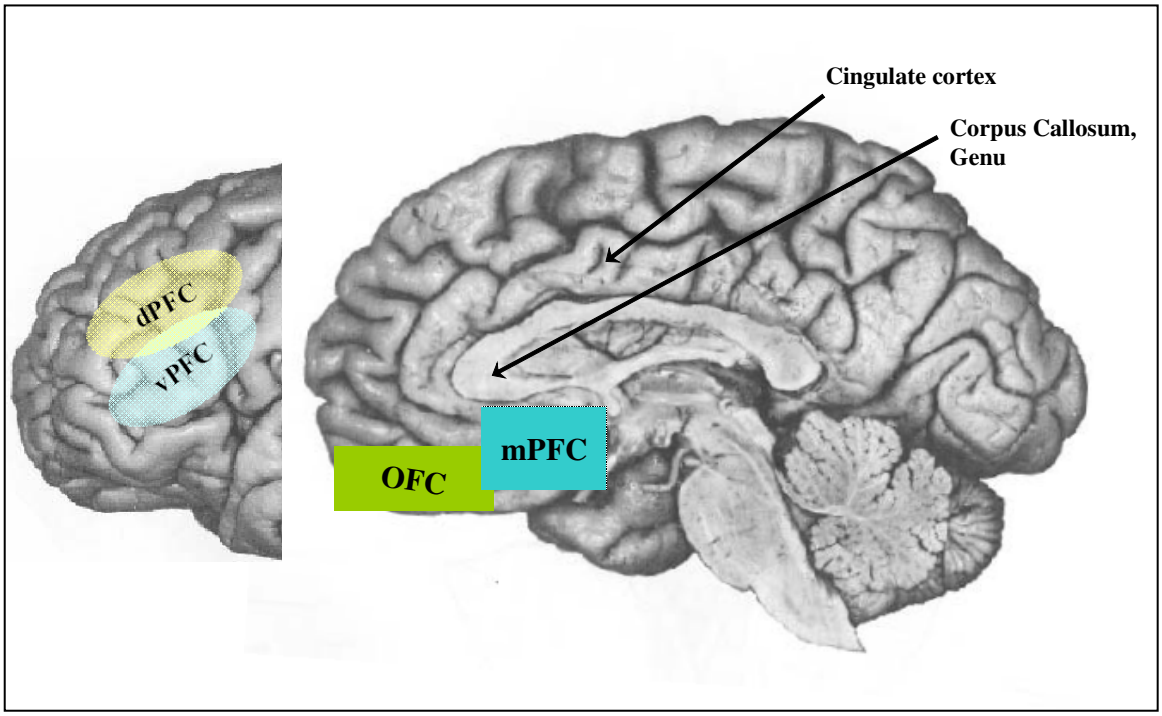


FIGURE 2: Limbic and Paralimbic Regions involved in GAD and MDD

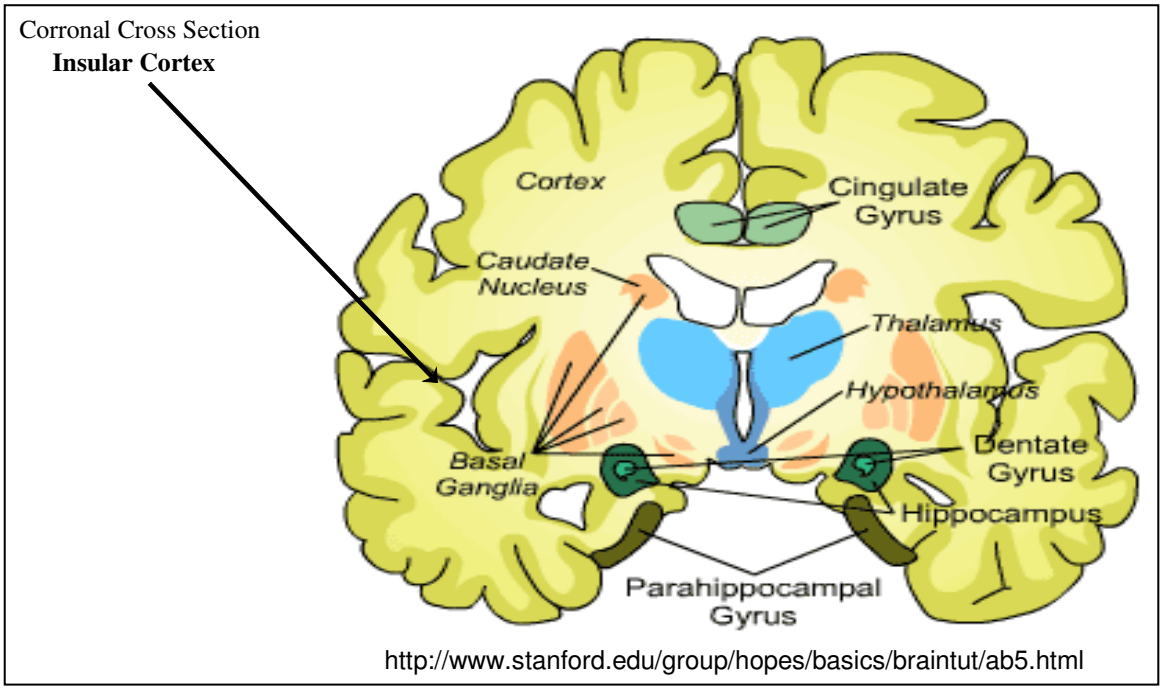
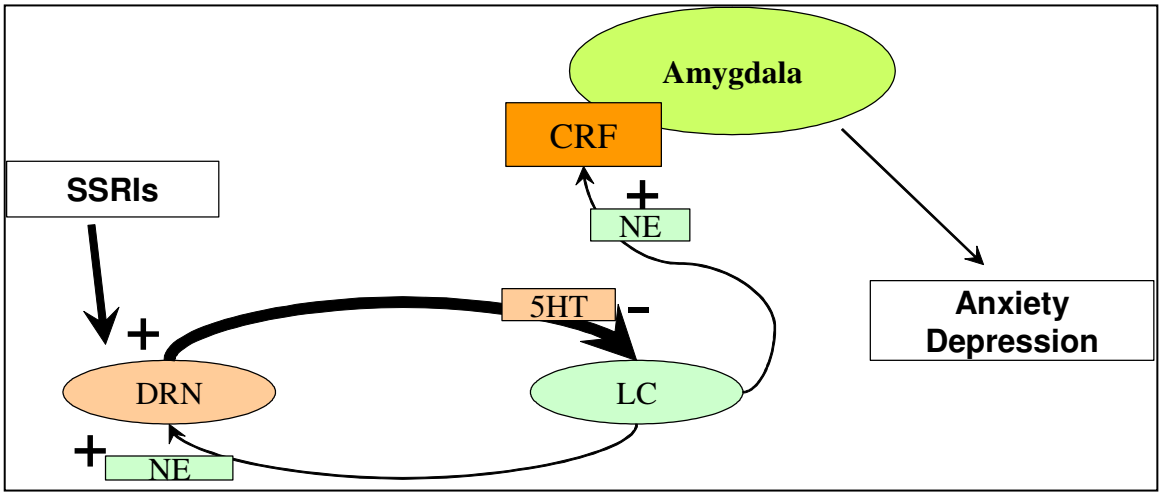


FIGURE 3: Hypothesized mechanism of action of SSRIs



Appendix D: Diurnal Regulation of Gene Expression in Select Brain Regions of Adult Male Sprague Dawley Rats

Elizabeth I. Martin, Terrell E. Brotherton, Becky Kinkead, K.V. Thrivikraman, Paul M. Plotsky, Charles B. Nemeroff, Michael J. Owens

Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta GA 30322, USA

Society for Neuroscience Convention, 2007 San Diego, CA

Supported by NARSAD, DA015040, MH58922, MH42088, NIH MH-77083, MH-69056, MH-58922, MH-39415, and MH-42088.

INTRODUCTION

Gene expression changes in the suprachiasmatic nucleus of the hypothalamus (SCN) are known to regulate circadian rhythmicity, other brain regions also exhibit diurnal changes in gene expression, and diurnal endocrine cycles such as the hypothalamic-pituitary-adrenal (HPA) axis contribute to, and are influenced by these gene-expression patterns. However, many questions remain regarding the relationship between these diurnal expression patterns and circadian activity. One potential confound for comparing results of neuroscience experiments between laboratories is the wide variability of protocols, making it difficult to replicate studies from other labs and to interpret disparate results between studies. In particular, the time of day in which behavioral experiments are performed and tissue samples are obtained varies between studies. The goal of this study was to identify changes in gene expression in morning and evening tissue samples from adult male Sprague Dawley rats. Future work will use this information to examine the role of these genes in homeostasis and circadian rhythms.

MATERIALS and METHODS

Sample Procurement: Adult male Sprague Dawley rats (n=5/time point) were killed 2hrs after lights on (0900) or 2hrs after lights off (2100). Brains were removed and frozen on dry ice until dissected into prefrontal cortex, amygdala, septum, hippocampus, and hypothalamus. Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen). Quality and quantity of RNA was determined by the absorbance method, followed by evaluation of the integrity of 28S and 18S RNA on a Bioanalyzer (Agilent). OD 260/280 values ranged from 1.99 to 2.05. Two or three samples from each time point were assayed using Affymetrix rat neurobiology U34 array (Cat# 900283).

Microarray*:* Affymetrix RN-U34 contained 1263 Oligo probes (25-mer) including 160 ESTs. Homology of the EST's determined using the public domain BLAST program from the NCBI web site. Affymetrix protocols were strictly followed in the preparation of labeled RNA

targets and hybridization. Expression analyses (absolute and comparison) were performed according to Affymetrix algorithms.

Data Analysis: For each region, expression tables display genes of interest that are increased (top row) or decreased (bottom row) in the AM samples relative to the PM samples. Within each table, the order in which the genes are listed reflects the magnitude of that difference. Genes of interest were identified based on three criteria:

- Detection Value: At least one of the two samples in an individual comparison must be identified as "present" with the Affymetrix algorithm. Comparison files in which both samples had an "absent" call are represented as N/A.
- Signal Log Ratio: A signal log ratio (SLR) of +/- 1 is equal to a two-fold change in gene expression.
- Difference: At least three of the four (Hypothalamus, Hippocampus, Septum) or six (PFC, Amygdala) comparisons were determined by Affymetrix to be increased or decreased.

SUMMARY and CONCLUSIONS

- Given the high quality of the samples and robust pattern of changes in duplicate oligos representing the same gene, the genes in the "other" category are likely due to individual variability between subjects.
- The observed down-regulation of gene expression as rats cycle from the active to the quiescent period could result from decreases in protein kinase expression.
- *Strong* differences between the AM and PM samples were identified in only a small number of genes.
    - In the hippocampus, Jun-D and copper/zinc SOD decreased by over two-fold in the AM relative to the PM samples (Average SLR -1.075 and -1.225).

    - In the hypothalamus, decreases were observed in transcripts for oxytocin (SLR -1.225) and vasopressin (SLR -0.925), potentially resulting from the peak in corticosterone before "lights-off".
    - Also in the hypothalamus, dopamine transporter expression was increased in the AM relative to the PM sample by nearly two-fold (SLR 0.975).
- *Moderate* gene expression changes were identified in NT receptors and related enzymes including monoamine oxidase-A and tyrosine hydroxylase as well as transporters, channels, and kinases.
- All five regions also showed moderate gene expression changes in mRNA for transcription factors and immediate early genes such as Jun-D and NGFI-B. The particularly short half-life for these gene products may account for much of their variability between time points.

TABLE 1: Number of Genes Detected

Expressed: Affymetrix detection "present"- above background in all samples
Not Expressed: Affymetrix detection "absent"- no detectable level of expression above background
Other: Detection level within group differed between chips.

	Amygdala		**Hippocampus**		**Hypothalamus**		**Prefrontal Cortex**		**Septum**	
	AM	*PM*	*AM*	*PM*	*AM*	*PM*	*AM*	*PM*	*AM*	*PM*
Expressed	518	544	575	568	522	537	489	525	524	565
Not Expressed	538	600	569	596	635	586	591	561	622	593
Marginal Expression	0	8	5	10	9	2	0	3	5	8
OTHER	207	111	111	89	97	138	183	174	112	97

TABLE 2: Signal log ratio (SLR) of comparisons between AM and PM samples in Individual Brain Regions

For each brain region the top table displays gene expression increases in AM vs. PM samples; the bottom table displays decreases in AM vs. PM samples.

*: These samples were part of a second run
N/A: neither sample had detectible levels of gene expression

= Increase = Decrease = No Change

TABLE 2A

HIPPOCAMPUS

INCREASE

Gene ID	Description	AM_4 vs. PM_3	AM_4 vs. PM_4	AM_5 vs. PM_3	AM_5 vs. PM_4
L09119_g_at	C kinase substrate calmodulin-binding protein (RC3)	0.2	0.3	0.2	0.3
M28648_s_at	Na,K-ATPase alpha-2 subunit	0.0	0.4	0.3	0.8
U37147_at	Sodium channel beta 2 subunit (SCNB2) gene	0.8	0.3	0.4	-0.1

DECREASE

Gene ID	Description	AM_4 vs. PM_3	AM_4 vs. PM_4	AM_5 vs. PM_3	AM_5 vs. PM_4
M60654_at	Alpha-1A-adrenergic receptor	-0.4	-0.4	-0.6	-0.8
Rc_AA892814_s_at	cDNA, 3 end /clone gb=AA892814	-0.4	-0.2	-0.4	-0.3
X60769mRNA_at	Silencer factor B, unknown cds	-0.7	-0.3	-0.5	-0.3
U11419_at	Glutamate receptor, Ionotropic, NMDA2B	-0.4	-0.2	-0.6	-0.4
U53859_at	Calpain small subunit (css1)	-0.4	-0.3	-0.3	-0.1
D26307cds_at	Rat jun-D gene, complete cds	-2.6	*A/A*	-0.6	0.3

TABLE 2B

HYPOTHALAMUS

INCREASE

Gene ID	Description	AM_6 vs. PM_5	AM_6 vs. PM_6	AM_7 vs. PM_5	AM_7 vs. PM_6
M80570_at	Dopamine transporter mRNA,	0.1	0.8	1.1	1.9
L00603_at	Vesicular monoamine transporter	0.5	0.5	0.9	1.0
M10244_at	Tyrosine hydroxylase	0.1	0.5	1.0	1.5
S76145_s_at	Dopamine transporter	0.1	0.6	0.8	1.1
Y00497_s_at	Manganese-containing superoxide dismutase (MnSoD)	0.8	0.4	0.5	0.2

DECREASE

Gene ID	Description	AM_6 vs. PM_5	AM_6 vs. PM_6	AM_7 vs. PM_5	AM_7 vs. PM_6
K01701_at	Oxytocin/neurophysin (Oxt) gene	-1.9	-1.7	-0.7	-0.6
M64785_g_at	Vasopressin (VP)	-1.0	-1.1	-0.8	-0.8
M25646_at	Vasopressin, complete cds	-0.7	-0.6	-0.6	-0.4

TABLE 2C

SEPTUM

INCREASE

Gene ID	Description	AM_{11} vs. PM_9	AM_{11} vs. PM_{10}	AM_{12} vs. PM_9	AM_{12} vs. PM_{10}
U09211_at	Acetylcholine transporter	0.5	0.7	0.5	0.6
X56306_s_at	Splicing variant of substance P	1.1	0.4	0.6	-0.2
X80395cds_s_at	Vesicular Acetylcholine transporter	0.5	0.4	0.5	0.6
J04486_at	Insulin growth binding protein	1.0	0.5	0.4	0.0
M15191_s_at	Beta-tachykinin	0.9	0.3	0.5	-0.1

DECREASE

Gene ID	Description	AM_{11} vs. PM_9	AM_{11} vs. PM_{10}	AM_{12} vs. PM_9	AM_{12} vs. PM_{10}
D26307cds_at	Jun-D	-0.8	-0.8	-4.7	-4.5
Rc_AI029183_s_at	3 end clone; gbAI029183	-0.7	-0.5	-0.5	-0.3
Rc_AI176456_at	3 end clone; gbAI176456	-0.4	-0.4	-0.2	-0.2
Rc_AI227647_s_at	3 end clone; gb AI227647	-0.4	-0.4	-0.1	-0.3

TABLE 2D

PREFRONTAL CORTEX

INCREASE

Gene ID	Description	AM_8 vs. PM_8	AM_{10} vs. PM_7*	AM_9* vs. PM_8	AM_{10} vs. PM_8	AM_8 vs. PM_7*	Am_{10} vs. PM_7*
L14851_at	neurexin III-alpha	0.3	0.0	1.4	0.4	-0.2	0.0
Rc_AA800602_s_at	cDNA, 3 end /clone gb=AA800602	0.5	0.0	0.3	0.5	0.3	0.1
Rc_AA900476_g_at	cDNA, 3 end /clone gb=AA900476	0.4	-0.8	1.3	1.1	-0.3	0.0
Rc_AI072060_s_at	cDNA, 3 end /clone gb=AA900476	0.4	0.3	0.5	0.3	-0.3	-0.1

DECREASE

Gene ID	Description	AM_8 vs. PM_8	AM_{10} vs. PM_7*	AM_9 * vs. PM_8	AM_{10} vs. PM_8	AM_8 vs. PM_7*	Am_{10} vs. PM_7*
AB013130_at	Synaptopodin, complete cds	-0.1	*N/A*	-0.7	-0.2	-0.6	-0.7
X62952_at	Vimentin	-0.5	-0.8	-0.7	-0.3	-0.2	-0.1
S75687_s_at	Glutamate/aspartate transporter	0.0	-0.1	-1.7	-0.9	-2.0	-1.8
S55933_i_at	GABA-A receptor alpha 4 subunit	0.0	-0.4	-1.6	-1.0	-0.5	-0.5
M91595exon_s_at	Insulin-like growth factor binding protein-2 gene, exon1	-0.7	-0.5	-0.9	-0.4	-0.3	-0.2
AF109405_s_at	GABA-B receptor 2	0.0	-0.1	-1.0	-0.5	-0.6	-2.1
AF030086UTR#1_at	Activity & neurotransmitter-induced early gene 1 (ania-1)	-0.8	-0.6	-0.3	-1.4	0.6	0.5
Z38067exon_at	c-myc, exon 2	-0.3	-0.1	-0.7	-0.2	-0.2	-0.2
M64300_at	Extracellular signal-related kinase (ERK2)	-0.2	-0.2	-1.5	-0.8	-0.4	-0.2
L18889_at	Calnexin	-0.1	-0.3	-1.3	-0.5	-0.6	-0.2
U17254_g_at	Immediate early gene transcription factor NGFI-B	-0.5	-0.8	-0.3	-0.3	0.7	0.2
M16960_s_at	Calcium-calmodulin-dependent protein kinase II, partial cds	-0.4	-0.5	-0.4	-0.6	0.2	0.3
D16817_g_at	Metabotropic glutamate receptor mGluR7	0.0	*N/A*	-0.4	-0.2	-0.6	-0.7
rc_AI231354_at	cDNA, 3 end /clone gb=AI227665	-0.5	0.4	0.8	0.2	-0.4	-0.7
X17012mRNA_s_at	IGFII gene for insulin-like growth factor II	-0.4	-0.6	-0.7	0.0	0.8	0.6

TABLE 2E

AMYGDALA

INCREASE

Gene ID	Transcript	AM_1 vs. PM_1	AM_1 vs. PM_2	AM_2 vs. PM_1	AM_3 vs. PM_1	AM_2 vs. PM_2 *	AM_3 vs. PM_2 *
M22357_at	Myelin-associated glycoprotein	-1.3	*N/A*	0.7	1.1	1.9	2.3
Rc_AI231354_at	cDNA, 3 end/cone gb: AI231354	-0.4	0.2	0.6	0.5	1.3	0.9
M91595exon_s_at	Insulin-like growth factor binding protein-2 gene, exon 1	-0.2	0.2	0.4	0.7	0.8	1.1
U38812_s_at	Olfactory inositol 1, 4, 5-trisphosphate receptor (InsP3R)	*N/A*	*N/A*	0.5	0.5	3.0	2.7
J04486_at	Insulin growth factor-binding protein	0.1	0.1	0.3	0.8	0.3	0.7
D38380_at	Transferrin	-0.1	-0.2	0.3	0.8	0.2	0.7
D38492_at	Neural adhesion molecule F3	-0.9	0.1	0.1	0.4	1.0	1.3
U56261_s_at	SNAP-25a	-0.6	0.1	0.6	0.2	1.3	1.0

DECREASE

Gene ID	Transcript	AM_1 vs. PM_1	AM_1 vs. PM_2	AM_2 vs. PM_1	AM_3 vs. PM_1	AM_2 vs. PM_2 *	AM_3 vs. PM_2 *
D26307cds_at	Jun-D	-2.9	-2.7	0.5	-2.6	0.5	-2.5
Rc_AA925495_at	cDNA, 3 end/clone; gb=AA925495	0.3	0.1	-1.0	-0.7	-1.0	-0.8
AF050659UTR1_at	Activity and neurotransmitter induced early gene 7 (ania-7)	0.2	0.4	-0.9	-0.8	-0.6	-0.5
M16960_s_at	Calcium-calmodulin-dependent protein kinase II	-0.2	-0.3	-0.1	-0.4	-0.2	-0.6
Rc_AI009268_at	cDNA, 3 end/clone; gb = AI009268	0.0	0.2	-0.9	-0.6	-0.7	-0.4
Rc_AI230211_at	Potassium voltage gated channel	0.3	-0.1	-0.3	-0.4	-0.8	-0.9
S59525_s_at	Gonadotropin-releasing hormone receptor	0.6	0.5	-0.7	-0.8	-0.7	-1.3
U88324_at	G protein beta 1 subunit (rGb1)	0.2	-0.3	-0.2	-0.2	-0.8	-0.8
AF-031430_at	Syntaxin 7	0.4	-0.4	-0.2	-0.1	-1.2	-1.1
L14851_at	Neurexin III-alpha gene	0.7	-0.1	-0.1	-0.3	-0.9	-1.0
Rc_AA900476_g_at	cDNA, 3 end/clone; gb=AA900476	1.2	-0.1	-0.5	-0.1	-1.7	-1.3
Rc_AI227660_s_at	cDNA, 3 end/clone; gb = AI227660	0.9	-0.5	-0.1	0.0	-1.5	-1.5
Rc_AI230404_s_at	cDNA, 3 end/clone; gb = AI230404	0.6	-0.5	-0.3	-0.2	-1.3	-1.2
S71570_s_at	Calcium-calmodulin-dependent protein kinase II isoform gamma-B	0.4	-0.2	-0.1	-0.1	-0.9	-0.8
U88324_g_at	G protein beta 1 subunit (rGb1)	0.7	-0.4	-0.3	-0.2	-1.4	-1.3

WORKS CITED

(2000) DSM-IV-TR. Washington, DC American Psychiatric Association.

Abraham G, Milev R, Stuart Lawson J (2006) T3 augmentation of SSRI resistant depression. J Affect Disord 91:211-215.

Adams MR, Kaplan JR, Koritnik DR (1985) Psychosocial influences on ovarian endocrine and ovulatory function in Macaca fascicularis. Physiol Behav 35:935-940.

Aguilera G (1994) Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol 15:321-350.

Akmaev IG, Kalimullina LB, Sharipova LA (2004) The central nucleus of the amygdaloid body of the brain: cytoarchitectonics, neuronal organization, connections. Neurosci Behav Physiol 34:603-610.

Anand A, Shekhar A (2003) Brain imaging studies in mood and anxiety disorders: special emphasis on the amygdala. Ann N Y Acad Sci 985:370-388.

Anisman H, Merali Z, Stead JD (2008) Experiential and genetic contributions to depressive- and anxiety-like disorders: clinical and experimental studies. Neurosci Biobehav Rev 32:1185-1206.

Ansseau M, Von Frenckell R, Cerfontaine JL, Papart P, Franck G, Timsit-Berthier M, Geenen V, Legros JJ (1988) Blunted response of growth hormone to clonidine and apomorphine in endogenous depression. Br J Psychiatry 153:65-71.

Arato M, Banki CM, Bissette G, Nemeroff CB (1989) Elevated CSF CRF in suicide victims. Biol Psychiatry 25:355-359.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999) The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1-12.

Asan E, Yilmazer-Hanke DM, Eliava M, Hantsch M, Lesch KP, Schmitt A (2005) The corticotropin-releasing factor (CRF)-system and monoaminergic afferents in the central amygdala: investigations in different mouse strains and comparison with the rat. Neuroscience 131:953-967.

Azima H, Vispo RH (1958) Imipramine; a potent new anti-depressant compound. Am J Psychiatry 115:245-246.

Baker DG, Ekhator NN, Kasckow JW, Hill KK, Zoumakis E, Dashevsky BA, Chrousos GP, Geracioti TD, Jr. (2001) Plasma and cerebrospinal fluid interleukin-6 concentrations in posttraumatic stress disorder. Neuroimmunomodulation 9:209-217.

Baker GB, Coutts RT, McKenna KF, Sherry-McKenna RL (1992) Insights into the mechanisms of action of the MAO inhibitors phenelzine and tranylcypromine: a review. J Psychiatry Neurosci 17:206-214.

Baker SL, Kentner AC, Konkle AT, Santa-Maria Barbagallo L, Bielajew C (2006) Behavioral and physiological effects of chronic mild stress in female rats. Physiol Behav 87:314-322.

Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, Kalin NH (2002) Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. J Neurosci 22:2926-2935.

Bale TL (2006) Stress sensitivity and the development of affective disorders. Horm Behav 50:529-533.

Bale TL, Vale WW (2004) CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol 44:525-557.

Bali B, Kovacs KJ (2003) GABAergic control of neuropeptide gene expression in parvocellular neurons of the hypothalamic paraventricular nucleus. Eur J Neurosci 18:1518-1526.

Barbany G, Persson H (1992) Regulation of Neurotrophin mRNA Expression in the Rat Brain by Glucocorticoids. Eur J Neurosci 4:396-403.

Barbas H (2007) Flow of information for emotions through temporal and orbitofrontal pathways. J Anat 211:237-249.

Barrera G, Echevarria DJ, Poulin JF, Laforest S, Drolet G, Morilak DA (2005) One for all or one for one: does co-transmission unify the concept of a brain galanin "system" or clarify any consistent role in anxiety? Neuropeptides 39:289-292.

Bartanusz V, Muller D, Gaillard RC, Streit P, Vutskits L, Kiss JZ (2004) Local gamma-aminobutyric acid and glutamate circuit control of hypophyseotrophic corticotropin-releasing factor neuron activity in the paraventricular nucleus of the hypothalamus. Eur J Neurosci 19:777-782.

Bedecs K, Berthold M, Bartfai T (1995) Galanin--10 years with a neuroendocrine peptide. Int J Biochem Cell Biol 27:337-349.

Berga SL, Loucks TL (2005) The diagnosis and treatment of stress-induced anovulation. Minerva Ginecol 57:45-54.

Binder EB, Kinkead B, Owens MJ, Nemeroff CB (2001) Neurotensin and dopamine interactions. Pharmacol Rev 53:453-486.

Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, Ressler KJ (2008) Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. Jama 299:1291-1305.

Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Kunzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Kohnlein O, Dabitz H, Bruckl T, Muller N, Pfister H, Lieb R, Mueller JC, Lohmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B (2004) Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat Genet 36:1319-1325.

Binneman B, Feltner D, Kolluri S, Shi Y, Qiu R, Stiger T (2008) A 6-week randomized, placebo-controlled trial of CP-316,311 (a selective CRH1 antagonist) in the treatment of major depression. Am J Psychiatry 165:617-620.

Bitran D, Shiekh M, Dowd JA, Dugan MM, Renda P (1998) Corticosterone is permissive to the anxiolytic effect that results from the blockade of hippocampal mineralocorticoid receptors. Pharmacol Biochem Behav 60:879-887.

Bonaz B, Rivest S (1998) Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. Am J Physiol 275:R1438-1449.

Bradley RG, Binder EB, Epstein MP, Tang Y, Nair HP, Liu W, Gillespie CF, Berg T, Evces M, Newport DJ, Stowe ZN, Heim CM, Nemeroff CB, Schwartz A, Cubells JF, Ressler KJ (2008) Influence of child abuse on adult depression: moderation by

the corticotropin-releasing hormone receptor gene. Arch Gen Psychiatry 65:190-200.

Bradley RG BE, Epstein MP, Tang Y, Nair HP, Liu W, Gillespie CF, Berg T, Evces M, Newport DJ, Stowe ZN, Heim CM, Nemeroff CB, Schwartz A, Cubells JF and Ressler KJ. (2007) Influence of Child Abuse on Adult Depression is Moderated by the Corticotropin Releasing Hormone Receptor Gene. Archives of General Psychiatry In Press.

Brawman-Mintzer O, Lydiard RB, Bradwejn J, Villarreal G, Knapp R, Emmanuel N, Ware MR, He Q, Ballenger JC (1997) Effects of the cholecystokinin agonist pentagastrin in patients with generalized anxiety disorder. Am J Psychiatry 154:700-702.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154:624-629.

Bugnon C, Hadjiyiassemis M, Fellmann D, Cardot J (1983) Reserpine-induced depletion of corticoliberin (CRF)-like immunoreactivity in the zona externa of the rat median eminence. Brain Res 275:198-201.

Butler PD, Weiss JM, Stout JC, Nemeroff CB (1990) Corticotropin-releasing factor produces fear-enhancing and behavioral activating effects following infusion into the locus coeruleus. J Neurosci 10:176-183.

Buwalda B, de Boer SF, Van Kalkeren AA, Koolhaas JM (1997) Physiological and behavioral effects of chronic intracerebroventricular infusion of corticotropin-releasing factor in the rat. Psychoneuroendocrinology 22:297-309.

Buwalda B, Van Kalkeren AA, de Boer SF, Koolhaas JM (1998) Behavioral and physiological consequences of repeated daily intracerebroventricular injection of corticotropin-releasing factor in the rat. Psychoneuroendocrinology 23:205-218.

Cameron OG, Nesse RM (1988) Systemic hormonal and physiological abnormalities in anxiety disorders. Psychoneuroendocrinology 13:287-307.

Campbell BM, Morrison JL, Walker EL, Merchant KM (2004) Differential regulation of behavioral, genomic, and neuroendocrine responses by CRF infusions in rats. Pharmacol Biochem Behav 77:447-455.

Carlson NR (2001) Physiology of Behavior, 7 Edition. Needham Heights, Massachusetts 02494: A Pearson Education Company.

Casacchia M, Carolei A, Barba C, Frontoni M, Rossi A, Meco G, Zylberman MR (1984) A placebo-controlled study of the antidepressant activity of moclobemide, a new MAO-A inhibitor. Pharmacopsychiatry 17:122-125.

Centeno ML, Sanchez RL, Cameron JL, Bethea CL (2007) Hypothalamic gonadotrophin-releasing hormone expression in female monkeys with different sensitivity to stress. J Neuroendocrinol 19:594-604.

Chalmers DT, Lovenberg TW, De Souza EB (1995) Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci 15:6340-6350.

Champagne D, Beaulieu J, Drolet G (1998) CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. J Neuroendocrinol 10:119-131.

Chao HM, McEwen BS (1994) Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. Brain Res Mol Brain Res 26:271-276.

Chappell PB, Smith MA, Kilts CD, Bissette G, Ritchie J, Anderson C, Nemeroff CB (1986) Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. J Neurosci 6:2908-2914.

Chaw D, Campls, FE, Eccleston, EG (1967) 5-Hydroxy tryptamine in the hidbrain of depressive suicides. Br J Psychiatry 113:1407-1411.

Chhatwal JP, Stanek-Rattiner L, Davis M, Ressler KJ (2006) Amygdala BDNF signaling is required for consolidation but not encoding of extinction. Nat Neurosci 9:870-872.

Chhatwal JP, Hammack SE, Jasnow AM, Rainnie DG, Ressler KJ (2007) Identification of cell-type-specific promoters within the brain using lentiviral vectors. Gene Ther 14:575-583.

Christianson JP, Thompson BM, Watkins LR, Maier SF (2008a) Medial prefrontal cortical activation modulates the impact of controllable and uncontrollable stressor exposure on a social exploration test of anxiety in the rat. Stress:1.

Christianson JP, Paul ED, Irani M, Thompson BM, Kubala KH, Yirmiya R, Watkins LR, Maier SF (2008b) The role of prior stressor controllability and the dorsal raphe nucleus in sucrose preference and social exploration. Behav Brain Res 193:87-93.

Claes SJ (2004) Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. Ann Med 36:50-61.

Cole RL, Sawchenko PE (2002) Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J Neurosci 22:959-969.

Contarino A, Dellu F, Koob GF, Smith GW, Lee KF, Vale WW, Gold LH (2000) Dissociation of locomotor activation and suppression of food intake induced by CRF in CRFR1-deficient mice. Endocrinology 141:2698-2702.

Conti AC, Kuo YC, Valentino RJ, Blendy JA (2004) Inducible cAMP early repressor regulates corticosterone suppression after tricyclic antidepressant treatment. J Neurosci 24:1967-1975.

Cooper MA, Ritchie EC (2000) Testosterone replacement therapy for anxiety. Am J Psychiatry 157:1884.

Coupland N, Glue P, Nutt DJ (1992) Challenge tests: assessment of the noradrenergic and GABA systems in depression and anxiety disorders. Mol Aspects Med 13:221-247.

Crane JW, Buller KM, Day TA (2003) Evidence that the bed nucleus of the stria terminalis contributes to the modulation of hypophysiotropic corticotropin-releasing factor cell responses to systemic interleukin-1beta. J Comp Neurol 467:232-242.

Crawley JN (1985) Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev 9:37-44.

Cryan JF, Valentino RJ, Lucki I (2005) Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev 29:547-569.

Curtis AL, Valentino RJ (1991) Acute and chronic effects of the atypical antidepressant, mianserin on brain noradrenergic neurons. Psychopharmacology (Berl) 103:330-338.

Curtis AL, Bello NT, Connolly KR, Valentino RJ (2002) Corticotropin-releasing factor neurones of the central nucleus of the amygdala mediate locus coeruleus activation by cardiovascular stress. J Neuroendocrinol 14:667-682.

Custro N, Scafidi V, Lo Baido R, Nastri L, Abbate G, Cuffaro MP, Gallo S, Vienna G, Notarbartolo A (1994) Subclinical hypothyroidism resulting from autoimmune thyroiditis in female patients with endogenous depression. J Endocrinol Invest 17:641-646.

Dallman MF, Akana SF, Jacobson L, Levin N, Cascio CS, Shinsako J (1987) Characterization of corticosterone feedback regulation of ACTH secretion. Ann N Y Acad Sci 512:402-414.

Dallman MF, Pecoraro N, Akana SF, La Fleur SE, Gomez F, Houshyar H, Bell ME, Bhatnagar S, Laugero KD, Manalo S (2003) Chronic stress and obesity: a new view of "comfort food". Proc Natl Acad Sci U S A 100:11696-11701.

Daniels WM, Richter L, Stein DJ (2004) The effects of repeated intra-amygdala CRF injections on rat behavior and HPA axis function after stress. Metab Brain Dis 19:15-23.

Dantzer R (2006) Cytokine, sickness behavior, and depression. Neurol Clin 24:441-460.

Davis M (2006) Neural systems involved in fear and anxiety measured with fear-potentiated startle. Am Psychol 61:741-756.

De Bellis MD, Gold PW, Geracioti TD, Jr., Listwak SJ, Kling MA (1993) Association of fluoxetine treatment with reductions in CSF concentrations of corticotropin-releasing hormone and arginine vasopressin in patients with major depression. Am J Psychiatry 150:656-657.

de Kloet CS, Vermetten E, Geuze E, Kavelaars A, Heijnen CJ, Westenberg HG (2006) Assessment of HPA-axis function in posttraumatic stress disorder: pharmacological and non-pharmacological challenge tests, a review. J Psychiatr Res 40:550-567.

de Kloet ER (2003) Hormones, brain and stress. Endocr Regul 37:51-68.

Delbende C, Tranchand Bunel D, Tarozzo G, Grino M, Oliver C, Mocaer E, Vaudry H (1994) Effect of chronic treatment with the antidepressant tianeptine on the hypothalamo-pituitary-adrenal axis. Eur J Pharmacol 251:245-251.

Dickie EW, Brunet A, Akerib V, Armony JL (2008) An fMRI investigation of memory encoding in PTSD: influence of symptom severity. Neuropsychologia 46:1522-1531.

Donaldson CJ, Sutton SW, Perrin MH, Corrigan AZ, Lewis KA, Rivier JE, Vaughan JM, Vale WW (1996) Cloning and characterization of human urocortin. Endocrinology 137:2167-2170.

Drevets WC (2001) Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. Curr Opin Neurobiol 11:240-249.

Drevets WC (2003) Neuroimaging abnormalities in the amygdala in mood disorders. Ann N Y Acad Sci 985:420-444.

Duman RS, Malberg J, Thome J (1999) Neural plasticity to stress and antidepressant treatment. Biol Psychiatry 46:1181-1191.

Dunn AJ, Berridge CW (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res Brain Res Rev 15:71-100.

Dunn AJ, Swiergiel AH (1999) Behavioral responses to stress are intact in CRF-deficient mice. Brain Res 845:14-20.

Eaton WW, Shao H, Nestadt G, Lee HB, Bienvenu OJ, Zandi P (2008) Population-based study of first onset and chronicity in major depressive disorder. Arch Gen Psychiatry 65:513-520.

Egashira N, Tanoue A, Matsuda T, Koushi E, Harada S, Takano Y, Tsujimoto G, Mishima K, Iwasaki K, Fujiwara M (2006) Impaired social interaction and reduced anxiety-related behavior in vasopressin V1a receptor knockout mice. Behav Brain Res.

Everett JW (1989) Neurobiology of Reproduction in the Female Rat. Berlin, Germany: Springerr-Verlag.

Fadda P, Pani L, Porcella A, Fratta W (1995) Chronic imipramine, L-sulpiride and mianserin decrease corticotropin releasing factor levels in the rat brain. Neurosci Lett 192:121-123.

Feldman S, Weidenfeld J (1998) The excitatory effects of the amygdala on hypothalamo-pituitary-adrenocortical responses are mediated by hypothalamic norepinephrine, serotonin, and CRF-41. Brain Res Bull 45:389-393.

Feldman S, Conforti N, Weidenfeld J (1995a) Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli. Neurosci Biobehav Rev 19:235-240.

Feldman S, Conforti N, Itzik A, Weidenfeld J (1995b) The role of limbic structures in the modulation of ACTH responses following adrenalectomy. Ann N Y Acad Sci 771:73-81.

Fink H, Rex A, Voits M, Voigt JP (1998) Major biological actions of CCK--a critical evaluation of research findings. Exp Brain Res 123:77-83.

Forster GL, Feng N, Watt MJ, Korzan WJ, Mouw NJ, Summers CH, Renner KJ (2006) Corticotropin-releasing factor in the dorsal raphe elicits temporally distinct serotonergic responses in the limbic system in relation to fear behavior. Neuroscience 141:1047-1055.

Fossey MD, Lydiard RB, Ballenger JC, Laraia MT, Bissette G, Nemeroff CB (1993) Cerebrospinal fluid thyrotropin-releasing hormone concentrations in patients with anxiety disorders. J Neuropsychiatry Clin Neurosci 5:335-337.

Fossey MD, Lydiard RB, Ballenger JC, Laraia MT, Bissette G, Nemeroff CB (1996) Cerebrospinal fluid corticotropin-releasing factor concentrations in patients with anxiety disorders and normal comparison subjects. Biol Psychiatry 39:703-707.

Frohlich J, Ogawa S, Morgan M, Burton L, Pfaff D (1999) Hormones, genes and the structure of sexual arousal. Behav Brain Res 105:5-27.

Gallager DW, Kehne JH, Wakeman EA, Davis M (1983) Development changes in pharmacological responsivity of the acoustic startle reflex: effects of picrotoxin. Psychopharmacology (Berl) 79:87-93.

Gallagher JP, Orozco-Cabal LF, Liu J, Shinnick-Gallagher P (2008) Synaptic physiology of central CRH system. Eur J Pharmacol 583:215-225.

Garcia-Lecumberri C, Ambrosio E (2000) Differential effect of low doses of intracerebroventricular corticotropin-releasing factor in forced swimming test. Pharmacol Biochem Behav 67:519-525.

Gemar MC, Segal ZV, Mayberg HS, Goldapple K, Carney C (2006) Changes in regional cerebral blood flow following mood challenge in drug-free, remitted patients with unipolar depression. Depress Anxiety.

Gilmor ML, Skelton KH, Nemeroff CB, Owens MJ (2003) The effects of chronic treatment with the mood stabilizers valproic acid and lithium on corticotropin-releasing factor neuronal systems. J Pharmacol Exp Ther 305:434-439.

Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629-683.

Givalois L, Naert G, Rage F, Ixart G, Arancibia S, Tapia-Arancibia L (2004) A single brain-derived neurotrophic factor injection modifies hypothalamo-pituitary-adrenocortical axis activity in adult male rats. Mol Cell Neurosci 27:280-295.

Goekoop JG, de Winter RP, de Rijk R, Zwinderman KH, Frankhuijzen-Sierevogel A, Wiegant VM (2006) Depression with above-normal plasma vasopressin: validation by relations with family history of depression and mixed anxiety and retardation. Psychiatry Res 141:201-211.

Goldapple K, Segal Z, Garson C, Lau M, Bieling P, Kennedy S, Mayberg H (2004) Modulation of cortical-limbic pathways in major depression: treatment-specific effects of cognitive behavior therapy. Arch Gen Psychiatry 61:34-41.

Goldstein LE, Rasmusson AM, Bunney BS, Roth RH (1996) Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. J Neurosci 16:4787-4798.

Grammatopoulos DK, Randeva HS, Levine MA, Kanellopoulou KA, Hillhouse EW (2001) Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. J Neurochem 76:509-519.

Green B (2003) Post-traumatic stress disorder: symptom profiles in men and women. Curr Med Res Opin 19:200-204.

Gregoire AJ, Kumar R, Everitt B, Henderson AF, Studd JW (1996) Transdermal oestrogen for treatment of severe postnatal depression. Lancet 347:930-933.

Griebel G (1999) Is there a future for neuropeptide receptor ligands in the treatment of anxiety disorders? Pharmacol Ther 82:1-61.

Grigoriadis DE, Pearsall D, De Souza EB (1989) Effects of chronic antidepressant and benzodiazepine treatment on corticotropin-releasing-factor receptors in rat brain and pituitary. Neuropsychopharmacology 2:53-60.

Groenink L, Dirks A, Verdouw PM, Schipholt M, Veening JG, van der Gugten J, Olivier B (2002) HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. Biol Psychiatry 51:875-881.

Gutman DA, Nemeroff CB (2003) Persistent central nervous system effects of an adverse early environment: clinical and preclinical studies. Physiol Behav 79:471-478.

Gysling K, Forray MI, Haeger P, Daza C, Rojas R (2004) Corticotropin-releasing hormone and urocortin: redundant or distinctive functions? Brain Res Brain Res Rev 47:116-125.

Harlow JM (1999) Passage of an iron rod through the head. 1848. J Neuropsychiatry Clin Neurosci 11:281-283.

Hashimoto H, Onishi H, Koide S, Kai T, Yamagami S (1996) Plasma neuropeptide Y in patients with major depressive disorder. Neurosci Lett 216:57-60.

Heilig M (2004) The NPY system in stress, anxiety and depression. Neuropeptides 38:213-224.

Heim C, Owens MJ, Plotsky PM, Nemeroff CB (1997a) The role of early adverse life events in the etiology of depression and posttraumatic stress disorder. Focus on corticotropin-releasing factor. Ann N Y Acad Sci 821:194-207.

Heim C, Owens MJ, Plotsky PM, Nemeroff CB (1997b) Persistent changes in corticotropin-releasing factor systems due to early life stress: relationship to the pathophysiology of major depression and post-traumatic stress disorder. Psychopharmacol Bull 33:185-192.

Heim C, Mletzko T, Purselle D, Musselman DL, Nemeroff CB (2008) The dexamethasone/corticotropin-releasing factor test in men with major depression: role of childhood trauma. Biol Psychiatry 63:398-405.

Heinrichs SC, Britton KT, Koob GF (1991) Both conditioned taste preference and aversion induced by corticotropin-releasing factor. Pharmacol Biochem Behav 40:717-721.

Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 12:656-670.

Hendrick V, Gitlin M, Altshuler L, Korenman S (2000) Antidepressant medications, mood and male fertility. Psychoneuroendocrinology 25:37-51.

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20:78-84.

Herman JP, Spencer R (1998) Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. J Neurosci 18:7462-7473.

Herman JP, Adams D, Prewitt C (1995) Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinology 61:180-190.

Herman JP, Schafer MK, Thompson RC, Watson SJ (1992) Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. Mol Endocrinol 6:1061-1069.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24:151-180.

Heuser I (1998) Anna-Monika-Prize paper. The hypothalamic-pituitary-adrenal system in depression. Pharmacopsychiatry 31:10-13.

Hikosaka O, Bromberg-Martin E, Hong S, Matsumoto M (2008) New insights on the subcortical representation of reward. Curr Opin Neurobiol 18:203-208.

Hisano S, Tsuruo Y, Katoh S, Daikoku S, Yanaihara N, Shibasaki T (1987) Intragranular colocalization of arginine vasopressin and methionine-enkephalin-octapeptide in CRF-axons in the rat median eminence. Cell Tissue Res 249:497-507.

Hobbs LF (1959) The use of phenelzine, and antidepressant, in general practice: a preliminary report of two hundred cases.]. Va Med Mon (1918) 86:692-695.
Hoehn-Saric R, McLeod DR (2000) Anxiety and arousal: physiological changes and their perception. J Affect Disord 61:217-224.
Hogg S (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 54:21-30.
Hokfelt T, Meister B, Villar MJ, Ceccatelli S, Cortes R, Schalling M, Everitt B (1989) Hypothalamic neurosecretory systems and their messenger molecules. Acta Physiol Scand Suppl 583:105-111.
Holmes A, Heilig M, Rupniak NM, Steckler T, Griebel G (2003) Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. Trends Pharmacol Sci 24:580-588.
Holsboer F (2000) The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23:477-501.
Holsboer F (2003) Corticotropin-releasing hormone modulators and depression. Curr Opin Investig Drugs 4:46-50.
Holsboer F, Barden N (1996) Antidepressants and hypothalamic-pituitary-adrenocortical regulation. Endocr Rev 17:187-205.
Honkaniemi J, Pelto-Huikko M, Rechardt L, Isola J, Lammi A, Fuxe K, Gustafsson JA, Wikstrom AC, Hokfelt T (1992) Colocalization of peptide and glucocorticoid receptor immunoreactivities in rat central amygdaloid nucleus. Neuroendocrinology 55:451-459.
Hotta M, Shibasaki T, Yamauchi N, Ohno H, Benoit R, Ling N, Demura H (1991) The effects of chronic central administration of corticotropin-releasing factor on food intake, body weight, and hypothalamic-pituitary-adrenocortical hormones. Life Sci 48:1483-1491.
Hou C, Jia F, Liu Y, Li L (2006) CSF serotonin, 5-hydroxyindolacetic acid and neuropeptide Y levels in severe major depressive disorder. Brain Res 1095:154-158.
Hugin-Flores ME, Steimer T, Aubert ML, Schulz P (2004) Mineralo- and glucocorticoid receptor mrnas are differently regulated by corticosterone in the rat hippocampus and anterior pituitary. Neuroendocrinology 79:174-184.
Hugin-Flores ME, Steimer T, Schulz P, Vallotton MB, Aubert ML (2003) Chronic corticotropin-releasing hormone and vasopressin regulate corticosteroid receptors in rat hippocampus and anterior pituitary. Brain Res 976:159-170.
Hwang BH, Guntz JM (1997) Downregulation of corticotropin-releasing factor mRNA, but not vasopressin mRNA, in the paraventricular hypothalamic nucleus of rats following nutritional stress. Brain Res Bull 43:509-514.
Ising M, Kunzel HE, Binder EB, Nickel T, Modell S, Holsboer F (2005) The combined dexamethasone/CRH test as a potential surrogate marker in depression. Prog Neuropsychopharmacol Biol Psychiatry 29:1085-1093.
Ising M, Horstmann S, Kloiber S, Lucae S, Binder EB, Kern N, Kunzel HE, Pfennig A, Uhr M, Holsboer F (2007) Combined dexamethasone/corticotropin releasing hormone test predicts treatment response in major depression - a potential biomarker? Biol Psychiatry 62:47-54.

Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118-134.

Jacobson L, Akana SF, Cascio CS, Shinsako J, Dallman MF (1988) Circadian variations in plasma corticosterone permit normal termination of adrenocorticotropin responses to stress. Endocrinology 122:1343-1348.

Jakobsson J, Lundberg C (2006) Lentiviral vectors for use in the central nervous system. Mol Ther 13:484-493.

Kalantaridou SN, Makrigiannakis A, Zoumakis E, Chrousos GP (2004) Stress and the female reproductive system. J Reprod Immunol 62:61-68.

Kalin NH, Shelton SE, Davidson RJ (2000) Cerebrospinal fluid corticotropin-releasing hormone levels are elevated in monkeys with patterns of brain activity associated with fearful temperament. Biol Psychiatry 47:579-585.

Kalin NH, Shelton SE, Davidson RJ (2004) The role of the central nucleus of the amygdala in mediating fear and anxiety in the primate. J Neurosci 24:5506-5515.

Kalueff AV, Nutt DJ (2006) Role of GABA in anxiety and depression. Depress Anxiety.

Kaneda Y, Fujii A (2001) Effects of tandospirone, a serotonin-1A agonist, on the hypothalamo-pituitary-gonadal axis of male patients. Neuro Endocrinol Lett 22:243-247.

Kapcala LP, Dicke JA (1992) Brain corticotropin-releasing hormone receptors on neurons and astrocytes. Brain Res 589:143-148.

Karl T, Herzog H (2006) Behavioral profiling of NPY in aggression and neuropsychiatric diseases. Peptides.

Karl T, Herzog H (2007) Behavioral profiling of NPY in aggression and neuropsychiatric diseases. Peptides 28:326-333.

Karlsson RM, Holmes A (2006) Galanin as a modulator of anxiety and depression and a therapeutic target for affective disease. Amino Acids 31:231-239.

Katz RJ (1982) Animal model of depression: pharmacological sensitivity of a hedonic deficit. Pharmacol Biochem Behav 16:965-968.

Keedwell PA, Andrew C, Williams SC, Brammer MJ, Phillips ML (2005) The neural correlates of anhedonia in major depressive disorder. Biol Psychiatry 58:843-853.

Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME (2008) Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol Psychiatry.

Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME (2009) Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol Psychiatry 14:37-50.

Keeney A, Jessop DS, Harbuz MS, Marsden CA, Hogg S, Blackburn-Munro RE (2006) Differential effects of acute and chronic social defeat stress on hypothalamic-pituitary-adrenal axis function and hippocampal serotonin release in mice. J Neuroendocrinol 18:330-338.

Keightley ML, Seminowicz DA, Bagby RM, Costa PT, Fossati P, Mayberg HS (2003) Personality influences limbic-cortical interactions during sad mood induction. Neuroimage 20:2031-2039.

Kendler KS, Karkowski LM, Prescott CA (1999) Causal relationship between stressful life events and the onset of major depression. Am J Psychiatry 156:837-841.
Kent JM, Mathew SJ, Gorman JM (2002) Molecular targets in the treatment of anxiety. Biol Psychiatry 52:1008-1030.
Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, Rush AJ, Walters EE, Wang PS (2003) The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). Jama 289:3095-3105.
Kilts C (2003) In vivo neuroimaging correlates of the efficacy of paroxetine in the treatment of mood and anxiety disorders. Psychopharmacol Bull 37 Suppl 1:19-28.
King BR, Nicholson RC (2007) Advances in understanding corticotrophin-releasing hormone gene expression. Front Biosci 12:581-590.
King BR, Smith R, Nicholson RC (2002) Novel glucocorticoid and cAMP interactions on the CRH gene promoter. Mol Cell Endocrinol 194:19-28.
Kitada Y, Miyauchi T, Satoh A, Satoh S (1981) Effects of antidepressants in the rat forced swimming test. Eur J Pharmacol 72:145-152.
Knapska E, Radwanska K, Werka T, Kaczmarek L (2007) Functional internal complexity of amygdala: focus on gene activity mapping after behavioral training and drugs of abuse. Physiol Rev 87:1113-1173.
Kompagne H, Bardos G, Szenasi G, Gacsalyi I, Harsing LG, Levay G (2008) Chronic mild stress generates clear depressive but ambiguous anxiety-like behaviour in rats. Behav Brain Res 193:311-314.
Koob GF, Bloom FE (1985) Corticotropin-releasing factor and behavior. Fed Proc 44:259-263.
Korte SM, De Boer SF, Bohus B (1999) Fear-potentiation in the elevated plus-maze test depends on stressor controllability and fear conditioning. Stress 3:27-40.
Koszycki D, Copen J, Bradwejn J (2004) Sensitivity to cholecystokinin-tetrapeptide in major depression. J Affect Disord 80:285-290.
Krishnan KR, Manepalli AN, Ritchie JC, Rayasam K, Melville ML, Daughtry G, Thorner MO, Rivier JE, Vale WW, Nemeroff CB, et al. (1988) Growth hormone-releasing factor stimulation test in depression. Am J Psychiatry 145:90-92.
Kudler H, Davidson J, Meador K, Lipper S, Ely T (1987) The DST and posttraumatic stress disorder. Am J Psychiatry 144:1068-1071.
Lacerda AL, Keshavan MS, Hardan AY, Yorbik O, Brambilla P, Sassi RB, Nicoletti M, Mallinger AG, Frank E, Kupfer DJ, Soares JC (2004) Anatomic evaluation of the orbitofrontal cortex in major depressive disorder. Biol Psychiatry 55:353-358.
Lang R, Gundlach AL, Kofler B (2007) The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. Pharmacol Ther 115:177-207.
Le Melledo JM, Baker G (2004) Role of progesterone and other neuroactive steroids in anxiety disorders. Expert Rev Neurother 4:851-860.
Leibowitz SF (1990) Hypothalamic neuropeptide Y in relation to energy balance. Ann N Y Acad Sci 611:284-301.
Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C (1997) Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. Front Neuroendocrinol 18:383-439.

Lewandoski M (2001) Conditional control of gene expression in the mouse. Nat Rev Genet 2:743-755.

Liebsch G, Landgraf R, Engelmann M, Lorscher P, Holsboer F (1999) Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. J Psychiatr Res 33:153-163.

Liebsch G, Landgraf R, Gerstberger R, Probst JC, Wotjak CT, Engelmann M, Holsboer F, Montkowski A (1995) Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats. Regul Pept 59:229-239.

Lifschytz T, Segman R, Shalom G, Lerer B, Gur E, Golzer T, Newman ME (2006) Basic mechanisms of augmentation of antidepressant effects with thyroid hormone. Curr Drug Targets 7:203-210.

Liotti M, Mayberg HS, McGinnis S, Brannan SL, Jerabek P (2002) Unmasking disease-specific cerebral blood flow abnormalities: mood challenge in patients with remitted unipolar depression. Am J Psychiatry 159:1830-1840.

Liotti M, Mayberg HS, Brannan SK, McGinnis S, Jerabek P, Fox PT (2000) Differential limbic--cortical correlates of sadness and anxiety in healthy subjects: implications for affective disorders. Biol Psychiatry 48:30-42.

Lipschitz DS, Rasmusson AM, Yehuda R, Wang S, Anyan W, Gueoguieva R, Grilo CM, Fehon DC, Southwick SM (2003) Salivary cortisol responses to dexamethasone in adolescents with posttraumatic stress disorder. J Am Acad Child Adolesc Psychiatry 42:1310-1317.

Liu HX, Hokfelt T (2002) The participation of galanin in pain processing at the spinal level. Trends Pharmacol Sci 23:468-474.

Liu Z, Zhu F, Wang G, Xiao Z, Tang J, Liu W, Wang H, Liu H, Wang X, Wu Y, Cao Z, Li W (2007) Association study of corticotropin-releasing hormone receptor1 gene polymorphisms and antidepressant response in major depressive disorders. Neurosci Lett 414:155-158.

Long JA, Evans, H.M. (1922) The oestrous cycle in the rat and its associated phenomena. Berkeley: University of California Press.

Lowry CA, Moore FL (2006) Regulation of behavioral responses by corticotropin-releasing factor. Gen Comp Endocrinol 146:19-27.

Lowry CA, Rose JD, Moore FL (1996) Corticotropin-releasing factor enhances locomotion and medullary neuronal firing in an amphibian. Horm Behav 30:50-59.

Lu A, Steiner MA, Whittle N, Vogl AM, Walser SM, Ableitner M, Refojo D, Ekker M, Rubenstein JL, Stalla GK, Singewald N, Holsboer F, Wotjak CT, Wurst W, Deussing JM (2008) Conditional mouse mutants highlight mechanisms of corticotropin-releasing hormone effects on stress-coping behavior. Mol Psychiatry 13:1028-1042.

Ma XM, Lightman SL, Aguilera G (1999) Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. Endocrinology 140:3623-3632.

Makino S, Gold PW, Schulkin J (1994a) Effects of corticosterone on CRH mRNA and content in the bed nucleus of the stria terminalis; comparison with the effects in

the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus. Brain Res 657:141-149.

Makino S, Gold PW, Schulkin J (1994b) Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Brain Res 640:105-112.

Malberg JE (2004) Implications of adult hippocampal neurogenesis in antidepressant action. J Psychiatry Neurosci 29:196-205.

Malhi GS, Parker GB, Gladstone G, Wilhelm K, Mitchell PB (2002) Recognizing the anxious face of depression. J Nerv Ment Dis 190:366-373.

Malison RT, Price LH, Berman R, van Dyck CH, Pelton GH, Carpenter L, Sanacora G, Owens MJ, Nemeroff CB, Rajeevan N, Baldwin RM, Seibyl JP, Innis RB, Charney DS (1998) Reduced brain serotonin transporter availability in major depression as measured by [123I]-2 beta-carbomethoxy-3 beta-(4-iodophenyl)tropane and single photon emission computed tomography. Biol Psychiatry 44:1090-1098.

Malkoski SP, Dorin RI (1999) Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. Mol Endocrinol 13:1629-1644.

Maron E, Kuikka JT, Ulst K, Tiihonen J, Vasar V, Shlik J (2004) SPECT imaging of serotonin transporter binding in patients with generalized anxiety disorder. Eur Arch Psychiatry Clin Neurosci 254:392-396.

Martin MC, ed (2004) Neuropeptide Y and related peptides. Heidelberg, Germany: springer.

Mayberg HS (1997) Limbic-cortical dysregulation: a proposed model of depression. J Neuropsychiatry Clin Neurosci 9:471-481.

Mayberg HS (2003) Modulating dysfunctional limbic-cortical circuits in depression: towards development of brain-based algorithms for diagnosis and optimised treatment. Br Med Bull 65:193-207.

Mayberg HS, Liotti M, Brannan SK, McGinnis S, Mahurin RK, Jerabek PA, Silva JA, Tekell JL, Martin CC, Lancaster JL, Fox PT (1999) Reciprocal limbic-cortical function and negative mood: converging PET findings in depression and normal sadness. Am J Psychiatry 156:675-682.

Mazure CM, Maciejewski PK, Jacobs SC, Bruce ML (2002) Stressful life events interacting with cognitive/personality styles to predict late-onset major depression. Am J Geriatr Psychiatry 10:297-304.

McDonald AJ, Mascagni F, Mania I, Rainnie DG (2005) Evidence for a perisomatic innervation of parvalbumin-containing interneurons by individual pyramidal cells in the basolateral amygdala. Brain Res 1035:32-40.

McEwen BS (1994) Corticosteroids and hippocampal plasticity. Ann N Y Acad Sci 746:134-142; discussion 142-134, 178-139.

McIlwain KL, Merriweather MY, Yuva-Paylor LA, Paylor R (2001) The use of behavioral test batteries: effects of training history. Physiol Behav 73:705-717.

Meltzer HY, Kolakowska T, Fang VS, Fogg L, Robertson A, Lewine R, Strahilevitz M, Busch D (1984) Growth hormone and prolactin response to apomorphine in

schizophrenia and the major affective disorders. Relation to duration of illness and depressive symptoms. Arch Gen Psychiatry 41:512-519.
Merali Z, Khan S, Michaud DS, Shippy SA, Anisman H (2004) Does amygdaloid corticotropin-releasing hormone (CRH) mediate anxiety-like behaviors? Dissociation of anxiogenic effects and CRH release. Eur J Neurosci 20:229-239.
Merlo Pich E, Lorang M, Yeganeh M, Rodriguez de Fonseca F, Raber J, Koob GF, Weiss F (1995) 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. J Neurosci 15:5439-5447.
Meyer-Lindenberg A (2008) Impact of prosocial neuropeptides on human brain function. Prog Brain Res 170:463-470.
Miller EK, Cohen JD (2001) An integrative theory of prefrontal cortex function. Annu Rev Neurosci 24:167-202.
Miller RJ, Pickel VM (1980) The distribution and functions of the enkephalins. J Histochem Cytochem 28:903-917.
Mitchell AJ (1998) The role of corticotropin releasing factor in depressive illness: a critical review. Neurosci Biobehav Rev 22:635-651.
Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirus vector. J Virol 72:8150-8157.
Monk CS, Nelson EE, McClure EB, Mogg K, Bradley BP, Leibenluft E, Blair RJ, Chen G, Charney DS, Ernst M, Pine DS (2006) Ventrolateral prefrontal cortex activation and attentional bias in response to angry faces in adolescents with generalized anxiety disorder. Am J Psychiatry 163:1091-1097.
Morley JE, Levine AS (1982) Corticotrophin releasing factor, grooming and ingestive behavior. Life Sci 31:1459-1464.
Munjack DJ, Palmer R (1988) Thyroid hormones in panic disorder, panic disorder with agoraphobia, and generalized anxiety disorder. J Clin Psychiatry 49:229-231.
Murakami S, Imbe H, Morikawa Y, Kubo C, Senba E (2005) Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. Neurosci Res 53:129-139.
Musselman DL, Nemeroff CB (1996) Depression and endocrine disorders: focus on the thyroid and adrenal system. Br J Psychiatry Suppl:123-128.
Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L (2007) A global double-fluorescent Cre reporter mouse. Genesis 45:593-605.
Nakano I (1998) The limbic system: An outline and brief history of its concept. Neuropathology 18:211-214.
Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996a) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A 93:11382-11388.
Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996b) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. PNAS 93:11382-11388.
Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996c) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267.

Natelson BH, Ottenweller JE, Cook JA, Pitman D, McCarty R, Tapp WN (1988) Effect of stressor intensity on habituation of the adrenocortical stress response. Physiol Behav 43:41-46.

Nemeroff CB (1988) The role of corticotropin-releasing factor in the pathogenesis of major depression. Pharmacopsychiatry 21:76-82.

Nemeroff CB (2003) The role of GABA in the pathophysiology and treatment of anxiety disorders. Psychopharmacol Bull 37:133-146.

Nemeroff CB (2004) Early-Life Adversity, CRF Dysregulation, and Vulnerability to Mood and Anxiety Disorders. Psychopharmacol Bull 38 Suppl 1:14-20.

Nemeroff CB, Owens MJ (2002) Treatment of mood disorders. Nat Neurosci 5 Suppl:1068-1070.

Nemeroff CB, Simon JS, Haggerty JJ, Jr., Evans DL (1985) Antithyroid antibodies in depressed patients. Am J Psychiatry 142:840-843.

Nemeroff CB, Owens MJ, Bissette G, Andorn AC, Stanley M (1988) Reduced corticotropin releasing factor binding sites in the frontal cortex of suicide victims. Arch Gen Psychiatry 45:577-579.

Nemeroff CB, Bremner JD, Foa EB, Mayberg HS, North CS, Stein MB (2005) Posttraumatic stress disorder: A state-of-the-science review. J Psychiatr Res.

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W (1984) Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science 226:1342-1344.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002) Neurobiology of depression. Neuron 34:13-25.

Nicholson RC, King BR, Smith R (2004) Complex regulatory interactions control CRH gene expression. Front Biosci 9:32-39.

Nikisch G, Mathe AA, Czernik A, Thiele J, Bohner J, Eap CB, Agren H, Baumann P (2005) Long-term citalopram administration reduces responsiveness of HPA axis in patients with major depression: relationship with S-citalopram concentrations in plasma and cerebrospinal fluid (CSF) and clinical response. Psychopharmacology (Berl) 181:751-760.

Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci U S A 93:11699-11704.

Nutt DJ (2001) Neurobiological mechanisms in generalized anxiety disorder. J Clin Psychiatry 62 Suppl 11:22-27; discussion 28.

Ogren SO, Kuteeva E, Hokfelt T, Kehr J (2006) Galanin receptor antagonists : a potential novel pharmacological treatment for mood disorders. CNS Drugs 20:633-654.

Olds J, Milner P (1954) Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol 47:419-427.

Olff M, Guzelcan Y, de Vries GJ, Assies J, Gersons BP (2006) HPA- and HPT-axis alterations in chronic posttraumatic stress disorder. Psychoneuroendocrinology 31:1220-1230.

Ortega E, Ruiz E, Rodriguez E, Frias J (1994) Effect of corticotropin releasing factor (CRF) in the median eminence on gonadotropins in ovariectomized rats with or without steroid priming: dose-response study. Neurochem Res 19:1225-1230.

Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev 43:425-473.

Owens MJ, Nemeroff CB (1999) Corticotropin-releasing factor antagonists in affective disorders. Expert Opin Investig Drugs 8:1849-1858.

Owens MJ, Bissette G, Nemeroff CB (1989) Acute effects of alprazolam and adinazolam on the concentrations of corticotropin-releasing factor in the rat brain. Synapse 4:196-202.

Owens MJ, Mulchahey JJ, Kasckow JW, Plotsky PM, Nemeroff CB (1995) Exposure to an antisense oligonucleotide decreases corticotropin-releasing factor receptor binding in rat pituitary cultures. J Neurochem 64:2358-2361.

Pace TW, Hu F, Miller AH (2007) Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain Behav Immun 21:9-19.

Palkovits M (2000) Stress-induced expression of co-localized neuropeptides in hypothalamic and amygdaloid neurons. Eur J Pharmacol 405:161-166.

Palkovits M, Young WS, 3rd, Kovacs K, Toth Z, Makara GB (1998) Alterations in corticotropin-releasing hormone gene expression of central amygdaloid neurons following long-term paraventricular lesions and adrenalectomy. Neuroscience 85:135-147.

Pande AC, Greiner M, Adams JB, Lydiard RB, Pierce MW (1999) Placebo-controlled trial of the CCK-B antagonist, CI-988, in panic disorder. Biol Psychiatry 46:860-862.

Papadimitriou GN, Kerkhofs M, Kempenaers C, Mendlewicz J (1988) EEG sleep studies in patients with generalized anxiety disorder. Psychiatry Res 26:183-190.

Patisaul HB, Luskin JR, Wilson ME (2004) A soy supplement and tamoxifen inhibit sexual behavior in female rats. Horm Behav 45:270-277.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press.

Paxinos G, Watson CR, Emson PC (1980) AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. J Neurosci Methods 3:129-149.

Paylor R, Spencer CM, Yuva-Paylor LA, Pieke-Dahl S (2006) The use of behavioral test batteries, II: effect of test interval. Physiol Behav 87:95-102.

Peeters PJ, Fierens FL, van den Wyngaert I, Goehlmann HW, Swagemakers SM, Kass SU, Langlois X, Pullan S, Stenzel-Poore MP, Steckler T (2004) Gene expression profiles highlight adaptive brain mechanisms in corticotropin releasing factor overexpressing mice. Brain Res Mol Brain Res 129:135-150.

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-167.

Pfeifer A, Brandon EP, Kootstra N, Gage FH, Verma IM (2001) Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. Proc Natl Acad Sci U S A 98:11450-11455.

Pike AC, Brzozowski AM, Walton J, Hubbard RE, Bonn T, Gustafsson JA, Carlquist M (2000) Structural aspects of agonism and antagonism in the oestrogen receptor. Biochem Soc Trans 28:396-400.

Pitchot W, Hansenne M, Moreno AG, Ansseau M (1996) Growth hormone response to apomorphine in obsessive-compulsive disorder. J Psychiatry Neurosci 21:343-345.

Pitts AF, Samuelson SD, Meller WH, Bissette G, Nemeroff CB, Kathol RG (1995) Cerebrospinal fluid corticotropin-releasing hormone, vasopressin, and oxytocin concentrations in treated patients with major depression and controls. Biol Psychiatry 38:330-335.

Plotsky PM, Owens MJ, Nemeroff CB (1998) Psychoneuroendocrinology of depression. Hypothalamic-pituitary-adrenal axis. Psychiatr Clin North Am 21:293-307.

Porsolt RD, Bertin A, Jalfre M (1978a) "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. Eur J Pharmacol 51:291-294.

Porsolt RD, Anton G, Blavet N, Jalfre M (1978b) Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol 47:379-391.

Porter RJ, Gallagher P, Watson S, Smith MS, Young AH (2003) Elevated prolactin responses to L-tryptophan infusion in medication-free depressed patients. Psychopharmacology (Berl) 169:77-83.

Prewitt CM, Herman JP (1998) Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. J Chem Neuroanat 15:173-185.

Price ML, Lucki I (2001) Regulation of serotonin release in the lateral septum and striatum by corticotropin-releasing factor. J Neurosci 21:2833-2841.

Price ML, Kirby LG, Valentino RJ, Lucki I (2002) Evidence for corticotropin-releasing factor regulation of serotonin in the lateral septum during acute swim stress: adaptation produced by repeated swimming. Psychopharmacology (Berl) 162:406-414.

Rainnie DG, Fernhout BJ, Shinnick-Gallagher P (1992) Differential actions of corticotropin releasing factor on basolateral and central amygdaloid neurones, in vitro. J Pharmacol Exp Ther 263:846-858.

Rainnie DG, Holmes KH, Shinnick-Gallagher P (1994) Activation of postsynaptic metabotropic glutamate receptors by trans-ACPD hyperpolarizes neurons of the basolateral amygdala. J Neurosci 14:7208-7220.

Rainnie DG, Mania I, Mascagni F, McDonald AJ (2006) Physiological and morphological characterization of parvalbumin-containing interneurons of the rat basolateral amygdala. J Comp Neurol 498:142-161.

Raison CL, Capuron L, Miller AH (2006) Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 27:24-31.

Rasmusson AM, Lipschitz DS, Wang S, Hu S, Vojvoda D, Bremner JD, Southwick SM, Charney DS (2001) Increased pituitary and adrenal reactivity in premenopausal women with posttraumatic stress disorder. Biol Psychiatry 50:965-977.

Rassnick S, Heinrichs SC, Britton KT, Koob GF (1993) Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res 605:25-32.

Rattiner LM, Davis M, French CT, Ressler KJ (2004) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. J Neurosci 24:4796-4806.

Rauch SL, Shin LM, Wright CI (2003) Neuroimaging studies of amygdala function in anxiety disorders. Ann N Y Acad Sci 985:389-410.

Ressler KJ, Nemeroff CB (2000) Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depress Anxiety 12 Suppl 1:2-19.

Ressler KJ, Paschall G, Zhou XL, Davis M (2002) Regulation of synaptic plasticity genes during consolidation of fear conditioning. J Neurosci 22:7892-7902.

Reul JM, Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2:23-33.

Reul JM, Bilang-Bleuel A, Droste S, Linthorst AC, Holsboer F, Gesing A (2000a) New mode of hypothalamic-pituitary-adrenocortical axis regulation: significance for stress-related disorders. Z Rheumatol 59 Suppl 2:II/22-25.

Reul JM, Gesing A, Droste S, Stec IS, Weber A, Bachmann C, Bilang-Bleuel A, Holsboer F, Linthorst AC (2000b) The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. Eur J Pharmacol 405:235-249.

Reyes BA, Valentino RJ, Xu G, Van Bockstaele EJ (2005) Hypothalamic projections to locus coeruleus neurons in rat brain. Eur J Neurosci 22:93-106.

Riedel WJ, Klaassen T, Griez E, Honig A, Menheere PP, van Praag HM (2002) Dissociable hormonal, cognitive and mood responses to neuroendocrine challenge: evidence for receptor-specific serotonergic dysregulation in depressed mood. Neuropsychopharmacology 26:358-367.

Risbrough VB, Stein MB (2006) Role of corticotropin releasing factor in anxiety disorders: a translational research perspective. Horm Behav 50:550-561.

Rivest S, Plotsky PM, Rivier C (1993) CRF alters the infundibular LHRH secretory system from the medial preoptic area of female rats: possible involvement of opioid receptors. Neuroendocrinology 57:236-246.

Romeo RD, Bellani R, Karatsoreos IN, Chhua N, Vernov M, Conrad CD, McEwen BS (2006) Stress history and pubertal development interact to shape hypothalamic-pituitary-adrenal axis plasticity. Endocrinology 147:1664-1674.

Roozendaal B, Brunson KL, Holloway BL, McGaugh JL, Baram TZ (2002) Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. Proc Natl Acad Sci U S A 99:13908-13913.

Rubinow DR (2005) Reproductive steroids in context. Arch Womens Ment Health 8:1-5.

Rubinow DR, Schmidt PJ (2006) Gonadal steroid regulation of mood: the lessons of premenstrual syndrome. Front Neuroendocrinol 27:210-216.

Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, Niederehe G, Thase ME, Lavori PW, Lebowitz BD, McGrath PJ, Rosenbaum JF, Sackeim HA, Kupfer DJ, Luther J, Fava M (2006) Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. Am J Psychiatry 163:1905-1917.

Sajdyk TJ, Shekhar A, Gehlert DR (2004) Interactions between NPY and CRF in the amygdala to regulate emotionality. Neuropeptides 38:225-234.

Sawchenko PE, Imaki T, Potter E, Kovacs K, Imaki J, Vale W (1993) The functional neuroanatomy of corticotropin-releasing factor. Ciba Found Symp 172:5-21; discussion 21-29.

Schaaf MJ, de Jong J, de Kloet ER, Vreugdenhil E (1998) Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. Brain Res 813:112-120.

Schatzberg AaN, CB, ed (2000) Textbook of Psychopharmacology, 3 Edition: The American Psychiatric Publishing.

Schiess MC, Asprodini EK, Rainnie DG, Shinnick-Gallagher P (1993) The central nucleus of the rat amygdala: in vitro intracellular recordings. Brain Res 604:283-297.

Schittecatte M, Garcia-Valentin J, Charles G, Machowski R, Pena-Othaitz MJ, Mendlewicz J, Wilmotte J (1995) Efficacy of the 'clonidine REM suppression test (CREST)' to separate patients with major depression from controls; a comparison with three currently proposed biological markers of depression. J Affect Disord 33:151-157.

Schmidt PJ, Nieman L, Danaceau MA, Tobin MB, Roca CA, Murphy JH, Rubinow DR (2000) Estrogen replacement in perimenopause-related depression: a preliminary report. Am J Obstet Gynecol 183:414-420.

Schule C, Baghai TC, Eser D, Zwanzger P, Jordan M, Buechs R, Rupprecht R (2006) Time course of hypothalamic-pituitary-adrenocortical axis activity during treatment with reboxetine and mirtazapine in depressed patients. Psychopharmacology (Berl).

Schulkin J, Gold PW, McEwen BS (1998) Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. Psychoneuroendocrinology 23:219-243.

Scott LV, Dinan TG (1998) Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. Life Sci 62:1985-1998.

Seasholtz AF, Thompson RC, Douglass JO (1988) Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. Mol Endocrinol 2:1311-1319.

Seidman SN, Rabkin JG (1998) Testosterone replacement therapy for hypogonadal men with SSRI-refractory depression. J Affect Disord 48:157-161.

Semeniuk T, Jhangri GS, Le Melledo JM (2001) Neuroactive steroid levels in patients with generalized anxiety disorder. J Neuropsychiatry Clin Neurosci 13:396-398.

Seminowicz DA, Mayberg HS, McIntosh AR, Goldapple K, Kennedy S, Segal Z, Rafi-Tari S (2004) Limbic-frontal circuitry in major depression: a path modeling metanalysis. Neuroimage 22:409-418.

Seth KA, Majzoub JA (2001) Repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) can act as an enhancer as well as a repressor of corticotropin-releasing hormone gene transcription. J Biol Chem 276:13917-13923.

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996) Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci U S A 93:3908-3913.

Shepard JD, Barron KW, Myers DA (2000) Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. Brain Res 861:288-295.

Shepard JD, Barron KW, Myers DA (2003) Stereotaxic localization of corticosterone to the amygdala enhances hypothalamo-pituitary-adrenal responses to behavioral stress. Brain Res 963:203-213.

Shepard JD, Schulkin J, Myers DA (2006) Chronically elevated corticosterone in the amygdala increases corticotropin releasing factor mRNA in the dorsolateral bed nucleus of stria terminalis following duress. Behav Brain Res 174:193-196.

Sherman JE, Kalin NH (1987) The effects of ICV-CRH on novelty-induced behavior. Pharmacol Biochem Behav 26:699-703.

Sichel DA, Cohen LS, Robertson LM, Ruttenberg A, Rosenbaum JF (1995) Prophylactic estrogen in recurrent postpartum affective disorder. Biol Psychiatry 38:814-818.

Simmons DM AJ, and Swanson LW (1989) A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. J Histotechnol 12:169–181.

Simon AB, Gorman JM (2006) Advances in the treatment of anxiety: targeting glutamate. NeuroRx 3:57-68.

Singh A, Petrides JS, Gold PW, Chrousos GP, Deuster PA (1999) Differential hypothalamic-pituitary-adrenal axis reactivity to psychological and physical stress. J Clin Endocrinol Metab 84:1944-1948.

Sirinathsinghji DJ, Rees LH, Rivier J, Vale W (1983) Corticotropin-releasing factor is a potent inhibitor of sexual receptivity in the female rat. Nature 305:232-235.

Skelton KH, Nemeroff CB, Knight DL, Owens MJ (2000) Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. J Neurosci 20:1240-1248.

Smagin GN, Heinrichs SC, Dunn AJ (2001) The role of CRH in behavioral responses to stress. Peptides 22:713-724.

Smialowska M, Wieronska JM, Wedzony K (2002) A search for colocalization of mglula receptors with CRF or NPY in the rat brain amygdala. Folia Histochem Cytobiol 40:153-154.

Smith MA, Makino S, Kim SY, Kvetnansky R (1995a) Stress increases brain-derived neurotropic factor messenger ribonucleic acid in the hypothalamus and pituitary. Endocrinology 136:3743-3750.

Smith MA, Makino S, Kvetnansky R, Post RM (1995b) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 15:1768-1777.

Smythe JW, Murphy D, Timothy C, Costall B (1997) Hippocampal mineralocorticoid, but not glucocorticoid, receptors modulate anxiety-like behavior in rats. Pharmacol Biochem Behav 56:507-513.

Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21:70-71.

Spengler D, Rupprecht R, Van LP, Holsboer F (1992) Identification and characterization of a 3',5'-cyclic adenosine monophosphate-responsive element in the human corticotropin-releasing hormone gene promoter. Mol Endocrinol 6:1931-1941.

Steckler T, Holsboer F (1999) Corticotropin-releasing hormone receptor subtypes and emotion. Biol Psychiatry 46:1480-1508.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 14:2579-2584.

Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Muller MB, Schmidt MV (2008) Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. Horm Behav 53:386-394.

Stout SC, Owens MJ, Nemeroff CB (2002) Regulation of corticotropin-releasing factor neuronal systems and hypothalamic-pituitary-adrenal axis activity by stress and chronic antidepressant treatment. J Pharmacol Exp Ther 300:1085-1092.

Stout SC, Owens MJ, Lindsey KP, Knight DL, Nemeroff CB (2001) Effects of sodium valproate on corticotropin-releasing factor systems in rat brain. Neuropsychopharmacology 24:624-631.

Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W (1982) Corticotropin releasing factor produces behavioural activation in rats. Nature 297:331-333.

Swaab DF, Bao AM, Lucassen PJ (2005) The stress system in the human brain in depression and neurodegeneration. Ageing Res Rev 4:141-194.

Swanson LW, Simmons DM (1989) Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. J Comp Neurol 285:413-435.

Swanson LW, Sawchenko PE, Rivier J, Vale WW (1983) Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36:165-186.

Swerdlow NR, Geyer MA, Vale WW, Koob GF (1986) Corticotropin-releasing factor potentiates acoustic startle in rats: blockade by chlordiazepoxide. Psychopharmacology (Berl) 88:147-152.

Tache Y, Brunnhuber S (2008) From Hans Selye's discovery of biological stress to the identification of corticotropin-releasing factor signaling pathways: implication in stress-related functional bowel diseases. Ann N Y Acad Sci 1148:29-41.

Tan H, Zhong P, Yan Z (2004) Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. J Neurosci 24:5000-5008.

Tanapat P, Galea LA, Gould E (1998) Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. Int J Dev Neurosci 16:235-239.

Taylor C, Fricker AD, Devi LA, Gomes I (2005) Mechanisms of action of antidepressants: from neurotransmitter systems to signaling pathways. Cell Signal 17:549-557.

Tezval H, Jahn O, Todorovic C, Sasse A, Eckart K, Spiess J (2004) Cortagine, a specific agonist of corticotropin-releasing factor receptor subtype 1, is anxiogenic and antidepressive in the mouse model. Proc Natl Acad Sci U S A 101:9468-9473.

Thompson RC, Seasholtz AF, Herbert E (1987) Rat corticotropin-releasing hormone gene: sequence and tissue-specific expression. Mol Endocrinol 1:363-370.

Tiscornia G, Singer O, Ikawa M, Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci U S A 100:1844-1848.

Tizabi Y, Skofitsch G, Jacobowitz DM (1985) Effect of chronic reserpine and desmethylimipramine treatment on CRF-like immunoreactivity of discrete brain areas of rat. Brain Res 335:389-391.

Torashima T, Yamada N, Itoh M, Yamamoto A, Hirai H (2006) Exposure of lentiviral vectors to subneutral pH shifts the tropism from Purkinje cell to Bergmann glia. Eur J Neurosci 24:371-380.

Toufexis DJ, Davis C, Hammond A, Davis M (2004) Progesterone attenuates corticotropin-releasing factor-enhanced but not fear-potentiated startle via the activity of its neuroactive metabolite, allopregnanolone. J Neurosci 24:10280-10287.

Treede RD, Kenshalo DR, Gracely RH, Jones AK (1999) The cortical representation of pain. Pain 79:105-111.

Treweek JB, Jaferi A, Colago EE, Zhou P, Pickel VM (2009) Electron microscopic localization of corticotropin-releasing factor (CRF) and CRF receptor in rat and mouse central nucleus of the amygdala. J Comp Neurol 512:323-335.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865-871.

Uphouse L, Selvamani A, Lincoln C, Morales L, Comeaux D (2005) Mild restraint reduces the time hormonally primed rats spend with sexually active males. Behav Brain Res 157:343-350.

Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394-1397.

Valentino RJ, Curtis AL (1991) Antidepressant interactions with corticotropin-releasing factor in the noradrenergic nucleus locus coeruleus. Psychopharmacol Bull 27:263-269.

Valentino RJ, Rudoy C, Saunders A, Liu XB, Van Bockstaele EJ (2001) Corticotropin-releasing factor is preferentially colocalized with excitatory rather than inhibitory amino acids in axon terminals in the peri-locus coeruleus region. Neuroscience 106:375-384.

Vamvakopoulos NC, Chrousos GP (1994) Hormonal regulation of human corticotropin-releasing hormone gene expression: implications for the stress response and immune/inflammatory reaction. Endocr Rev 15:409-420.

van Londen L, Goekoop JG, van Kempen GM, Frankhuijzen-Sierevogel AC, Wiegant VM, van der Velde EA, De Wied D (1997) Plasma levels of arginine vasopressin elevated in patients with major depression. Neuropsychopharmacology 17:284-292.

Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol 428:191-212.

Vaswani M, Linda FK, Ramesh S (2003) Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. Prog Neuropsychopharmacol Biol Psychiatry 27:85-102.

Veith RC, Lewis N, Langohr JI, Murburg MM, Ashleigh EA, Castillo S, Peskind ER, Pascualy M, Bissette G, Nemeroff CB, et al. (1993) Effect of desipramine on

cerebrospinal fluid concentrations of corticotropin-releasing factor in human subjects. Psychiatry Res 46:1-8.
Veldhuis HD, De Wied D (1984) Differential behavioral actions of corticotropin-releasing factor (CRF). Pharmacol Biochem Behav 21:707-713.
Venihaki M, Majzoub J (2002) Lessons from CRH knockout mice. Neuropeptides 36:96-102.
Vesga-Lopez O, Schneier FR, Wang S, Heimberg RG, Liu SM, Hasin DS, Blanco C (2008) Gender Differences in Generalized Anxiety Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). J Clin Psychiatry.
Vogt BA, Finch DM, Olson CR (1992) Functional heterogeneity in cingulate cortex: the anterior executive and posterior evaluative regions. Cereb Cortex 2:435-443.
Walf AA, Frye CA (2006) A review and update of mechanisms of estrogen in the hippocampus and amygdala for anxiety and depression behavior. Neuropsychopharmacology 31:1097-1111.
Wallach MB, Winters WD, Mandell AJ, Spooner CE (1968) A neuropharmacological comparison of some tricyclic anti-depressant agents. Proc West Pharmacol Soc 11:61-65.
Wallen K (1990) Desire and ability: hormones and the regulation of female sexual behavior. Neurosci Biobehav Rev 14:233-241.
Watts AG (1996) The impact of physiological stimuli on the expression of corticotropin-releasing hormone (CRH) and other neuropeptide genes. Front Neuroendocrinol 17:281-326.
Weissman MM, Olfson M (1995) Depression in women: implications for health care research. Science 269:799-801.
Welberg LA, Kinkead B, Thrivikraman K, Huerkamp MJ, Nemeroff CB, Plotsky PM (2006) Ketamine-xylazine-acepromazine anesthesia and postoperative recovery in rats. J Am Assoc Lab Anim Sci 45:13-20.
Wernicke JF (1985) The side effect profile and safety of fluoxetine. J Clin Psychiatry 46:59-67.
Wiersma A, Bohus B, Koolhaas JM (1993) Corticotropin-releasing hormone microinfusion in the central amygdala diminishes a cardiac parasympathetic outflow under stress-free conditions. Brain Res 625:219-227.
Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, Liu D, Kresse A, Neumann ID, Holsboer F, Plotsky PM, Landgraf R (2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. Neuropsychopharmacology 29:1-14.
Wilson ME, Gordon TP, Bernstein IS (1978) Timing of births and reproductive success in rhesus monkey social groups. J Med Primatol 7:202-212.
Wilson TJ, Kola I (2001) The LoxP/CRE system and genome modification. Methods Mol Biol 158:83-94.
Wolter HJ (1985) Corticotropin-releasing factor: immunohistochemical colocalization with adrenocorticotropin and beta-endorphin, but not with Met-enkephalin, in subpopulations of duodenal perikarya of rat. Biochem Biophys Res Commun 128:402-410.

Yamamori E, Asai M, Yoshida M, Takano K, Itoi K, Oiso Y, Iwasaki Y (2004) Calcium/calmodulin kinase IV pathway is involved in the transcriptional regulation of the corticotropin-releasing hormone gene promoter in neuronal cells. J Mol Endocrinol 33:639-649.

Yao M, Denver RJ (2007) Regulation of vertebrate corticotropin-releasing factor genes. Gen Comp Endocrinol 153:200-216.

Yao M, Schulkin J, Denver RJ (2008) Evolutionarily conserved glucocorticoid regulation of corticotropin-releasing factor expression. Endocrinology 149:2352-2360.

Yehuda R (2001) Biology of posttraumatic stress disorder. J Clin Psychiatry 62 Suppl 17:41-46.

Yehuda R, Brand S, Yang RK (2006) Plasma neuropeptide Y concentrations in combat exposed veterans: relationship to trauma exposure, recovery from PTSD, and coping. Biol Psychiatry 59:660-663.

Yehuda R, Giller EL, Southwick SM, Lowy MT, Mason JW (1991) Hypothalamic-pituitary-adrenal dysfunction in posttraumatic stress disorder. Biol Psychiatry 30:1031-1048.

Yehuda R, Halligan SL, Grossman R, Golier JA, Wong C (2002) The cortisol and glucocorticoid receptor response to low dose dexamethasone administration in aging combat veterans and holocaust survivors with and without posttraumatic stress disorder. Biol Psychiatry 52:393-403.

Yehuda R, Southwick SM, Nussbaum G, Wahby V, Giller EL, Jr., Mason JW (1990) Low urinary cortisol excretion in patients with posttraumatic stress disorder. J Nerv Ment Dis 178:366-369.

Young EA, Lopez JF, Murphy-Weinberg V, Watson SJ, Akil H (2003) Mineralocorticoid receptor function in major depression. Arch Gen Psychiatry 60:24-28.

Young EA, Altemus M, Lopez JF, Kocsis JH, Schatzberg AF, DeBattista C, Zubieta JK (2004) HPA axis activation in major depression and response to fluoxetine: a pilot study. Psychoneuroendocrinology 29:1198-1204.

Yuuki N, Ida I, Oshima A, Kumano H, Takahashi K, Fukuda M, Oriuchi N, Endo K, Matsuda H, Mikuni M (2005) HPA axis normalization, estimated by DEX/CRH test, but less alteration on cerebral glucose metabolism in depressed patients receiving ECT after medication treatment failures. Acta Psychiatr Scand 112:257-265.

Zarate CA, Jr., Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, Charney DS, Manji HK (2006) A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. Arch Gen Psychiatry 63:856-864.

Zhao D, Pothoulakis C (2006) Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. Peptides 27:2434-2444.

Ziegler DaH, JP (2002) Neurocircuitry of Stress Integration: Anatomical Pathways Regulating the Hypothalamo-Pituitary-Adrenocortical Axis of the Rat. Integrative and Comparative Biology 42:541–551.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M, Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J Psychiatr Res 34:171-181.

Zufferey R, Donello JE, Trono D, Hope. TJ (1999) Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. J Virol 73:2886-2892.

Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72:9873-9880.