OPTOGENETIC AND PHARMACOLOGICAL DISSECTION OF A NEUROPEPTIDE CIRCUIT THAT GATES SUSTAINED FEAR

by

Arun Asok

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

Spring 2016

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

OPTOGENETIC AND PHARMACOLOGICAL DISSECTION OF A NEUROPEPTIDE CIRCUIT THAT GATES SUSTAINED FEAR

by

Arun Asok

Approved: _____

Robert F. Simons, Ph.D.
Chair of the Department of Psychological and Brain Sciences

Approved: _____

George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____

Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Jeffrey B. Rosen, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Mark Stanton, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Mary Dozier, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Murali Temburni, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Jay Schulkin, Ph.D.
Member of dissertation committee

ACKNOWLEDGMENTS

There are so many people I am grateful for and without whom I would not have been able to succeed during graduate school. I am forever indebted to the individuals that I have interacted with during my time at the University of Delaware. Those that have so freely contributed to my intellectual, personal, and professional development – graduate students and faculty alike.

First and foremost, I would like to thank my mentor Jeffrey B. Rosen. His compassion, understanding, and openness to ideas and innovation are characteristics that are truly remarkable and that I will strive to exemplify throughout my career. Without his willingness to take me into his lab, my career (and views about science) would be very different. One of the most important lessons I have learned from him through speech and action is to follow the data, wherever it may take me.

Second, I would like to thank Tania L. Roth. Her willingness to freely share equipment and resources, scientific ideas, and provide emotional support when needed, have helped me to survive and flourish during my graduate years. Without her help I would have never been able to accomplish many of the things I have.

Third, I would like to thank Mark E. Stanton. Our numerous discussions on the philosophical foundations of science, theoretical constructs for establishing linear causality, (and strong encouragement to include the right control groups), are fundamental to the type of science I will conduct and the scientist I will become.

Fourth, I would like to thank Mary Dozier. Her willingness to explore new ideas (telomeres) and provide the necessary resources and support to be successful is

something I am forever indebted to her for. Most of all, her willingness to encourage me (and others) to challenge her if we did not agree is a truly remarkable quality – a quality I will try to embrace throughout my career.

Fifth, I would like to thank Jay Schulkin. His contagious enthusiasm for science, phone calls and e-mail at any time day or night, and persistence on completing projects, have strongly impacted my work ethic and productivity. I am extremely grateful for his presence in my life.

Finally I would like to thank my family (father Asok, mother Vathsala, aunt Lakshmi, brother Aravind, and partner Amber) for their support in times of uncertainty, stress, and disenchantment with science. It is through their support that I have been able to succeed and flourish in my scientific pursuits. But, more importantly, it is their support I will depend on throughout the rest of my professional and personal life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAV	adeno associated virus
ACTH	adrenocortictropic releasing hormone
ANOVA	analysis of variance
ArchT	archaerhodopsin tp009
BLA	basolateral amygdala complex
BMA	basomedial amygdala
BNST	bed nucleus of the stria terminalis
$_{L}$BNST	dorsolateral division of the bed nucleus of the stria terminalis
BOLD	blood-oxygen level dependent signal
cf	confer; “compare”
CA	Cornu Ammonis
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
CeA	central nucleus of the amygdala
$_{L}$CeA	lateral part of the central nucleus of the amygdala
$_{M}$CeA	medial part of the central nucleus of the amygdala
CoA	cortical amygdala
CeA	central nucleus of the amygdala
c-fos	cellular feline osteosarcoma gene
cm	centimeter

CORT	corticosterone
CREB	cAMP response element-binding protein
CR	conditioned response
CRF	corticotropin-releasing factor
CRF-ArchT	corticotropin-releasing factor promoter driven ArchT
CRF-EGFP	corticotropin-releasing factor promoter driven enhanced green fluorescent protein
CRFr1	corticotropin-releasing factor type-1 receptor
CRFr2	corticotropin-releasing factor type-2 receptor
CS	conditioned stimulus
e.g.	exempli gratia, “for example”
EGFP	enhanced green fluorescent protein
Egr-1	early growth response gene 1
fMRI	functional magnetic resonance imaging
g	gram
GABA	γ-aminobutyric acid
GECI	genetically encoded calcium indicator
GCaMP6f	green fluorescent protein, calmodulin, myosin light chain kinase peptide
GPCR	g-protein coupled receptor

HPA	hypothalamic-pituitary-adrenal
hr	hour
IACUC	Institutional Animal Care and Use Committee
i.e.	id est, “that is”
IL	infralimbic region of the medial prefrontal cortex
$_{V}$LA	ventrolateral division of the lateral nucleus of the amygdala
mA	milliamperes
MeA	medial amygdala
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
OLAM	Office of Laboratory Animal Medicine
PAG	periaqueductal gray
PL	prelimbic region of the medial prefrontal cortex
PVT	paraventricular nucleus of the thalamus
PVN	paraventricular nucleus of the hypothalamus
RSC	retrosplenial cortex
SEM	standard error of the mean
sec, s	second
US	unconditioned (unconditional) stimulus

µg	microgram
µl	microliter
+	positive

ABSTRACT

This dissertation focuses on how corticotropin-releasing factor (CRF), a 41 amino-acid neuropeptide, within an extended amygdala microcircuit gates fear learning and memory. Chapter 1 frames fear and anxiety within a historical perspective. It also describes how a reductionist approach using rodent models has been useful for deciphering the neural circuitry and neurobiology of fear memory formation and retrieval. Chapter 2 briefly describes how stress can modulate fear learning and memory. It also outlines a theoretical framework for the experiments of my dissertation. Chapter 3 is an empirical study that examines how blockade of CRFs primary receptor (CRFr1s) in a region of the extended amygdala, the bed nucleus of the stria terminalis (BNST), impacts discrete phases (acquisition, consolidation, or retrieval) of contextual fear learning and memory. Chapter 4 expands on the experiments of chapter 3 to investigate how optogenetically inhibiting a CRF pathway to the BNST regulates the retention of short and long-lasting fear. Finally, Chapter 5 highlights a few critical future experiments and frames the empirical findings of my dissertation within the context of its public health relevance.

Chapter 1

THE NEURAL BASIS OF LEARNED FEAR: A SYSTEMS VIEW

"…it is certain that the problem of fear is the meeting point of many important questions, an enigma whose complete solution would cast a flood of light upon psychic life" (Freud, 1920).

1.1 A Century of Fear

"The intention is to furnish a psychology that shall be a natural science: that is, to represent psychical processes as quantitatively determinate states of specifiable material particles…the neurones are to be taken as the material particles" (Freud, 1895). More than a century ago, Freud expounded on his view that psychological processes were represented by precise neuronal states – a view widely regarded as a fundamental truth by many modern day neuroscientists and psychologists (Kandel, 2005). While Freud is well-known for his psychoanalytic contributions to psychology, his ideas on the human manifestations of fear and anxiety (e.g., nervousness; (Freud, 1920)) helped to pave the way for scientific inquiry into their neural correlates. Freud's investigation of fear and anxiety was by no means a novel endeavor during the early 1900's. In fact, his work expanded on the observations of Charles Darwin across humans and animals some decades earlier (Darwin, 1872). However, what is most evident since the age of Freud and Darwin (and Pavlov) is a continuing pursuit to

understand the psychological, behavioral, and cellular constituents of fear and anxiety - the goal of which is to better diagnose and treat fear and anxiety disorders.

Research in the last 50 years has made great strides towards describing the physiological changes associated with fear and anxiety disorders – progress which has greatly advanced our understanding of how the brain is involved. This progress has often been accompanied by the development of a number of treatments (e.g., exposure therapy, pharmacological anxiolytics, etc.) aimed towards easing the burden on the millions who suffer from fear and anxiety disorders each year (Kessler et al., 2005). However, there is still much to be understood with regard to how normal fear/anxiety can turn to pathological dysfunction (Rosen & Schulkin, 1998) and how we can provide more efficacious treatments. It is this gap in our understanding of fear/anxiety that deserves much needed attention.

From the outset, I should make it clear that my principal goal is to understand the neural basis of fear. My dissertation adds a small piece to this enduring puzzle. I make no attempt to clearly differentiate fear from anxiety. However, I do, at times, distinguish between fear that is learned and innate (Chapter 3) in addition to fears that are short and long-lasting (Chapter 4; (Davis, Walker, Miles, & Grillon, 2010); for an interesting read see transdiagnostic constructs of anxious apprehension and anxious arousal (Sharp, Miller, & Heller, 2015). While my dissertation cuts across varying levels of analysis, it is important to note that the reductionist approach, behavioral paradigms, pharmacological compounds, genetic manipulations, and molecular analyses, are all simply tools used for gaining a greater insight into how the brain

processes fear. I believe that advancing our fundamental knowledge of how different types of fear are processed at behavioral, anatomical, and molecular levels will lead to a better framework for classifying (see RDoC (NIMH, 2013), conceptualizing (Perusini & Fanselow, 2015), and treating fear and anxiety disorders (as discussed in Chapter 5).

1.1.1 A Reductionist Approach to Exploring Fear

A number of human studies have examined the acquisition and expression of fear in response to various stimuli such as facial expressions, scenes, and aversive agents (Whalen & Phelps, 2009). Individuals who learn and express fear to these stimuli display a number of common physiological changes including: cardiac acceleration, more muscle potentials, increased galvanic skin conductance, and increased respiration, to name a few (Ax, 1953; Garfinkel & Critchley, 2015; Van Diest, Bradley, Guerra, Van den Bergh, & Lang, 2009). In addition to changes measured in the periphery, recent work has shown that during the acquisition of fear, that is where an association between a conditioned stimulus (CS; e.g., a tone, light, or environment) and unconditioned stimulus (US; e.g., a shock or air-puff) is formed, there is increased neural activity (blood-oxygenation level dependent activity) within the amygdala (LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998). These changes, both physiological and neural, are thought to reflect preparatory defensive actions (or inaction; (Van Diest, et al., 2009)). Importantly, it is here, at the intersection of physiological and neural activity, that a reductionist approach using model organisms have been highly informative.

The fear response, while differing in form, is thought to be preserved in function (i.e., survival) across species (Adolphs, 2013). Rodent models of fear learning and expression have been vital for elucidating the neural bases of fear for two key reasons. First, they allow for the causal investigation of the neural substrates that regulate the acquisition and expression of fear. Second, they allow for the precise control of environmental variables (i.e., the CS and the US). The notion of the amygdala as a critical brain region important for processing "fear" was evident from studies using a reductionist approach, well before the advent of fMRI (circa 1992) and its use for investigating "fear-related" brain activity in humans. In fact, initial studies in primates and rodents some decades earlier identified the medial temporal lobe in general, and the amygdala more specifically, as areas important for integrating and regulating emotionality to fearful CS – US information (Goddard, 1964; Grossman, Grossman, & Walsh, 1975; Klüver & Bucy, 1939). Particularly, rodent models of fear have been beneficial for (1) initially identifying the functional importance of the amygdala during fear learning and expression and (2) in continuing to identify how different types of fear are processed across select brain regions and within specific cells.

1.1.2 Contextual Fear Conditioning

In a typical fear conditioning experiment an animal is presented with a precisely controlled CS (e.g., generally a tone, light, or context) that is paired with a foot-shock. While the CS can be presented through discrete auditory and visual sensory modalities, it can also consist of a multisensory CS such as a context (Figure

1.1). This type of contextual CS (cf. (Holland & Bouton, 1999)) is thought to be a conjunction of the individual sensory features of the environment (Rudy, 2009; Rudy & O'Reilly, 2001). In contextual fear conditioning, the rodent learns to associate a foot-shock with a context CS. The context, as whole, is comprised of its multisensory parts including: the tactile feel of the grid bars, the visual components of lighting and objects, the olfactory stimuli, spatial layout, and the auditory background noise (see Figure 1.1; (Murawski & Asok, 2015)).

Before forming an association between the context-CS and the foot-shock-US, animals must first form a representation of the context. This generally occurs through random exploration of the environment (i.e., a pre-exposure to the environment) where the rodent incidentally (i.e., without reinforcement) learns about the context or environment. This exploration is thought to facilitate the formation of a conjunctive or unitary contextual representation by binding together individual features or elements (Rudy, 2009; Rudy & O'Reilly, 2001). The animal must explore the environment for a minimum amount of time (~30 seconds) in order for this contextual representation to be formed (Fanselow, 1990).

Following the initial period of context exploration, rodents are given a brief shock US of a specific intensity (measured in mA) and duration (measured in seconds). During this period, rodents associate the contextual representation (or CS) with the shock US information. The exposure to the context prior to shock , either on the same or on a separate occasion prior to receiving the foot-shock, is critical for forming the CS-US association (Fanselow, 1986, 1990). Animals that form an

association between the context and the shock exhibit a number of species specific defensive reactions (SSDRs), the most well studied of which is freezing (Fanselow, 1980). This SSDR of freezing is generally used as a proxy for measuring the strength of the CS-US association and overall fear (Wood & Anagnostaras, 2011). Operationally, freezing is defined as the cessation of all movement except that necessary for respiration (Blanchard & Blanchard, 1969; Bolles, 1970) and, following conditioning, is robust and long-lasting (Fanselow, 2000). That is, the acquired fear memory can last on the order of days, weeks, months, and years (Gale et al., 2004). When fear conditioned rodents are exposed to the context in the future (without a shock), they reliably express freezing that is specific to that context. An interesting quality of contextual conditioning is that even when animals are conditioned to discrete CSs such as tones or lights, they almost always form an association with the background context– suggesting that the *acquisition* of contextual information is inextricably linked to acquired fear of differing sensory modalities.

Contextual fear conditioning is an excellent tool for understanding how fears are learned and expressed in particular environments. I will use freezing behavior in my dissertation as a proxy for measuring the strength of the fear association during discrete phases of learning and memory (e.g., acquisition, consolidation/storage and retrieval/expression).

1.1.3 Fear Acquisition, Consolidation and Retrieval

Acquisition of contextual fear memories can be separated into two distinct phases: (1) acquisition of the context representation (described above) and (2)

acquisition of the context-shock association. These phases of memory do not rely on the synthesis of new proteins, but do rely on intact synaptic transmission across areas that are (1) important for forming a representation of the context (i.e., the hippocampus), (2) important for relaying US information (i.e., somatosensory and thalamic areas), and, critically, (3) areas where the CS and US converge (i.e., the amygdala). A number of studies have shown that pre-training electrolytic lesions (i.e., lesions destroying cell bodies and fibers of passage; summarized in greater detail in Chapter 1.2) that ultimately affect information transmission across the amygdala and hippocampus disrupt fear acquisition (e.g., (Kim & Davis, 1993; Maren & Fanselow, 1997; Phillips & LeDoux, 1992)). Acquisition of fear is measured by freezing during the period immediately following the shock (post-shock freezing). Given that rodents must learn about the context before they can associate the aversive US with it (as mentioned above), drugs administered before training that interfere with post-shock freezing can be assumed to disrupt the acquisition of fear (Johansen, Cain, Ostroff, & LeDoux, 2011).

Consolidation of contextual fear memories can be defined as a protein synthesis dependent process that relies on intact synaptic transmission (i.e., acquisition) and consequently affects nuclear processes and synaptic morphology. Although rodents can exhibit intact freezing in the short-term after conditioning (i.e., on the order of hours), blocking protein synthesis or signaling cascades (e.g., NMDA receptors, mRNA, MAPKs, etc.) interfere with long-term retention of freezing when tested 24 hours later (for review see (Abel & Lattal, 2001). Thus, the disruption of

consolidation is *assumed* to interfere with the prevailing mechanism of memory consolidation– long-term potentiation (LTP; for a recent in vivo demonstration involving the amygdala see (Nabavi et al., 2014)). The synthesis of new proteins occurs on the order of hours (and days) and functions to stabilize neurons and strengthen synaptic connections (Bourtchouladze et al., 1998; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999). However, consolidation has no direct behavioral consequence except for what is observed at the retention test ($\geq$ 24 hours later).

I define consolidation as the time-period following acquisition during which the acquired CS-US association is encoded and 'stored' in a stable manner for long-term retrieval. A more in-depth analysis of memory consolidation can be found in a review by Jim McGaugh (McGaugh, 2000) and a review on consolidation in fear conditioning by Marie Monfils (Seo & Monfils, 2012). Manipulations after animals exhibit post-shock freezing (i.e., after acquisition) are thought to impact memory consolidation (Johansen, et al., 2011). However, consolidation of the fear memory (enhanced freezing to the context-CS) and failures in consolidation (reduced freezing to the context-CS despite intact acquisition) are evident in the non-reinforced context-alone retention test given 24 hours later that measures fear expression (Figure 1.2).

Expression (or retrieval) is the time at the retention test during which rodents retrieve the stored CS-US association that was (1) acquired and (2) consolidated in order to guide freezing behavior in the specific context. Destruction of the cells and information transmission pathways involved in storage can disrupt retrieval of the consolidated context-US association (for an interesting recent study using temporally-

precise cell-type specific toxin lesions in the dentate gyrus see ((Matsuo, 2015). In my dissertation, manipulations occurring immediately before the long-term memory retention test (i.e., 24 hours after acquisition) are geared towards understanding if there are failures in retrieving the stored association and expressing freezing behavior.

In summary, freezing at specific time points during contextual fear conditioning can be used to measure discrete phases of memory (acquisition, consolidation, and retrieval/retention/expression). Although other time-points are commonly used to separate out short-term from long-term memories (or protein synthesis independent vs. dependent, respectively or even NMDA independent vs. dependent long-term memory; see (Bliss & Collingridge, 1993)), this description provides a simple framework for understanding the experiments in this dissertation. A large amount of research has accumulated over the last half-century to provide a significant evidence base for these theoretical constructs of learning and memory. However, only in recent decades have studies unraveled a common neuroanatomical and neurobiological framework for fear learning.

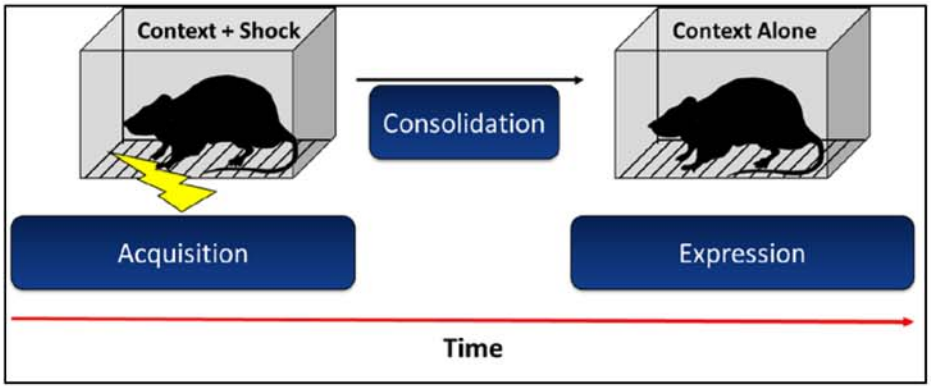


Figure 1.1 Schematic representation of contextual fear conditioning paradigm mapped onto phases of learning and memory. Note that memory phases are mapped onto discrete time points of behavior. Acquisition is the time period when the context-shock association is formed. Consolidation occurs during the time period after the shock. Expression is the period when animals are re-exposed to the context without presentation of the shock. Animals that learn context fear express freezing behavior at the expression test following the prior context-shock pairing.

1.2 Neural Networks of Contextual Fear

The discrete phases of fear learning and memory in contextual fear conditioning are thought to rely on specific brain regions. These brain regions form a neural network (see Figure 1.3) for storing and retrieving information related to fearful experiences. My dissertation examines the contributions of an extended amygdala microcircuit consisting of projections from the lateral part of the central nucleus of the amygdala ($_{L}$CeA) to the dorsolateral division of the bed nucleus of the stria terminalis ($_{I}$BNST). However, it is important to understand how this microcircuit is simply a module in a larger network that regulates fear learning and expression.

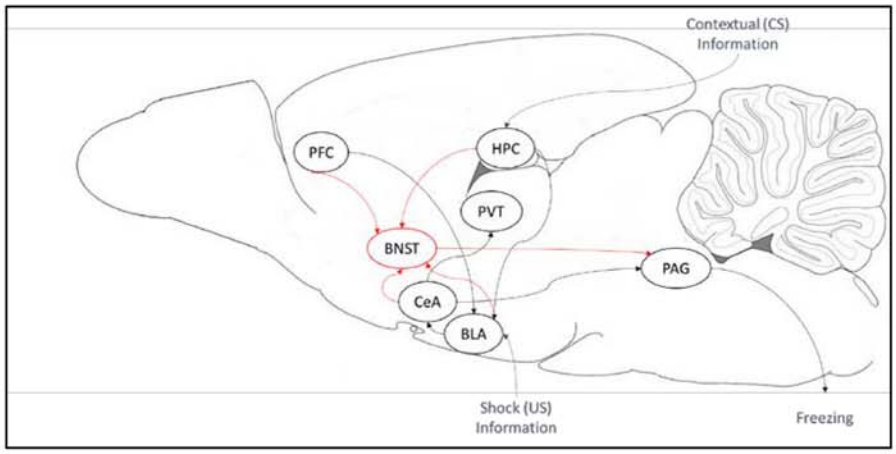


Figure 1.2. Simplified schematic of the neural pathways associated with contextual fear conditioning. Contextual information is processed by the hippocampus and then relayed to the basolateral amygdala (BLA). In the BLA contextual information converges with shock US information, before being relayed through the central nucleus of the amygdala (CeA) to the ventrolateral periaqueductal grey (PAG) that mediates the behavioral response of freezing. The bed nucleus of the stria terminals (BNST; outlined in red) is ideally positioned to integrate information across behavioral, mnemonic, and neuroendocrine and domains.

1.2.1 The Hippocampus

Spatial and contextual information is primarily processed by the hippocampus – a region, as mentioned earlier, that functions to bind individual features of the environment into a unitary representation during contextual fear conditioning (Rudy, 2009). The hippocampus is divided into two major divisions along a septotemporal axis – the dorsal and ventral portions (with a third often referred to as intermediate; Fanselow and Dong, 2010). The ventral hippocampus is important for processing information related to stress and emotion. It is generally accepted that multisensory information (likely selected based off of saliency; see discussion in (Fanselow, Zelikowsky, Perusini, Barrera, & Hersman, 2014)) about the context is relayed from

the sensory areas through the entorhinal cortex, and fimbria/fornix, into the dorsal dentate gyrus. Indeed, electrolytic lesions of the entorhinal cortex, fimbria/fornix, or dorsal hippocampus (1 week prior to training; for an important caveat about dorsal hippocampal lesions see (Zelikowsky, Bissiere, & Fanselow, 2012)) in addition to disrupting neurotransmission in the dentate gyrus (via the axonal transport inhibitor colchicine) disrupts acquisition and retention of contextual fear (Hernandez-Rabaza et al., 2008; Maren & Fanselow, 1997).

Anatomically, the flow of information into the hippocampal formation is generally believed to proceed from the dentate gyrus to the cornu ammonis (CA) subfields, specifically from CA3 to CA1. These subfields are important for processing distinct aspects contextual/spatial information. For example, the dentate gyrus and CA3 are important for contextual pattern separation (Leutgeb et al., 2007) and CA1 is important for pattern completion (Leutgeb et al., 2004) - as evidenced by the fact that exposure to similar contexts produces similar electrophysiological activity patterns. Furthermore, contextual information is distributed across the dorsal and ventral axis of CA1. Specifically, dorsal CA1 is thought to be important for contextual fear acquisition and ventral CA1 is thought to be important for contextual fear expression (Hunsaker and Kesner, 2008). While there is strong support for the idea that context-CS information, and the contextual representation itself, is formed and stored in dorsal CA1, studies have shown that ventral CA1 is also necessary (Rudy and Matus-Amat, 2005). More recent work has expanded upon these studies using cutting-edge genetic techniques to show how specific neurons active at the time of acquisition, within the

dorsal dentate gyrus and select dorsal CA subfields (using experience-dependent c-fos tagging), are also necessary for consolidating contextual information and modulating freezing behavior (Matsuo, 2015; Redondo et al., 2014; Tonegawa, Liu, Ramirez, & Redondo, 2015). Thus, while it is clear that the hippocampus is critical for contextual processing contextual information, the functional distinctions between the dorsal and ventral portions during contextual fear conditioning are still being unraveled.

Following overall contextual/emotional information processing by the hippocampal formation, information is then thought to reach the BLA from ventral CA1. However, this pathway is not unidirectional in that BLA inputs to the ventral hippocampus modulate so-called "anxiety"-related behaviors by decreasing the time spent in the center of the open field and increasing the time spent in the open-arms of elevated plus maze tests (Felix-Ortiz et al., 2013). Interestingly, this pathway uniquely contributes to context-US learning and not just contextual information processing or fear expression (as shown by Kesner's group), but how this works has yet to be determined (Huff, Emmons, Narayanan, & LaLumiere, 2016). Irrespective of the directionality of BLA-CA1 information transmission, the amygdala remains as a critical center for fear learning and memory.

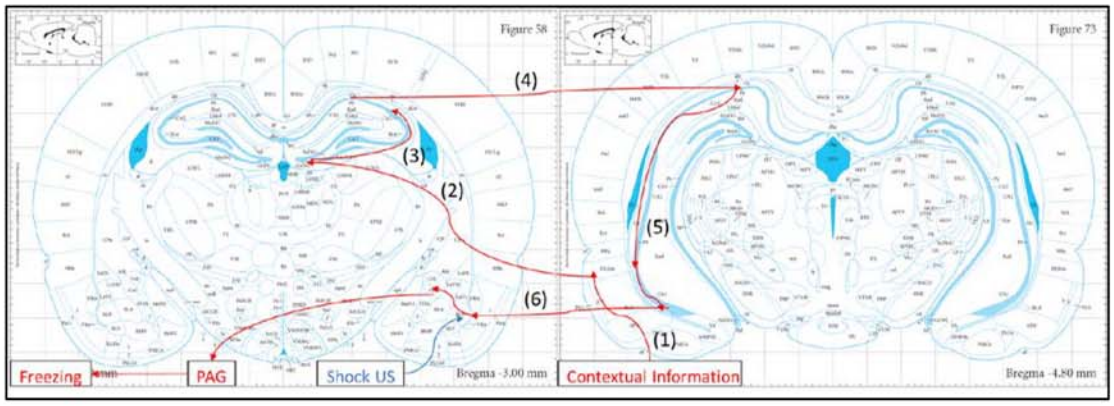


Figure 1.3 Flow of contextual information into the amygdala during contextual fear conditioning (serial processing view). Contextual information is relayed from (1) the entorhinal cortex, to (2) the dentate gyrus, before traversing through (3) dorsal CA3 and CA1. Information is then (possibly) relayed across the hippocampal commissure and septotemporal axis of the hippocampus to (5) ventral CA1. This projects to (6) the posterior part of the basolateral amygdala complex, before reaching the medial part of the central nucleus of the amygdala. Information from the medial part of the central nucleus of the amygdala is then relayed to the ventrolateral periaqueductal grey to mediate the behavioral response of freezing. The laterality of information transmission is not implied. Figure adapted from (Paxinos & Watson, 1982; Paxinos & Watson, 2007).

1.2.2 The Amygdala

The amygdala consists of over 13 subnuclei (Aggleton, Everitt, Cardinal, & Hall, 2000), with five major divisions: the basolateral amygdala (BLA), basomedial amygdala (BMA), medial amygdala (MeA), cortical amygdala (CoA), and the central amygdala (CeA). Different subnuclei of the BLA and CeA have various functions in fear conditioning, (e.g., the dorsolasteral division of the lateral nucleus of the amygdala within the BLA or the medial and lateral parts of the CeA; for review see (Josselyn, 2010; Jeffrey B Rosen, 2004). Contextual information from ventral CA1 is thought reach the anterior portion of the BLA and BMA, as neurotoxic lesions of the

posterior BLA and BMA have no effect on contextual fear (Figure 1.3; (Goosens & Maren, 2001)). It is here, in cells of the anterior BLA/BMA that contextual information is thought to converge with shock US information relayed from the thalamus (Lanuza, Nader, & Ledoux, 2004; J. LeDoux, 2003).

The CS-US association is acquired and consolidated across a sparse network of cells in the BLA, BMA, and CeA (Hashikawa et al., 2013). In fact, it was long thought that projections from the BLA signal the medial part of the central nucleus ($_{m}$CeA) to passively regulate fear expression (LeDoux, 2003; Tovote, Fadok, & Lüthi, 2015). However, this "passive" view of the $_{m}$CeA has recently been discounted by a number of studies showing that the CeA is, in fact, important for consolidating the CS-US association (cf. (Wilensky, Schafe, Kristensen, & LeDoux, 2006)). Indeed, post-training (i.e., after acquisition) manipulations of these different amygdala nuclei (e.g., the BLA and CeA). that block (1) activity in cell bodies, (2) different neurotransmitters, (3) receptors, (4) key molecular signaling pathways (e.g.,, mitogen activated protein kinases, extracellular regulated kinases), (5) transcription factors (i.e., cyclic adenosine monophosphate response element binding protein), (6) protein synthesis, and (7) RNA transcription and translation, disrupt contextual fear memory consolidation (for a detailed review see (Maren, 2001)). Finally, the last leg of information transmission is thought to occur via CeA projections to the ventrolateral periaqueductal grey (PAG) to modulate behavioral output (i.e., fear conditioned freezing) - as evidenced by the fact that electrolytic lesions of the PAG disrupt contextually conditioned freezing (Kim, Rison, & Fanselow, 1993).

It is worth mentioning that although the amygdala is critical for storing and retrieving contextual fear memories, it also plays a role in regulating behavior to rewarding stimuli and unconditioned fears (Salzman & Fusi, 2010; Tovote, et al., 2015). Not only has recent work shown that subsets of neurons within the BLA modulate reward-learning (Namburi et al., 2015), but specific neurons within the cortical nucleus (Root, Denny, Hen, & Axel, 2014) and MeA also modulate unconditioned fears to predators and predator odors (Canteras, 2002). An in depth analysis of innate fear to predator odors can be found in our recent review (Rosen, Asok, & Chakraborty, 2015). Studies continue to paint an ever more detailed picture of how amygdala microcircuits gate various types of behavior.

1.2.3 Other Regions Important for Contextual Fear Conditioning

In addition to the hippocampus and amygdala, a number of other brain regions have been implicated in the acquisition and expression of fear memories. For example, the medial prefrontal cortex (mPFC) plays an important role. Lesions of the prelimbic (PL) region of medial prefrontal cortex disrupts the expression of conditioned fear (Corocoran and Quirk, 2007), whereas lesions of the infralimbic (IL) region disrupts the extinction of conditioned fear (Quirk et al., 2006; Sotres-Bayon et al., 2010). Anterograde tracing studies (using phaseolus vulgaris-leucoagglutinin or PHAL-L) have shown that the IL projects to the intercalated cell masses (not discussed in detail; although see (Adhikari et al., 2015) for IL projections to BMA) of the amygdala and the PL projects to the basomedial amygdala (Vertes, 2004). In fact, it is thought that converging inputs from the ventral hippocampus (presumably CA1) and prefrontal

cortex (presumably PL) regulate contextual fear learning and extinction in a subset of basal nuclei cells (e.g., BMA and BLA; (Adhikari, et al., 2015; Orsini, Kim, Knapska, & Maren, 2011)) - fear extinction as defined by a reduction in freezing brought about by non-reinforced presentation of the CS and involving new learning (Quirk et al, 2008).

Recent studies using cell-type specific optogenetics with viral-mediated genetic targeting are beginning to rewrite some of the decades-long held beliefs about projections and regional functionality. For example, it was recently shown that monosynaptic projections from the medial prefrontal cortex (i.e., the anterior cingulate which is important for contextual fear (Einarsson & Nader, 2012)) to dorsal CA3 and CA1 exist, and regulate the retrieval of contextual fear (Rajasethupathy et al., 2015). Additionally, the IL was shown to control aspects of contextual fear specificity and generalization (Zelikowsky et al., 2013) presumably through its projections through the nucleus reuniens (a midline thalamic nucleus; (Xu & Südhof, 2013)). Interestingly, the paraventricular nucleus of the thalamus (PVT), another midline thalamic nucleus, controls retrieval of long-term fear memories (>24 hours up to 28 days) – a phenomena involving the PL (Do-Monte, Quiñones-Laracuente, & Quirk, 2015). These studies are beginning to reveal how midline thalamic nuclei have a much more important function in contextual fear learning than previously believed.

Other areas such as the retrosplenial cortex (RSC) are believed to serve as higher-order cortical hubs important for processing contextual fear (Cowansage et al., 2014; Keene & Bucci, 2008). This is not surprising given that CA1 has robust

projections to the RSC (for a beautiful interactive map of hippocampal and parahippocampal RSC connectivity see (Sugar, Witter, van Strien, & Cappaert, 2011). These recent mPFC and RSC studies have helped to illuminate the function of new areas in the age-old fear-circuit. As newer techniques and strategies for pathway and cell-type specific neuronal targeting emerge, the canonical neural circuits of contextual fear will evolve into a more complete picture.

1.2.4 The Bed Nucleus of the Stria Terminalis

One area that has received limited attention for its role in contextual fear conditioning is the bed nucleus of the stria terminalis (BNST). This is surprising given that research over a decade ago demonstrated that post-training (i.e., after acquisition) electrolytic lesions disrupt the expression of contextual fear (Sullivan et al., 2004) – pointing to a role for the BNST in consolidation (with a caveat that not all post-training effects reflect consolidation). However, the function of the BNST may be more complex than what was once thought. Recent work has shown that neurotoxic lesions of the BNST disrupt the expression of contextual fear memories, but only when the context is learned about for a considerable time (i.e., 10 min but not 1 min (Hammack, Todd, Kocho-Schellenberg, & Bouton, 2015)). Furthermore, lesions have no effect on fear (i.e., freezing) to short-duration (lasting on the order of seconds) discrete cues such as tones (LeDoux, Iwata, Cicchetti, & Reis, 1988) – an effect reported with other fear and anxiety paradigms (Davis, et al., 2010). Together, these studies suggest that while the BNST may be important for contextual fear, its function may relate more to the temporal nature of the conditioned stimuli.

Optogenetic studies have found that distinct BNST sub-regions (dorsolateral and anterodorsal), and projections, modulate select components of anxiety-like behavior (e.g., respiratory rate (S.-Y. Kim et al., 2013)). The lateral portions of the BNST may be especially important for fear learning. Although, neurons here decrease their firing rate during the expression of contextual fear (Haufler, Nagy, & Pare, 2013) – possibly alluding to a role in exerting inhibitory control. What is most striking about the BNST is that it can compensate for the critical role of the BLA during contextual fear learning, that is, when the BLA is lesioned and rodents are over-trained (Ponnusamy, Poulos, & Fanselow, 2007; Poulos, Ponnusamy, Dong, & Fanselow, 2010). This suggests that the BNST may have an important role in acquiring and consolidating contextual fear memories.

While I am framing my discussion of the BNST in terms of contextual fear conditioning, I should mention that it is also implicated in a number of other behaviors including: maternal neglect (Klampfl, Brunton, Bayerl, & Bosch, 2015), innate fear (Fendt, Endres, & Apfelbach, 2003), alcohol addiction (Lovinger & Kash, 2015), and many more (see (Dumont, 2009) for an overview of a special issue in Progress in Neuro-Psychopharmacology and Biological Psychiatry). These other functions and behaviors are not experimentally or theoretically addressed in this dissertation, but see (Lebow & Chen, 2016) for a comprehensive review.

1.2.4.1 BNST Anatomy

The BNST is clustered near the midline and located close to the lateral ventricles and the anterior commissure (Figure 1.4). It is almost 1.5 mm from the

amygdala nuclei in rodents (personal observations) and is highly interconnected (Figure 1.2). The BNST, like the amygdala, is comprised of over 13 subnuclei (Dumont, 2009) and can be divided into two major divisions (anterior and posterior; (Bayer, 1987)) and three primary subdivisions: the dorsolateral, the medial, and the ventrolateral (Figure 1.4). Each of these subdivisions has different inputs, outputs, and hypothesized functions. Given the similarity in morphology, chemical content, and efferent projections with the CeA, the BNST and CeA are commonly referred to as the extended amygdala (Alheid, De Olmos, & Beltramino, 1995). My dissertation examines the anterior portion of the dorsolateral division of the BNST (Figure 1.4 Number 1).

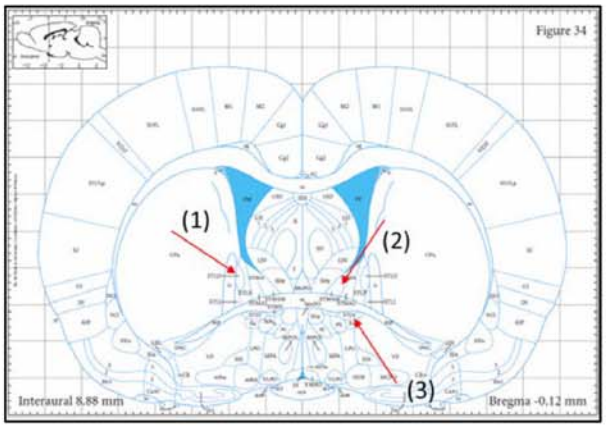


Figure 1.4. Coronal section outlining the BNST in the rat brain. (1) The dorsolateral division of the BNST is closest to the internal capsule and lateral ventricles. It receives inputs from the lateral part of the central nucleus of the amygdala. (2) The medial BNST receives input from the medial nucleus of the amygdala. (3) The ventrolateral portion is also thought to receive inputs from the amygdala, but also receives adrenergic inputs from the locus coeruleus (not shown). The BNST cells in (1) are thought to project to magnocellular GABAergic cells surrounding the parvocellular CRH producing cells of the paraventricular nucleus of the hypothalamus to control neuroendocrine function. However these BNST cell groups also project to areas necessary for respiration, vagal control, and freezing. Figure adapted from (Paxinos & Watson, 1982; Paxinos & Watson, 2007)

A number of studies have characterized afferent and efferent BNST pathways. Projections to the BNST from the amygdala are segregated. That is, the BLA and CeA project to lateral portions of the BNST (Alheid, et al., 1995) whereas the MeA, a nucleus thought to be involved in innate predator fear (C.-I. Li, Maglinao, & Takahashi, 2004), predominantly projects to medial portions of the BNST. The BNST also receives projections from the PFC (Radley, Gosselink, & Sawchenko, 2009) and ventral CA1, but the function of these pathways in contextual fear have not been studied. The CeA and the BNST both project to the parabrachial nucleus (respiration),

nucleus of the solitary tract (vagal control), and periaqueductal grey (passive and active avoidance) – an anatomical similarity that is thought to parallel a functional one (Walker, Toufexis, & Davis, 2003). The BNST also has projections to the paraventricular nucleus of the hypothalamus (PVN) and it is through the BNST by which the CeA is thought to exert control over the HPA-axis during stressful experiences (discussed in Chapter 2). Given that "stress" in intimately tied to aversive experiences, it is not surprising that disrupting hormones and steroids implicated in the stress response also impacts contextual fear conditioning (see (Pugh, Tremblay, Fleshner, & Rudy, 1997)).

1.2.4.2 BNST and CeA: Cellular Profiles and Connectivity

Both the CeA and BNST synthesize and express a number of the same peptides including: corticotropin-releasing factor, enkephalin, galanin, neuropeptide Y, orexin, and somatostatin, to name a few (Alheid, et al., 1995). Furthermore, both regions are primarily GABAergic, and provide feedforward inhibitory inputs at the intra and inter-circuit level. Tract tracing studies have indicated that the $_{L}$CeA and $_{L}$BNST are also intricately connected – suggesting that they may bi-directionally regulate activity of each other (Dong, Petrovich, & Swanson, 2001; Petrovich & Swanson, 1997; Swanson, Sawchenko, Rivier, & Vale, 1983). While it has long been known that the $_{L}$CeA sends strong projections to the dorsal $_{L}$BNST (Dong, et al., 2001; Petrovich & Swanson, 1997; Sakanaka, Shibasaki, & Lederis, 1986), recent work has demonstrated that $_{L}$BNST → $_{L}$CeA projections exist, although these projections have not been well studied in relation to behavior (Gungor, Yamamoto, & Pare, 2015). Given that the

ventrolateral division receives noradrenergic inputs from the locus coeruleus (LC), it has also been suspected that the LC → BNST pathway can provide tonic inhibition over both excitatory hypothalamic-pituitary-adrenal (HPA) axis activity (Forray & Gysling, 2004). Connectivity aside, what is most interesting is the projections from the LCeA to the LBNST. It is this pathway that has long been suspected to modulate the switch from phasic to sustained fear (Davis, et al., 2010; D. L. Walker & M. Davis, 2008).

1.3 Microcircuits of the Central Nucleus of the Amygdala

Although the focus of my dissertation is on the LCeA and LBNST, it is worth noting how some newer studies have elaborated upon the function of distinct microcircuits within these nuclei during fear learning. Research has greatly evolved both technologically and mechanistically from the gross lesion and pharmacological methods so widely used in the 20th century. It was recently shown that an inhibitory microcircuit important for fear within the CeA is regulated by the LCeA. Molecular phenotyping has identified distinct populations of cells within the LCeA containing protein kinase C delta (PKC-δ+) cells which are distinct from corticotropin releasing factor (CRF+) and dynorphin+ cells (Haubensak et al., 2010). These GABAergic PKC-δ+ cells within the LCeA directly inhibit the MCeA and shut off (decrease their firing) in response to the CS during fear conditioning (termed "fear-off" cells). Other studies have identified a population of LCeA cells that turn on during fear conditioning (termed "fear-on" cells; (H. Li et al., 2013)). These cells are somatostatin positive (Som+), are thought to receive direct inputs from the BLA and thalamus (Ciocchi et

al., 2010), are distinct from PKC-δ+ cells, and enhance their response during fear conditioning (to a tone). Additionally, activity in these Som+ cells are predominantly driven by direct projections from specific subdivisions of the lateral nucleus of the amygdala (i.e., the ventrolateral division; in contrast to the dorsolateral division where early growth responses gene 1 (egr-1), an immediate early gene necessary for contextual fear, is highly expressed; (Asok, Schreiber, Jablonski, Rosen, & Stanton, 2013; Malkani, Wallace, Donley, & Rosen, 2004)). Activation of this $_{V}$LA → $_{L}$CeA pathway drives increased AMPA-mediated excitatory post synaptic currents (H. Li, et al., 2013). Surprisingly, these $_{L}$CeA Som+ "fear-on" cells (in addition to the canonical $_{M}$CeA cells) have direct projections to the PAG and midline thalamic nuclei (Penzo, Robert, & Li, 2014) – a feature that may contribute to the function of the paraventricular thalamic nuclei (mentioned above) in fear memories. A wonderful, in-depth, review of amygdala microcircuits that control fear and reward was recently published by Patricia Janak and Kay Tye (Janak & Tye, 2015).

The properties of the $_{L}$CeA Som+ cells are important because it is likely that these overlap with CRF+ cells (Pomrenze, Maiya, et al., 2015). Furthermore, CRF+ cells in these GABAergic cells of the $_{L}$CeA contain NMDA-NR1 containing receptors (Beckerman, Van Kempen, Justice, Milner, & Glass, 2013) - a feature which may be important given the role of NR1's in the amygdala during fear conditioning (e.g., long-term depression associated with the prevention of excitotoxicity (Zinebi et al., 2003)). In fact, selective deletion of Grin1 (i.e., the gene that codes for the NR subunits) in CRF+ cells of the CeA enhances consolidation of auditory fear memories,

suggesting that glutamatergic inputs to LCeA CRF neurons may regulate fear memory formation (Gafford, Jasnow, & Ressler, 2014).

Importantly, these CRF+ cells also project to the LBNST (Beckerman, et al., 2013), suggesting these cells may be the long-sought after CRF neurons important for transmitting fear/anxiety related information to the LBNST during aversive experiences (Walker & Davis, 2008). However, given that CRF is a slow-acting neuropeptide (relative to glutamate and GABA), the possibility also arises that these cells may be important for the maintenance of long-term contextual fear memories – a speculation that future studies should fully aim to address. The next chapter focuses on how understanding the role of CRF during discrete phases of contextual fear-learning is critical for understanding its function in fear and anxiety-like behaviors.

Chapter 2

STRESS, CORTICOTROPIN RELEASING FACTOR AND FEAR LEARNING

"Anything that causes stress endangers life, unless it is met by adequate adaptive responses; conversely, anything that endangers life causes stress and adaptive responses" (Selye, 1950).

2.1 Stress and Fear Learning – A Short Summary

Stress can be thought of as the body's response to demand (i.e., homeostatic challenge), and a "stress response" is elicited during fear-learning (Rodrigues, LeDoux, & Sapolsky, 2009; Sapolsky, 2015). In general, stress provokes the release of hormones (or "factors") as a mechanism for adaptation (Selye, 1950) which can influence neural activity and modulate fear learning and memory (Rodrigues, et al., 2009).

Corticotropin-releasing factor (CRF) is one neuropeptide that has received substantial attention over the last few decades for its role within the central nervous system in modulating the stress response (Lupien, McEwen, Gunnar, & Heim, 2009). CRF (within the paraventricular nucleus of the hypothalamus) is generally acknowledged for its function in regulating the stress, or hypothalamic-pituitary-adrenal (HPA), axis. However, CRF is also produced in key limbic areas like the amygdala, hippocampus, and BNST which are (1) important for fear-learning (Chapter 1), but (2) also play a role in modulating the HPA-axis (detailed below; (Herman, Ostrander, Mueller, & Figueiredo, 2005; Lee & Davis, 1997). While I am focused on examining how CRF within specific brain regions can modulate contextual fear

memories, it is important to mention that CRF is known to influence a wide-range of behaviors including: feeding, grooming, reproduction, and depression, to name a few (for a review see (Bale & Vale, 2004)). Additionally, CRF expression is not solely restricted to the brain and central nervous system (the primary focus of my dissertation), but it and its receptors are also found throughout the peripheral nervous system (Bileviciute et al., 1997).

2.2 Corticotropin-Releasing Factor, Corticosterone, and Fear

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide widely implicated in the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is commonly acknowledged for its regulation of physiological (and neural) processes associated with stress reactivity. CRF can exert top-down control over the HPA axis. That is, parvocellular cells of the paraventricular nucleus of the hypothalamus release CRF onto CRF type 1 receptors located in the anterior pituitary (Herman & Cullinan, 1997). The anterior pituitary then releases adrenocorticotropic hormone (ACTH), on the order of seconds, into the blood stream. ACTH binds to receptors in the adrenal cortex to stimulate the release of glucocorticoids (i.e., corticosterone in the rat). Glucocorticoids then penetrate the blood-brain-barrier to bind to fast-acting (mineralocorticoid) and slow-acting (glucocorticoid) receptors to regulate the HPA-axis. A substantial amount of work has suggested that glucocorticoids within the amygdala and hippocampus can have opposing functions on HPA-axis activity – whereby the amygdala can provide feedforward excitation and the hippocampus can provide negative feedback/inhibition (Schulkin, Morgan, & Rosen, 2005).

Corticosterone, the principal stress-related glucocorticoid released in the periphery of rats, can modulate fear learning via actions in the brain (Korte, 2001). Depletion of CORT (via adrenalectomy) can reduce the retention of contextually conditioned fear (Pugh, et al., 1997) whereas increasing CORT (chronically) can have the opposite effect (Marks, Fenton, Guskjolen, & Kalynchuk, 2015; Skórzewska et al., 2014; Thompson, Erickson, Schulkin, & Rosen, 2004). Furthermore, CORT (administered chronically) increases CRF mRNA within the LCeA and LBNST (Shinya Makino, Gold, & Schulkin, 1994a, 1994b).

Electrolytic lesions of both the CeA and BNST not only disrupt contextually conditioned fear (as mentioned in Chapter 1), but also disrupt increases in CORT evoked during the expression of contextual fear (Sullivan, et al., 2004). Thus, CORT, along with CRF in the LCeA and the LBNST, may have an especially important, but poorly understood, role in modulating contextually conditioned fear. Recent work has started to examine how CRF within the amygdala may regulate discrete phases (i.e., consolidation) of learning and memory in contextual fear conditioning (Pitts & Takahashi, 2011; Pitts, Todorovic, Blank, & Takahashi, 2009). However, studies have not systematically assessed the role of CRF within the LBNST during different phases of contextual fear learning (Chapter 3).

2.2.1 CRF and CRF Receptors: Distribution and Function in Fear

CRFs cellular effects occur via its binding to two primary CRF receptors: the type 1, or CRFr1, and the type 2, or CRFr2, receptors. CRF preferentially binds to CRFr1s (>10x higher-affinity than CRFr2s; (Perrin et al., 1995)), whereas urocortin 2

and 3 preferentially bind to CRFr2s. However, urocortin 1 can bind to both types of CRF receptors (Bale & Vale, 2004)). CRF receptors are a type of class B g-protein coupled receptors (GPCRs). That is, they are characterized by a large N-terminal extracellular domain and a seven transmembrane domain spanning segment (Hollenstein et al., 2014). The g-proteins on CRFr1 and CRFr2s typically activate adenylate cyclase to increase levels of cyclic adenosine monophosphate (Owens & Nemeroff, 1991) – a process which can affect gene transcription via CREB (Aguilera & Liu, 2012). Antagonists of CRF receptors like antalarmin used in Chapter 3 act as negative allosteric modulators (Wootten, Christopoulos, & Sexton, 2013). Specifically, CRF antagonists tend to bind within an intra-cytoplasmic allosteric site and not at a traditional orthosteric site (Hollenstein, et al., 2014). .

CRFr1s and CRFr2s are thought to have opposing roles in fear and anxiety where antagonism of CRFr1s reduce fear and anxiety and antagonism of CRFr2s increase fear and anxiety (Takahashi, 2001). CRF and CRFr1s are spread throughout the brain, but are expressed in many of the key areas responsible for contextual fear learning such as the prefrontal cortex, entorhinal cortex, dentate gyrus, hippocampal CA subfields, BLA, and BNST (Olschowka, O'Donohue, Mueller, & Jacobowitz, 1982; Radulovic, Sydow, & Spiess, 1998).

2.3 CRF, the Extended Amygdala, and Fear

CRF is synthesized and expressed in GABAergic cells of the LCeA (Chapter 1.3; see (Pomrenze, Millan, et al., 2015) and these CRF+ cells in the LCeA project to the LBNST (Dong, et al., 2001; Sakanaka, et al., 1986; Swanson, et al., 1983).

Furthermore, CRF (Asok, Ayers, Awoyemi, Schulkin, & Rosen, 2013) is expressed in the LCeA (and LBNST), but CRFr1s are expressed in the LBNST and not the LCeA (see Figure 2.1 for graphical clarification). Thus, it has long been hypothesized that CRF released from the LCeA onto terminals in the LBNST affect fear-learning. In fact, this hypothesis was proposed by Michael Davis's group beginning in the 1980s (for review see (Davis, et al., 2010)). Specifically, Davis's group believed that CRF released from the LCeA, onto glutamatergic terminals in the LBNST arising from the BLA, modulate fear and anxiety (Walker, et al., 2003). My dissertation is a first step towards functionally examining the role of this CRF pathway in contextual fear learning. Furthermore, I *believe* this pathway modulates the strength of the CS-US association formed in contextual fear conditioning. The experiments in my dissertation lay the foundation for future experiments that will fully test this hypothesis.

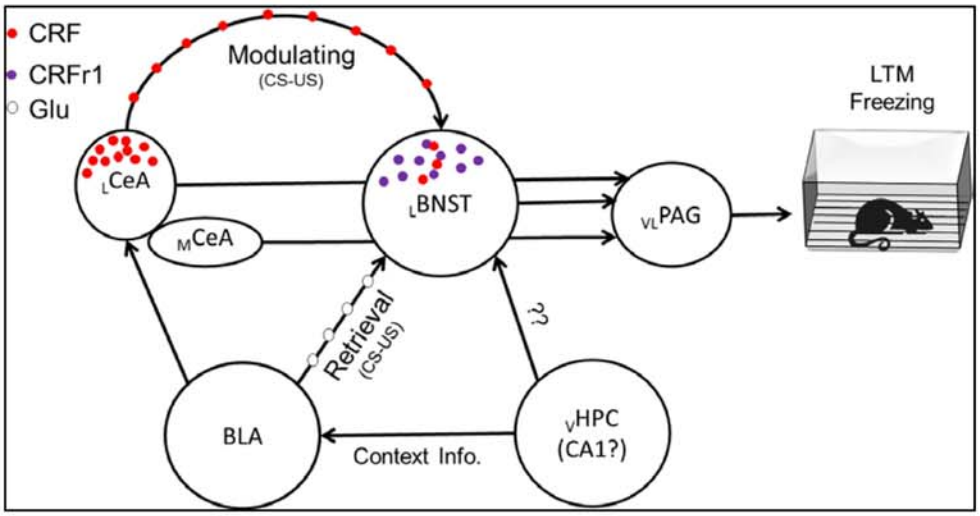


Figure 2.1. Model for CRF regulation of contextual fear memories within the extended amygdala. Whereas the ventral hippocampus is thought to encode the CS, the BLA/CeA encodes the CS-US association. However, CRF from the LCeA released onto the LBNST is ideally positioned to integrate information from all of these nodes to modulate contextual fear memories.

2.4 Dissertation Hypothesis

My dissertation is focused on experimentally testing three fundamental questions (Figure 2.2):

1. What is the role of CRFr1s in the LBNST during the acquisition, consolidation, and expression of contextual fear (Chapter 3)?
2. What is the function of LCeA CRF cells (and their projections) during the acquisition of contextual fear (Chapter 4)?
3. What is the function of the LCeA → LBNST CRF pathway during the acquisition of contextual fear conditioning (Chapter 4)?

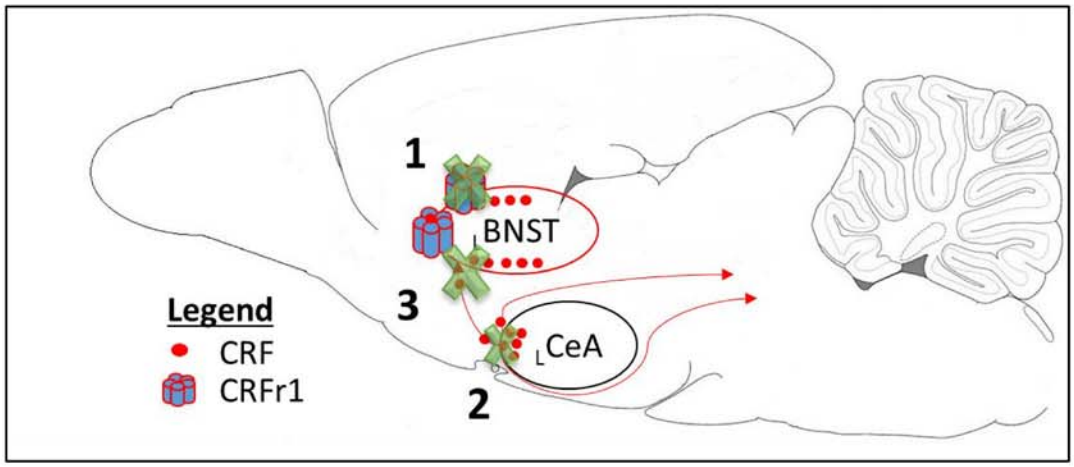


Figure 2.2. Simplified schematic of the extended amygdala CRF circuit evaluated in my dissertation. The first experiments (1) block the target of CRF at CRFr1s in the BNST during different phases of contextual fear conditioning. The next experiments selectively inhibit (2) CRF cells in the LCeA and (3) the LCeA → LBNST CRF pathway during contextual fear acquisition.

2.4.2 Basic Pharmacology (Chapter 3) vs. Optogenetics (Chapter 4)

For the experiments in chapter 3, I use a pharmacological approach to examine the role of CRFr1s. For the experiments in chapter 4, I use a cell-type specific optogenetic approach that I developed to selectively inhibit that activity of CRF cells and their projections. Previous limitations in technology have prevented the examination of this pathway. Thus, the use of optogenetics is fundamental to my dissertation. However, it is important to make the point that optogenetics is *not* a pharmacological manipulation (although see variations like OptoXR that manipulate receptor-coupled signaling cascades (Airan, Thompson, Fenno, Bernstein, & Deisseroth, 2009)). That is, no receptor is antagonized, no intracellular signaling pathway is directly modulated, and no alternative drug-induced state is produced.

I use a specific rhodopsin variant, archaerhodopsin tp009 (ArchT) for the experiments in Chapter 4. ArchT allows for the selective hyperpolarization of a particular group of cells within a region by the insertion of an outward proton pump that is sensitive to green-light. The temporal kinetics of ArchT (hyperpolarization and return to normal activity) are on the order of milliseconds (Chow, Han, & Boyden, 2012). Furthermore, green-light stimulation of ArchT has no known lasting effects, given that cells immediately return to their native state once illumination is removed. The combination of pharmacological and optogenetic techniques in recent years have proven extremely informative for elucidating how specific cell types and their pathways are important for various behaviors.

Chapter 3

CORTICOTROPING RELEASING FACTOR IN THE BED NUCELUS OF THE STRIA TERMINALIS REGULATES CONTEXTUAL FEAR MEMORIES

3.1 Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide widely studied for its role in the neuroendocrine stress response (Bale & Vale, 2004; Kovács, 2013; Smagin, Heinrichs, & Dunn, 2001; Vale, Spiess, Rivier, & Rivier, 1981). CRF is expressed in a number of limbic structures including the hippocampus, amygdala, and bed nucleus of the stria terminals (Makino et al., 1995; Wong, Licinio, Pasternak, & Gold, 1994). Although these limbic structures have been intensively studied for their role in conditioned and unconditioned fears (Antoniadis & McDonald, 2001; Campeau et al., 1991; Walker & Davis, 1997), our understanding of how CRF functions within these areas during fearful experiences is continually expanding.

Over the last few decades, CRF within the bed nucleus of the stria terminalis (BNST), a part of the extended amygdala, has received substantial attention for its role in mediating fear and anxiety-like behaviors ((D. L. Walker & M. Davis, 2008; Walker, et al., 2003). More recently, research has shed light on the function of the BNST in associative learning using contextual fear conditioning paradigms (Haufler, et al., 2013; Nijsen, Croiset, Diamant, De Wied, & Wiegant, 2001; Poulos, Ponnusamy, Dong, & Fanselow, 2010; Resstel et al., 2008; Sullivan, et al., 2004). In this paradigm, a neutral context (CS) is paired with a foot-shock (US) to produce a conditioned response (CR) such as freezing. Post-training electrolytic lesions of the

BNST disrupt long-term fear to a context, but not fear to discrete cues such as a tone (LeDoux, et al., 1988; Sullivan, et al., 2004). Furthermore, the BNST can compensate for contextual, but not auditory, fear learning when the basolateral amygdala is inactivated – suggesting that the BNST may provide an alternative pathway for contextual fear learning (Poulos, et al., 2010; Zimmerman & Maren, 2011). This role of the BNST in contextual fear complements other studies showing its involvement in fear to sustained environmental threats (Davis, et al., 2010). While there has been a great deal of progress in understanding the role of CRF in anxiety (Davis, et al., 2010; Walker, Miles, & Davis, 2009), the role of CRF within the BNST across different phases of memory (i.e., acquisition/consolidation/expression) has yet to be examined.

Similar to its function in contextual fear learning, the BNST is also important for behavioral and endocrine responses to unconditioned fear/threat stimuli such as predator odors (Fendt, et al., 2003; Rosen, et al., 2015; Walker & Davis, 1997). Predator odors are advantageous for investigating unconditioned fear for two reasons. First, although laboratory rats have never encountered the odor, they still exhibit robust defensive responses upon the first exposure. Second, predator odors are ethologically relevant stimuli for rodents relative to footshocks. Inactivation of the BNST, but not key nuclei of the amygdala important for fear conditioning, disrupts freezing to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; (Fendt, et al., 2003; Rosen, 2004; Wallace & Rosen, 2001)), a synthesized compound derived from the anal secretions of the red fox. Furthermore, TMT exposure increases immediate-early-genes and CRF mRNA in distinct brain regions (Asok, Ayers, et al.,

2013), while also increasing corticosterone secretion (Day, Masini, & Campeau, 2004). Taken together, these studies suggest that the BNST, and possibly CRF within the BNST, may be important for unconditioned fear behavior. How CRF modulates defensive behaviors (i.e., freezing) to different types of fear (i.e., conditioned and unconditioned predator fear) has not been investigated. Therefore, to better understand the role of CRF within the BNST in both conditioned and unconditioned fears, we evaluated the effects of blocking the CRF type 1 receptor (CRFr1) with a CRFr1 antagonist, antalarmin, administered intracerebroventricularly (ICV) or in the LBNST during different phases (i.e., acquisition, consolidation, or expression) of contextual fear conditioning or exposure to the predator odor TMT. We also evaluated if CRF antagonism produced state-dependent learning to determine if any deficits observed with CRFr1 antagonism were simply a result of the ability to learn while under drug, but a failure to retrieve the fear memory because the drug was not present at testing.

3.2 Methods

3.2.1 Subjects

216 male Sprague-Dawley rats (8 -11 weeks of age) obtained from Harlan breeders (Indianapolis, IN) and weighing between 280-330 g were used for all experiments. A representative sample of groups were assigned to more than one experiment (i.e., contextual fear conditioning, drug-free retraining, etc.) Rats were maintained on a 12h light/dark cycle (lights on at 7:00 A.M.) at constant temperature with free access to food and water. Following arrival in the animal colony, rats were left undisturbed for seven days prior to the start of any experimental procedures. Rats

were pair-housed in opaque polycarbonate cages with wood shavings for the duration of the study. All procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee.

3.2.2 Surgery

Rats were anesthetized with a ketamine/xylazine cocktail prior to stereotaxic surgery. For rats that received ICV surgery, a single 26-gauge 5mm guide cannula (Plastics One, Roanoke, VA) was implanted 1mm above the rat's right lateral ventricle using the following coordinates: AP = - 0.1, ML = -1.8, DV = -3.2. For rats that received cannula implanted into the dorsolateral division of the bed nucleus of the stria terminalis (LBNST), two 26-gauge guide cannula were angularly implanted targeting the following coordinates: AP = -0.1 mm, ML = ± 3.8 mm, DV = - 5.4 mm, at a 19º angle. Following all surgery, a dummy cannula that extended 1 mm beyond the tip of the guide cannula was inserted to prevent blockage.

3.2.3 Drug Preparation and Delivery

The selective CRFr1 receptor antagonist antalarmin hydrochloride (Sigma, St. Louis, MO) was used for all experiments (Zorrilla, Valdez, Nozulak, Koob, & Markou, 2002). Antalarmin was dissolved in dimethyl sulfoxide (DMSO) as a vehicle for all experiments. For ICV experiments, rats received either 3 µL of DMSO vehicle or 3 µL of DMSO vehicle containing 20 µg antalarmin. This dose was selected because it was in range with previous ICV and peripheral studies (Deak et al., 1999; Zorrilla et al., 2002; Zorrilla, Valdez, et al., 2002). For BNST infusions, rats received either 0.2 µL of DMSO vehicle or 0.2 µL DMSO containing antalarmin. Three doses

were tested with antalarmin dissolved in DMSO to a final concentration of 10 µg/µL (for 2 µg dose), 1 µg/µL (for 0.2 µg dose), or 0.01 µg/µL (for 0.02 µg dose). These doses were selected because the BNST is part of the extended amygdala and other studies have used a similar dose range for antalarmin infused into the amygdala (Vicentini, Céspedes, Nascimento, Bittencourt, & Viana, 2014; Wellman, Yang, Ambrozewicz, Machida, & Sanford, 2013).

Antalarmin or vehicle was administered 30 minutes prior to fear conditioning (training and/or expression), TMT exposure, or shock responsivity testing using an electronic infusion pump (Harvard Apparatus, Holliston, MA). 1 µL Hamilton syringes were connected to polyethylene tubing, and capped with a cannula injector that extended 1mm below the end of the guide cannula. Post-training infusions of antalarmin were conducted immediately (~5 min.) following single-trial contextual fear conditioning. Solutions were infused at a rate of 1 µL/min for ICV and 0.2 µL/min for BNST. The vehicle and administration time point were chosen based off of previous studies evaluating the pharmacokinetic profile of antalarmin (Sanghvi et al., 2009).

3.2.4 Contextual Fear Conditioning

Contextual fear conditioning was conducted in four identical Plexiglas/metal chambers (25cm x 31cm x 32cm) containing metal grid floors (19 stainless steel bars, 0.5 cm in diameter, and 1.25 cm apart). For conditioning, each animal was placed in the chamber for 180s (baseline freezing measurement), followed by a single 1s 1.5mA shock, followed by a 300s shock-free period (post-shock freezing measurement). 24

hours later, animals were returned to the same chamber and tested for freezing to the context for 300s (retention freezing measurement). A camera positioned at the top of each chamber recorded behavior for each animal and transmitted the signal to computer running FreezeFrame software (Actimetrics, Wilmette, IL). Freezeframe was configured to score freezing as 0.75s bouts without changes in pixel luminance and then verified offline by an experimenter (see Asok et al. 2014).

3.2.5 Contextual Fear Re-training in an Alternate Context

Rats were re-trained without any drug under identical contextual fear conditioning parameters (e.g., 180s baseline, a single 1s 1.5 mA shock, 300s measure of post-shock freezing, and 300s retention test) in an alternate context composed of four rectangular Plexiglas chambers (16.5 cm × 12.1 cm × 21.6 cm) with metal grid floors (9 stainless steel bars, 4 mm in diameter, and 1 cm apart) inside a fume hood.

3.2.6 Shock Responsivity Testing

Eight identical SR Lab ventilated startle chambers (San Diego Instruments, San Diego, CA) with clear Plexiglas cylinders (8.6 cm in diameter and 20 cm in length) were used for shock sensitivity testing similar to those previously reported in our lab (Thompson et al., 2004). Eight currents were tested (0 mA, 0.1 mA, 0.4 mA, 0.8 mA, 1.2 mA, 1.5 mA, 1.8 mA, and 2.1 mA) and responsivity was represented as arbitrary units (AU; (Thompson, et al., 2004)). Subjects were acclimated for 300s, then presented with a shock every 120s. Each current was presented twice in ascending order. Given that contextual fear conditioning was conducted using an AC current with a peak current of 1.5 mA, we adjusted the DC current levels presented to

rats using its transformed AC equivalent by computing the root mean square of the AC waveform. All rats were trained in the dark with white noise (65 dB) in the background

3.2.7 Predator Odor Exposure

Subjects were tested for freezing to the synthetic predator 2, 5-dihydro-2, 4, 5-trimethylthiazoline (TMT) 30 minutes after administration antalarmin. The context was the same as that used for Contextual Fear Re-training (see above). Procedures for TMT exposure were identical to Asok et al. (2013). Briefly, rats were acclimated to the context 10 minutes/day for three days (baseline) prior to TMT exposure on day four. A 300 µmole concentration of TMT (150 µmole/19.4µl per filter paper) was used because this amount consistently produces robust freezing and CRF expression in the LBNST (Asok, Ayers, et al., 2013; Wallace & Rosen, 2000). Freezing was scored by Freezeframe using 1s bouts similar to Asok et al. (2013).

3.2.8 Cannula Verification

For ICV infusions, cannula placement was verified at both the time of surgery and following behavioral testing. During surgery, sterile physiological saline was drawn into polyethylene tubing connected to an injector extending 1mm beyond the tip of the guide cannula. After achieving the targeted coordinates, a stop was removed from the tube to allow a small amount of saline to enter the ventricles via gravity flow. Given that saline will not perfuse into the ventricles if the injector tip is not within the ventricles, we appropriately adjusted the cannula depth as needed in those instances. Additionally, following behavioral testing, the rat was anesthetized, infused with 1 µL

of Indian ink, rapidly decapitated, and brain removed. The brain was coronally sliced with a razor blade and the presence of Indian ink in the ventricles was visually confirmed by the experimenter.

For BNST infusions, cannula placement was verified post-mortem. Following decapitation, brains were flash frozen in isopentane and stored at -80ºC until slicing. Brains were cut on a cryostat and sections corresponding to the cannula site were stored at -80ºC until staining. All brains were post-fixed in a 4% paraformaldehyde solution (pH 7.2) prior to staining with cresyl violet. Brain images were captured via a Dage CCD video camera and captured sections were overlain against corresponding sections in the Paxinos and Watson rat atlas (Paxinos & Watson, 1982; Paxinos & Watson, 2007). Accurate cannula placement was defined as within ~1.5 mm of target. This criteria was set given that the injector extended 1mm beyond the cannula tip and to account for a ~ 1mm diffusion diameter (0.5mm radius) of the drug (See Supplementary Figure 1 for cannula placement). In total, 28 rats out of a total of 160 were excluded (~17.5%).

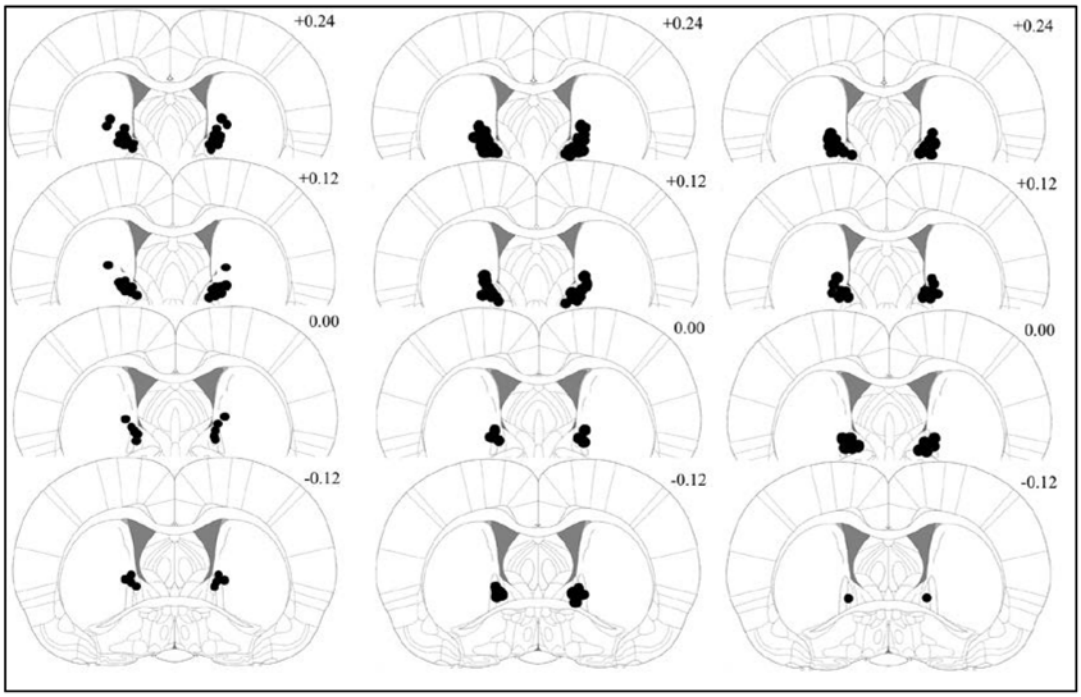


Figure 3.1. Representative LBNST Cannula Placements. Each black dot represents the reconstructed injection site. The distance from Bregma is located in the top right of each image. (A) Cannula placements for pre-training CRFr1 antagonism. (B) Cannula placements for post-training CRFr1 antagonism. (C) Cannula placements for expression and state-dependent testing of CRFr1 antagonism.

3.2.9 Statistical Analyses

For contextual fear conditioning, a group (i.e., drug) X phase (i.e., baseline, post-shock, and retention test) repeated measures ANOVA was used. Following significant main-effects and interactions, Fisher's LSD (for ICV analyses) or a Dunnett's test (for LBNST dose response analyses) was used. For TMT induced freezing and post-training antalarmin with only two doses, a group X phase (i.e., baseline and TMT exposure) two-way ANOVA was used for both ICV and BNST infusions. For shock responsivity testing, the startle amplitude score for each animal

was averaged across both presentations of the same current. Subsequently, a drug-group X startle amplitude paired samples t-test was used. Any animal that exhibited freezing scores above or below 2 S.D. of the group mean at the retention test was removed prior to statistical analyses. The final number of animals included in analyses are listed below.

3.3 Results

3.3.1 Pre-training ICV CRFr1 antagonism disrupts retention of contextual fear, but not unconditioned predator odor fear

ICV antalarmin at a dose of 20 µg disrupted contextually conditioned fear, but did not affect baseline or post-shock freezing or freezing to TMT (Figure 3.2 and 3.3).

For contextual fear conditioning, 24 subjects were used ($n_{\text{surgery control}} = 8$, $n_{20ug} = 8$, and $n_{\text{vehicle control}} = 8$). One animal from each group was removed as an outlier. A 3 x 3 repeated measures ANOVA contrasting groups across the measured phases revealed a main effect of group ($F(2, 21) = 3.88$, $p < .05$), a main effect of phase ($F(1, 21)=31.66$, $p < .001$) and a significant group X phase interaction ($F(2, 21) = 4.75$, $p < .05$). Separate ANOVAs performed on each phase showed that drug-groups did not differ at baseline or during the post-shock interval (p's $> .05$), but significantly differed at the retention test ($F(2, 21) = 11.68$, $p < .001$). Post-hoc LSD analyses showed that the 20 µg antalarmin group significantly differed from both the vehicle control and surgery control groups (p's $< .05$; Figure 3.2). Surgery control and vehicle control groups did not differ at retention testing ($p > .05$).

For TMT exposure, 16 subjects were used ($n_{20\mu g}$ = 5, and $n_{vehicle\ control}$ = 11). A two-way ANOVA contrasting groups (vehicle and 20 µg antalarmin) across the phases (acclimation and TMT exposure testing) revealed no main effect of group during acclimation ($F(1,14)=.878$, $p > .05$) and, more importantly, no effect of group on during TMT testing ($F(1,14)=.162$, $p > .05$; Figure 3.2B). Pairwise comparisons confirmed these results. Similarly, a high-dose of antalarmin (100 µg) did not affect freezing to TMT, confirming that the lack of an effect was not a result of ineffective dosage ($p > .05$; Figure 3.3).

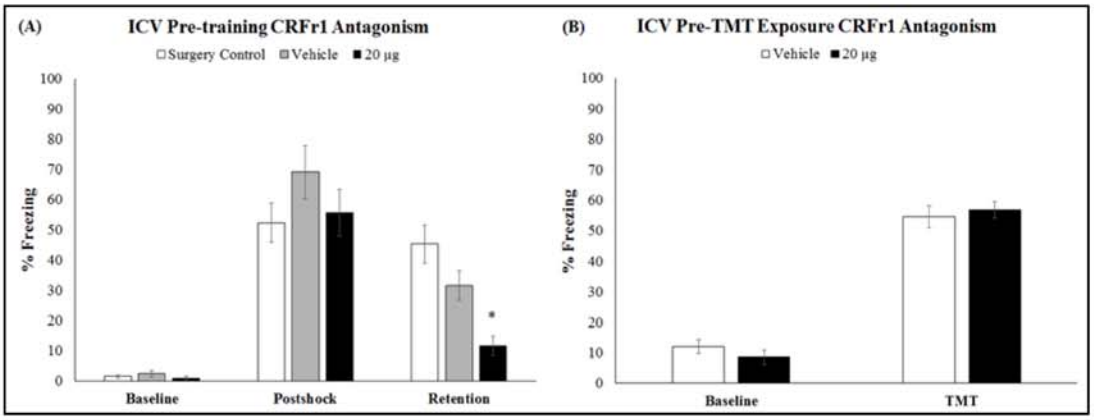


Figure 3.2 ICV CRFr1 antagonism disrupts retention of contextual fear. (A) Pre-training ICV CRFr1 antagonism disrupts retention of contextual fear, but has no effect on baseline or post-shock freezing. (B) Pre-TMT exposure ICV CRFr1 antagonism has no effect on freezing to TMT. Error bars are ± S.E.M., *$p < .05$.

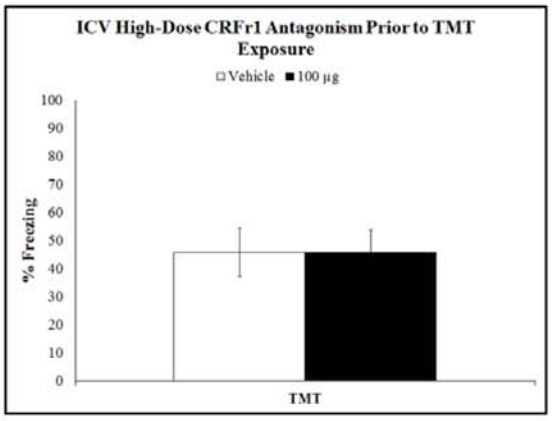


Figure 3.3. High-dose ICV CRFr1 antagonism has no effect on freezing to TMT. CRFr1 antagonism at a dose of 100 µg (5x greater than what affects contextual fear) does not affect freezing to TMT. Error bars are ± S.E.M.

3.3.2 Pre-training LBNST CRFr1 antagonism disrupts retention of contextual fear, but not unconditioned predator odor fear

LBNST CRFr1 antagonism at a dose of 2 µg prior to training disrupted retention of contextually conditioned fear, but not baseline or post-shock freezing. LBNST CRFr1 antagonism did not disrupt animal's ability to re-learn context-fear or the ability to freeze to TMT ((Figures 3.4 and 3.5).

For contextual fear conditioning, 47 subjects were included ($n_{\text{vehicle control}} = 12$, $n_{0.02\mu g} = 11$, $n_{0.2\mu g} = 12$, $n_{2\mu g} = 12$). Five animals were excluded for outlying retention data or cannula placement (two from the 0.02 µg group, one from the 0.2 µg group, and two from the 2 µg group). A 4 x 3 repeated measures ANOVA contrasting groups across the phases revealed a significant main effect of phase ($F(2,86) = 161.22$, $p < .001$), no main effect of group $F(3,43)=1.056$, $p > .05$) , but a significant group X phase interaction ($F(6,86) = 2.38$, $p < .05$). Step-down ANOVAs showed that drug-

groups did not differ at baseline or during the post-shock interval (p's > .05), but significantly differed at the retention test ($F(3,43) = 3.41$, $p < .05$). A post-hoc dunnett's test revealed that 0.02 µg did not significantly differ from vehicle controls ($p > .05$), 0.2 µg marginally differed from vehicle controls ($p = .08$), and 2 µg significantly differed from vehicle controls ($p < .01$; Figure 3.4A).

A subset of animals were re-trained in an alternate context to examine if antalarmin permanently disrupted rats' ability to learn contextual fear ($n_{\text{vehicle control}} = 8$, $n_{0.02ug} = 11$, $n_{0.2ug} = 12$, $n_{2ug} = 8$). Eight animals were lost due to damaged cannula (4 in the vehicle control group and 4 in the 2 µg). We detected a main effect of phase ($F(2,70)=89.572$, $p <.001$), but no main effect of group $F(3,35)=.304$, $p > .05$, and no group by phase interaction ($F(6,70)=0.546$, $p > .05$). One-way ANOVAs confirmed that drug-groups did not differ from one another at baseline, post-shock, or retention testing (p's > .05; Figure 3.5).

Finally, we examined the effects of $_{L}$BNST CRFr1 antagonism on freezing to TMT ($n_{\text{vehicle control}} = 4$, $n_{2ug} = 6$). Two animals from the vehicle control group were excluded due to damaged cannula. A two-way ANOVA contrasting groups (vehicle control and 2 µg) across the measured phases (baseline and TMT exposure) revealed no main effect of group at acclimation ($F(1,8)=2.497$, $p > .05$) or at the TMT exposure session ($F(1,8)=0.326$, $p > .05$; Figure 2B).

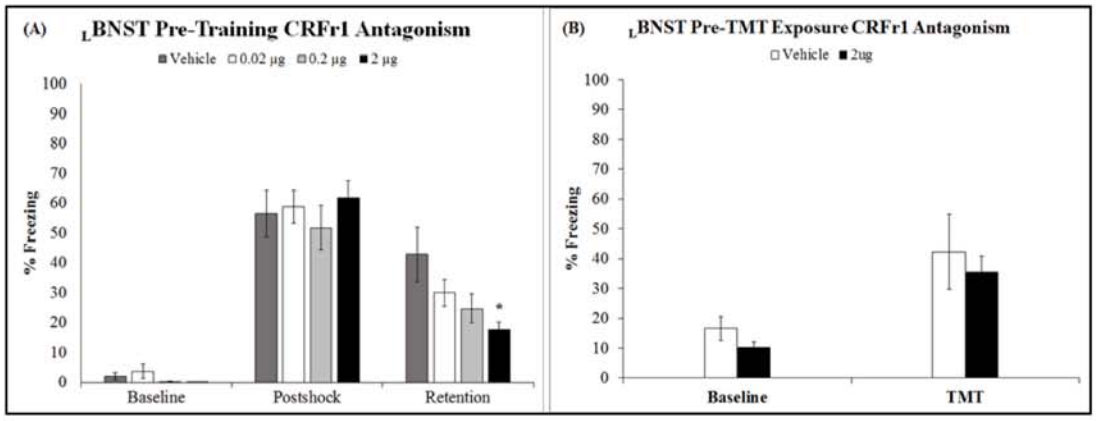


Figure 3.4. Pre-training LBNST CRFr1 antagonism disrupts retention of contextual fear. (A) Pre-training LBNST CRFr1 antagonism dose-dependently disrupts retention of contextual fear, but has no effect on baseline or post-shock freezing. (B) Pre-TMT exposure LBNST CRFr1 antagonism has no effect on freezing to TMT. Error bars are ± S.E.M., *p < .05.

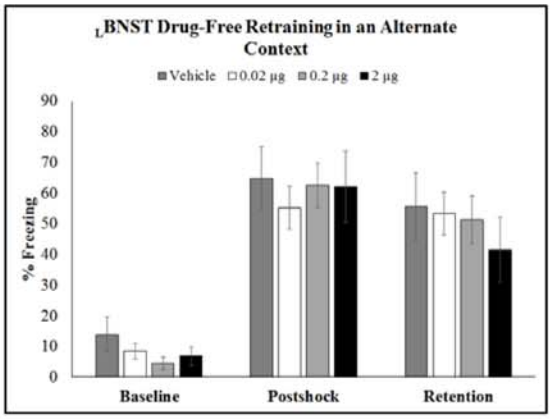


Figure 3.5. Animals exhibit freezing during a contextual fear retention test when re-trained without antalarmin. Animals that previously exhibited disrupted retention test freezing following pre-training CRFr1 antagonism are able to normally acquire and express contextual fear. Error bars are ± S.E.M.

3.3.3 Post-training $_{L}$BNST CRFr1 antagonism disrupts retention of contextual fear

Post-training $_{L}$BNST CRFr1 antagonism at a dose of 2 µg after training disrupted contextually conditioned fear (Figure 3.6).

Forty subjects were included in analyses ($n_{vehicle\ control}$ = 19, $n_{2\mu g}$ = 21). Four animals were excluded from analyses because of outlying retention-test data (1 animal from vehicle controls and 3 animals from the 2 µg group) and 12 animals were excluded for improper cannula placements (8 from the vehicle control group and 4 from the 2 µg group). A 2 x 3 repeated measures Greenhouse-Geisser corrected ANOVA revealed a significant main effect of phase ($F(1.595,60.591) = 68.651$, $p < .001$), a main effect of group ($F(1,38)=6.98$, $p < .05$), and a significant group X phase interaction ($F(1.595, 60.591) = 3.922$, $p < .05$). A variance corrected independent samples t-test at each time-point revealed groups did not differ at baseline ($t(22.715)=-0.849$, $p > .05$), or the post-shock period ($t(37.683)=1.803$, $p > .05$), but significantly differed at the retention test ($t(3.246)=29.534$, $p < .01$; Figure 3.4A).

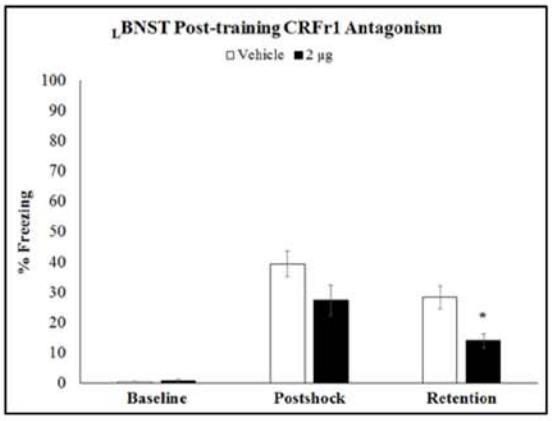


Figure 3.6. Post-training LBNST CRFr1 antagonism disrupts retention of contextual fear. Error bars are ± S.E.M., *p < .05.

3.3.4 LBNST CRFr1 antagonism does not affect shock responsivity

Antalarmin infused into the BNST did not affect animals' ability to respond to the shock (Figure 3.7).

Fourteen subjects were included in shock sensitivity training analyses (n_{2ug} = 8 and $n_{\text{vehicle control}}$ = 6). Two animals were excluded to due damaged cannula. A 2x8 repeated measures t-test contrasting groups(vehicle and antalarmin) across shock intensities (0 mA, 0.1 mA, 0.4 mA, 0.8 mA, 1.2 mA, 1.5 mA, 1.8 mA, and 2.1 mA) revealed no main effect of group ($F(1,12)=1.87$, $p > .05$, a main effect of shock intensity ($F(7, 84) =46.60$, $p < .001$), and no group by shock intensity interaction ($F(7,84)=0.83$, $p > .05$). Given visual differences at specific shock intensities (.08mA, 1.2mA, and 1.5 mA), we conducted exploratory t-tests to determine if groups differed. Rats did not differ at 0.8mA ($t(12) = -1.80$, $p > .05$), 1.2mA ($t(12) = -1.77$, $p > .05$), or 1.5mA ($t(12) = -1.53$, $p > .05$; Figure 3.4B).

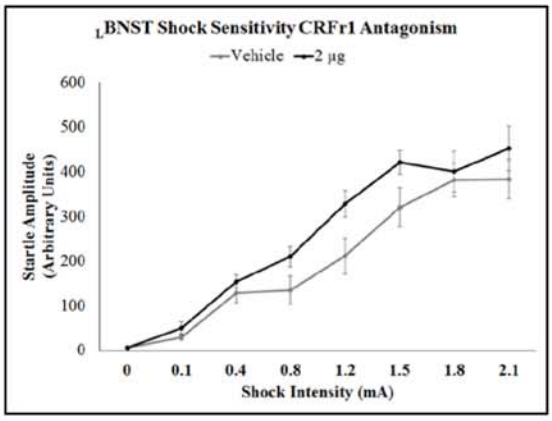


Figure 3.7. Pre-training $_{L}$BNST CRFr1 antagonism does not affect animal's ability to respond to the shock even at a shock intensity that matched what they were trained under for contextual fear. Error bars are ± S.E.M.

3.3.5 Pre-expression $_{L}$BNST CRFr1 antagonism marginally affects contextual fear, but CRFr1 antagonism does not produce state-dependent learning

Pre-expression BNST CRFr1 antagonism marginally affected retention of contextual fear. However, CRFr1 antagonism did not produce state-dependent learning. Results are described below (Figure 3.8).

Forty-four animals were included in the final analyses ($n_{\text{vehicle - vehicle}} = 10$, $n_{0ug - 2ug} = 11$, $n_{2ug - 0ug} = 11$, $n_{2ug - 2ug} = 12$). Prior to analyses, eights animals were excluded due to improper cannula placement (1 animal in the vehicle control group, 1 animal in the 0µg – 2µg group, 1 animal in the 2µg – 0µg group, and 5 animals in the 2µg – 2µg group), three animals were excluded from analyses because of outlying retention-test data (1 animals in the vehicle control group, 1 animal in 0µg – 2µg group, and 1 animal in the 2µg -2µg group), and two were excluded because of unusually high *increases* from post-shock to retention testing (1 animal in the vehicle control group and 1 animal in the 2µg - 0µg group). A 4 x 3 repeated measures ANOVA contrasting

group (vehicle control, 0µg – 2µg, 2µg – 0µg, and 2µg -2µg antalarmin) across the measured phases (baseline, post-shock, and retention testing) revealed a main effect of phase ($F(1.645, 46.070) = 64.544$, $p < .001$), a marginal group X phase interaction ($F(6, 80) = 2.002$, $p = .075$), and no effect of group ($F(3, 40) = .873$, $p > .05$).

Given the marginal interaction and visual differences apparent in the graphed data at the retention test, we conducted a post-hoc contrast comparing groups at each freezing period. Fisher's post-hoc analysis revealed that groups did not differ at baseline or during the post-shock period (p's > .05), but marginally differed from vehicle controls at the retention test. In particular, the 2µg – 0µg group ($p = .098$), the 0µg – 2µg group ($p = .074$), and the 2µg – 2µg group ($p = .02$) marginally differed from vehicle controls. However, the 2µg – 0µg group, 0µg – 2µg group, and 2µg – 2µg group did not differ from one another.

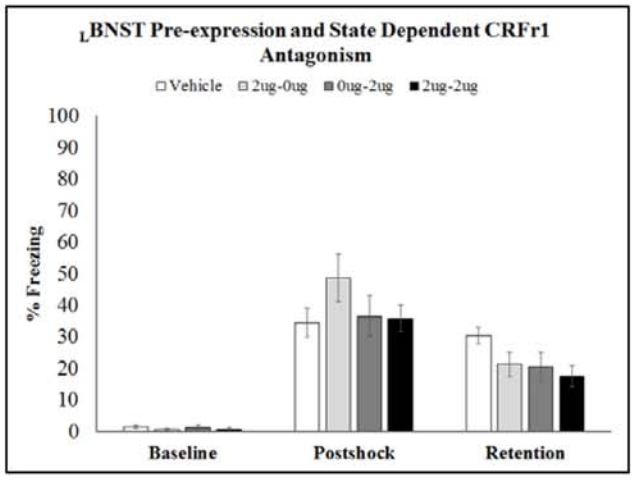


Figure 3.8. CRFr1 Antagonism marginally affects expression of contextual fear, but does not produce state-dependent learning. Expression of contextual fear (0µg – 2µg vs. vehicle controls) was marginally reduced by antalarmin prior to the retention test. Animals administered antalarmin before both contextual fear training and testing (2µg -2µg) exhibited reduced freezing relative to vehicle controls. The expression (0µg – 2µg), acquisition (2µg – 0µg), and state-dependent (2µg -2µg) groups did not differ from each other. Error bars are ± S.E.M.

3.4 Discussion

In the present study we examined the role of CRF type 1 receptors during two different types of fear - contextually conditioned fear and unconditioned fear to a predator odor. Pre-training antagonism of CRFr1s globally (ICV) and selectively in the LBNST with antalarmin disrupted the retention of contextual fear, but had no effect on unconditioned fear to the predator odor TMT. Antalarmin also did not change foot-shock sensitivity indicating that shock sensation was unaffected. Furthermore, post-training antalarmin disrupted freezing at the retention test whereas pre-expression antalarmin did so marginally. Our results highlight a role for CRFr1s in the LBNST during the consolidation (and possibly expression) of contextual fear memories.

The BNST is known to play a crucial role in fear to discrete cues of long-duration (i.e., cues being tones and lights (Walker, et al., 2009)) and recent work has shown that contextual stimuli may be processed in a similar way (Radke, 2009; Sullivan, et al., 2004); but only when presented for a long duration before receiving a shock (Hammack, et al., 2015). Thus, the BNST's role in processing aversive stimuli may be constrained along a temporal domain rather than a cue-related qualitative domain – a phenomenon that our study may be tapping into given that animals received three minutes of context exposure prior to shock. Our results suggest that the role of the BNST during long duration fear stimuli, and CRFr1s, is restricted to conditioned fear in that antalarmin had no effect on freezing during the 10 minutes of TMT exposure. Importantly, post-training antalarmin disrupted freezing during a retention test 24 hours later, suggesting that consolidation mechanisms of contextual fear learning in the LBNST are disrupted by CRFr1 antagonism.

CRFr1s in the LBNST are modulated by both local (occurring within the BNST) and distal (from the CeA) CRF release. The LBNST is densely innervated by CRF projections from the CeA (Sakanaka, et al., 1986; Swanson, et al., 1983). While CRF in the CeA, and CRF receptors in the BLA, are important for fear memory consolidation, we speculate that CeA CRF provides a critical signal to CRFr1s in the LBNST that are necessary for the acquisition/consolidation (and possibly expression) of contextual fear memories. This hypothesis is supported by studies showing that CRF knockdown in the CeA disrupts the consolidation of long-duration contextual

fear memories in a time-limited manner (Pitts & Takahashi, 2011; Pitts, et al., 2009) and also sensitizes CRFr1s in the BNST (Regev, Tsoory, Gil, & Chen, 2012).

Broadly, CRF administration increases fear and anxiety-like behaviors (for reviews see; Bale and Vale, 2004; Seckler, Kalin, and Reul, 2005) and CRF antagonists block many of these effects (Bale and Vale, 2004). CRFs primary receptors, the type 1 and type 2 receptors, have opposing roles in fear and anxiety (Bale and Vale, 2004; Takahashi, 2001), but blocking CRFr1s, in particular, produces anxiolytic effects. The importance of CRF within the (extended) amygdala is highlighted by recent studies showing that (1) non-selective CRF receptor antagonism in the basolateral amygdala (BLA; i.e., the major local amygdala sub-region that contains many CRFr1s) disrupts the consolidation of inhibitory avoidance memories (Roozendaal et al., 2002), (2) non-selective CRF blockade in the BNST disrupts CRF-induced freezing (Nijsen, et al., 2001), and (3) knockdown of CRF in the central nucleus of the amygdala (CeA; i.e., the CRF synthesizing region in the amygdala that sends CRF to both the BLA and BNST) disrupts the consolidation of contextual fear (Pitts & Takahashi, 2011; Pitts, et al., 2009). While the present study did not evaluate the effect of CRFr2 blockade, our findings expand on these studies to show that CRFr1s in the $_{L}$BNST are necessary for the consolidation of contextual fear memories. These effects seem to be restricted to associative learning as performance (i.e., responding to the foot-shock US and freezing to TMT) was largely unaffected by CRFr1 antagonism.

Whereas contextually conditioned defensive responses are thought to rely on CRF (Radulovic, Rühmann, Liepold, & Spiess, 1999) and corticosterone (CORT) signaling (Pugh, et al., 1997), unconditioned predator odor responses may not be regulated by CRF and CORT – an important distinction shown by a number of studies (for review see (Rosen, 2004; Rosen, et al., 2015)). This is puzzling given that both types of fear (1) increase CORT (Cordero, Merino, & Sandi, 1998; Day, et al., 2004), (2) increase CRF in the CeA and BNST (Asok, Ayers, et al., 2013; Lehner et al., 2008), and (3) CORT alone increases CRF expression in the CeA and BNST (Shinya Makino, et al., 1994a, 1994b). Additionally, lesions of the BNST also disrupt both types fear (Fendt, et al., 2003; Sullivan, et al., 2004). However, chronic CORT only affects contextually conditioned fear (Thompson, et al., 2004), not unconditioned fear to TMT (J.B. Rosen et al., 2008). While CRFr1 antagonism (peripherally and in the BNST) disrupts conditioned fear (Deak, et al., 1999; Kalin & Takahashi, 1990; Nijsen, et al., 2001), our work adds an important piece to this puzzle by showing that CRFr1s within the LBNST are important for conditioned, but not unconditioned, fear.

It is important to note some key limitations with our study. First, the post-shock and retention test freezing levels were substantially lower in our post-training, pre-expression, and state-dependent CRFr1 experiments – a factor which makes it difficult to draw any definitive conclusions from these data. Second, although we examined the effect of CRFr1 antagonism on contextual fear expression and in state-dependent learning, we only observed a marginal difference in our pre-training acquisition (2µg - 0µg) and expression (0µg -2 µg) groups relative to controls. The

state-dependent group (2µg – 2µg) demonstrated significantly reduced freezing relative to vehicle controls, but did not differ from the pre-training antalarmin group. This group was included as a contrast to our pre-training and post-training experiments to evaluate the possibility that animals could learn while under drug, but were simply unable to express fear conditioned freezing when the drug was not on-board at testing. This lack of state-dependent learning with CRFr1antagonism is consistent with studies using other CRF antagonists and fear conditioning paradigms (Waddell, Bouton, & Falls, 2008). Third, CRFr1 antagonism marginally reduced freezing at the retention test – a result that was inconclusive in the present study despite adequate *a priori* statistical power with the sample size used. Future studies are needed to more fully clarify the unique role of CRFr1s in the BNST on the expression of contextual fear.

Given its connectivity with core amygdala structures, the BNST is ideally situated to control both behavioral and endocrine function (Schulkin, et al., 2005) under situations of sustained threat. Since we did not investigate CRF in the CeA, it is difficult to conclude whether local CRF within the BNST or CRF release from the CeA CRF pathway regulate (1) activity at CRFr1s in the BNST or (2) the behavioral effects of reduced freezing during retention testing. The next chapter examines the role of CeA CRF projections during discrete phases of contextual fear learning using an optogenetic approach (Gafford & Ressler, 2015).

Chapter 4

OPTOGENETIC DISSECTION OF AN EXTENDED AMYGDALA CORTICROPING RELEASING FACTOR PATHWAY

4.1 Introduction

Rodent models have been valuable for understanding how distinct brain networks and cellular signaling cascades regulate fear and anxiety-like behaviors (Davis, 1992; Maren, 2001; Tovote, et al., 2015). In a typical fear conditioning experiment, a neutral conditioned stimulus (CS) such as a tone, light or context is paired with an aversive unconditioned stimulus (US) such as a foot-shock to produce a long-lasting conditioned response (CR) of freezing to future presentations of the CS alone. This fear CR can last on the order of days, weeks, and months (Gale, et al., 2004) after the initial learning has occurred and, thus, has been highly informative for understanding how key brain structures, like the amygdala, are involved in different phases (e.g., acquisition, consolidation, retrieval, etc.) of long-term memory (Pape & Pare, 2010).

Recent technological advances (e.g., optogenetics, cell-type specific viral targeting) have facilitated the shift towards understanding how phenotypically distinct neuronal subpopulations (e.g., Som+, PKC-δ, CREB+ (Botta et al., 2015; Haubensak, et al., 2010; J. Kim, Kwon, Kim, Josselyn, & Han, 2014; H. Li, et al., 2013)) and their long-range projections (Penzo, et al., 2014) within the amygdala contribute to the acquisition, consolidation (Huff, Miller, Deisseroth, Moorman, & LaLumiere, 2013), and retrieval of fear memories (Janak & Tye, 2015; Kwon et al., 2015). In particular,

the extended amygdala, comprised of the lateral part of the central nucleus of the amygdala (LCeA) and lateral bed nucleus of the stria terminalis (LBNST), controls distinct types of fear and anxiety-like behavior (Davis, et al., 2010; Tye et al., 2011). Only in the last few years have studies shown how select projections from the LBNST control discrete physiological (e.g., respiration rate) and behavioral (e.g., avoidance) components of anxiety (Kim, et al., 2013), in addition to how the LCeA and LBNST are functionally linked (Gungor, et al., 2015; Haufler, et al., 2013).

The LCeA and LBNST are some of the few limbic areas that express large amounts of the 41 amino acid neuropeptide corticotropin-releasing factor (CRF). CRF containing cells in the LCeA and LBNST display some unique properties relative to other CRF cell populations in the brain. First, they are primarily GABAergic, in contrast to CRF cells in the paraventricular nucleus of the hypothalamus which are glutamatergic (Sun & Cassell, 1993). Second, these GABAergic CRF cells also contain NMDA receptors (Beckerman, et al., 2013; G. M. Gafford & Ressler, 2015). They are not traditional fast-spiking pravalbumin+ neurons and display unique electrophysiological characteristics (Dabrowska, Hazra, Guo, DeWitt, & Rainnie, 2013; Nagano et al., 2015),. Finally, they co-express a large number of the same neuropeptides (Alheid, et al., 1995).

It has long been suspected that CRF within limbic areas like the LCeA and LBNST modulate aversive learning. Antisense knockdown of CRF mRNA within the LCeA disrupts the consolidation of contextual fear memories (Pitts & Takahashi, 2011) and sensitizes CRF's primary receptor (the type 1 receptor) in the LBNST

(Regev, et al., 2012) – suggesting the existence of an important, but unexplored, CRF fear-signaling pathway. For well over three decades, this $_{L}$CeA → $_{L}$BNST CRF pathway (Sakanaka, et al., 1986; Swanson, et al., 1983) has been suspected to play a role in modulating behavior to sustained environmental threats.

In particular, the CeA controls phasic (short-duration) fear whereas the BNST controls sustained fear (long-duration; (Walker & Davis, 2008)), lasting on the order of minutes. Importantly, the CRF pathway from the $_{L}$CeA to the $_{L}$BNST has been hypothesized to regulate the BNST under situations of sustained threat. Recent work has attempted to examine this pathway using a transgenic CRE approach, optogenetics, and DREADDs, (Pomrenze, Millan, et al., 2015), but functional questions still remain.

In the present study we used a cell-type specific AAV approach to inhibit $_{L}$CeA CRF neurons and $_{L}$CeA → $_{L}$BNST CRF projections during the acquisition of contextual fear. We examined the effect of optical inhibition during the retention of contextual fear – splitting the retention test session in half to evaluate short-duration (first-half) and long-duration (2nd half) fear. We developed a CRF promoter driven archaerhodopsin (CRF-ArchT) viral construct in order to selectively manipulate the activity of CRF cells and projections in real-time. First, we found that our CRF-ArchT construct selectively targeted CRF+ cells. Second, green-light stimulation functionally inhibited activity in $_{L}$CeA CRF-ArchT+ cell bodies. Third, optical inhibition of $_{L}$CeA CRF+ during acquisition disrupted the phasic and sustained components of contextual fear retention. Finally, optical inhibition of the $_{L}$CeA → $_{L}$BNST CRF pathway

uniquely disrupted the sustained, but not phasic, component of contextual fear retention.

4.2 Methods

4.2.1 Subjects

Male Sprague-Dawley rats (10 -16 weeks of age) obtained from Harlan breeders (Indianapolis, IN) were used for all experiments. Rats were maintained on a 12h light/dark cycle (lights on at 7:00 A.M.) at constant temperature with free access to food and water. Following arrival in the animal colony, rats were left undisturbed for seven days prior to the start of any experimental procedures. Rats were pair-housed in opaque polycarbonate cages with wood shavings for the duration of the study. All procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee.

4.2.2 Surgery

Rats received two surgeries: one for viral infusions and another for implantation of fiber optic ferrule. Prior to all stereotaxic surgeries rats were anesthetized with a weight appropriate dose of a ketamine/xylazine cocktail. Following surgeries, rats were given a weight appropriate dose of buprenorphine (for pain) and baytril (antibiotic). In the first surgery, all animals received bilateral 1 uL stereotactic injections (via a Pump11 Elite Nanomite Infusion System, Harvard Apparatus, Holliston, MA= of either the control or archaerhodopsin tp009 (ArchT) viral construct (see Viral construct below) into the CeA using the following coordinates: AP = - 2.5, ML = ± 4.4, DV = - 8.0. The syringe was lowered to the

target site, left in place for 1 minute prior to injection and 2 minutes following injections. Injections were delivered at a rate of 200nL/min over a five minute period.

For CeA targeting, one group was brought back 3 weeks after viral injections and implanted with fiber optic cannula (200 uM diameter, 0.39 nA, manufactured in-house) 0.4 mm above the site of viral injections. For axonal targeting in the LBNST, one group was brought back 5 weeks after viral infusions and implanted with cannula using the following coordinates: AP = -0.1 mm, ML = ± 2.2 mm, DV = - 7.2 mm, at a 4º angle. Behavioral testing was conducted one week after the cannula surgery.

4.2.3 Viral Constructs

Two viral plasmids were constructed: pAAV-CRF-ArchT-EGFP-WPRE-SV40 (abbreviated CRF-ArchT) and the control construct pAAV-CRF-EGFP-WPRE-HGH (abbreviated CRF-EGFP; Figure 1A). Both constructs contained a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a polyadenylation signal (SV40 or HGH). The CRF-ArchT construct was derived from a base plasmid containing the CamKII-ArchT-EGFP sequence that was a gift from Edward Boyden (Addgene plasmid # 37807). The CRF-EGFP construct was derived from a base plasmid containing the Syn-EGFP sequence that was also a gift from a gift from Edward Boyden (Addgene plasmid # 58867). The CRF promoter was derived from a pUC18 plasmid containing a ~2.2kb full length promoter, generously provided as gift from Audrey Seasholtz (University of Michigan). Sequence analysis (via Eukaryotic Promoter Database: http://epd.vital-it.ch/) and transcription factor binding analysis (via TransFac public database: http://www.gene-regulation.com/pub/databases.html)

confirmed that the promoter contained sites for all major transcriptional elements and spanned -2200 to + 94 of the promoter region upstream of exon 1.

For each construct, we excised the existing promoter (CAMKII or Synuclein) using restriction enzymes specific to each plasmid and purified gel extracted fragments. Next we conducted PCR to (1) first amplify the CRF promoter from the pUC18 plasmid and (2) add restriction sites to each promoter that was complimentary to the remaining overhangs present in each optogenetic plasmid. We ligated the promoter into the respective pAAV backbone plasmids and confirmed DNA integrity via DNA sequence analysis (not shown). SMA1 digests revealed intact SMA1 sites necessary for viral production (Figure 4.1). Finally, each construct was packaged into an AAV2/2 (Penn Vector Core, Philadelphia, PA).

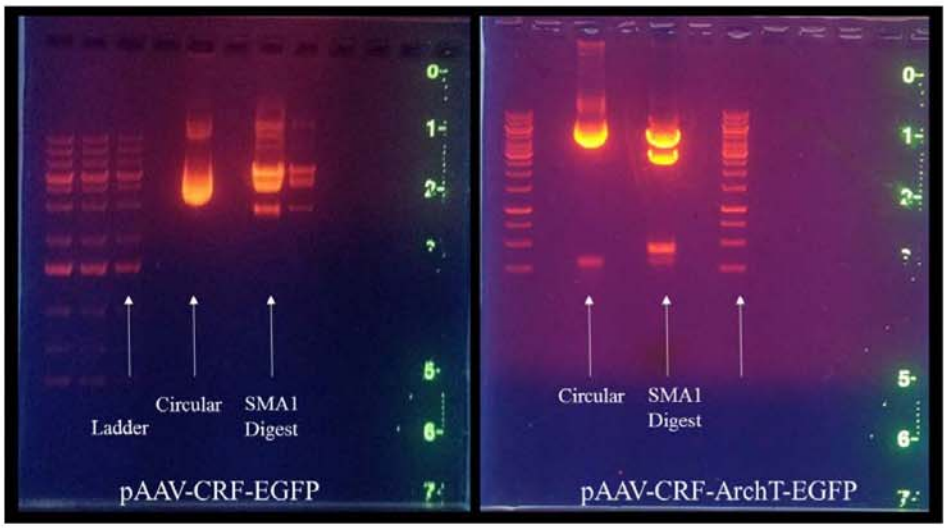


Figure 4.1. 1% Agarose gels showing final plasmid restriction digests prior to AAV packaging. Note the three varying coiled states in the circular plasmid. SMA1 digests produced more bands. The ladder used was a 1kb GeneRule Plus ladder where bright bands represent 500bp, 1500bp, and 5000bp DNA fragments.

4.2.4 Cellular and Molecular Verification

4.2.4.1 Immunohistochemistry

For initially confirming expression of target labeling, we used immunohistochemical techniques. Briefly, 4 weeks or 6 weeks after viral infusion, a group of animals were transcardially perfused with a 4% paraformeldahyde (PFA) solution in 1x phosphate-buffered saline (PBS). We also included viral infusions within the paraventricular nucleus (PVN) of the hypothalamus to evaluate expression within a site that expresses high levels of CRF. Brains were post-fixed in a 4% PFA solution for 24 hours, followed by two 48 hour incubations in a 30% sucrose 4% PFA solution at 4ºC prior to sectioning. Brains were sectioned on a cryostat at 40 uM, placed on charged slides, and stored with desiccant at -20ºC prior to staining. Staining was conducted over two days. Slides were washed 3 times in 1x PBS, followed by 1 hour incubation in a blocking solution containing 5% normal goat serum, 0.4% triton x-100, and 1X PBS. Following blocking, sections were incubated under gentle agitation with the primary antibodies in the blocking solution for 24 hours at 4ºC. A 1:80 dilution was used for mouse monoclonal anti-CRF (Abnova, 154-196), a 1:500 dilution for rabbit polyclonal anti-GFP (Life technologies, A-6455). The next day slices were washed 3 times in 1X PBS and then incubated for 1 hour with alex-fluor secondary antibodies specific to mouse (Alex-fluor 568, goat anti-mouse, 1:300 dilution) and rabbit (Alexa-Fluor 488, goat anti-rabbit, 1:500 dilution) in blocking solution at room temperature in the dark. Slides were then washed three times in 1x

PBS, briefly dried, and mounted with Prolong Gold antifade mountant containing DAPI (Life technologies, P36935). Slides were stored at -20ºC until imaging

4.2.4.2 RNAscope (Fluorescent In situ Hybridization)

Given the low levels of CRF-labeling observed in the CeA with immunohistochemical techniques (Palkovits, Brownstein, & Vale, 1985), we chose to validate the specificity of our construct at the transcriptional level. We used a novel method of in situ hybridization termed RNAscope. This method allows for the single-cell resolution of target mRNA using DNA Z-blocks containing a complimentary 13-20bp DNA sequence, genomic spacer, and oligonucleotide tail that is used to build a genomic scaffold which selectively binds targeted fluorophores with unparalleled specificity (F. Wang et al., 2012).

Animals that received viral infusions were rapidly decapitated, brains removed, flash frozen in isopentane, and stored at -80ºC until sectioning. Brains were sectioned at 16 µM and stored in a slide box at -80C until labeling. Labeling was conducted via manufacturer's instructions (Advanced Cell Diagnostics) using probes targeting CRF and GFP. CRF was labeled on channel 1 using Atto550 and GFP was labeled on channel 3 using Atto647 (Amp4-Alt-B solution). These channels were chosen in order to still visualize intrinsic EGFP fluorescence (~509 nm) and DAPI (~461 nm). Slides were then counterstained with DAPI, mounted with Prolong Gold antifade reagent, and stored at 4ºC until confocal imaging.

4.2.4.3 Radiolabeled In Situ Hybridization

In situ hybridization was conducted identically to our previous reports (Asok, Ayers, et al., 2013). Sections corresponding to the CeA and PVN were used to identify the expected CRF mRNA expression pattern and validate against our CRF-ArchT construct. Briefly, a CRF antisense RNA probe (riboprobe) was transcribed from a linearized (w/HINDIII) plasmid containing a sense cDNA sequence coding for a 1063 base pair sequence of CRF. S^{35} UTP was incorporated into the transcribed riboprobes using an SP6 RNA polymerase Maxiscript kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). 1×10^6 dpm of S^{35} labeled riboprobe was added to hybridization buffer and then to each slide. Slides were incubated overnight at 55C. Following hybridization, treatment with RNase, and washing, the dry slides were exposed to Kodak Biomax MR Film for three days (See Imaging for analysis parameters.

4.2.5 Imaging

All Three-dimensional z-stacks (1 µm slices) of immunostained tissue were captured on a Zeiss 780 laser-scanning Confocal microscope (Zeiss, Inc.). Images were captured at varying magnifications: 10x air, 20x 0.75 nA air, 40x 1.4 nA oil, and 63x 1.46 nA oil. Post-hoc image processing for adding scale bars and adjusting contrast was conducted using standard Zeiss software (Zen 2.1, Zeiss, Inc.).

For radiolabeled in situ hybridization, autoradiograms were captured and digitized to 8-bit gray values via a Dage CCD video camera controlled by ImageJ 1.45

(Wayne Rasband, NIMH) similar to our previous reports (Asok et al., 2013). ImageJ was used to subtract the background (2D-rolling ball radius of 50.0 pixels).

4.2.6 Behavior

4.2.6.1 Contextual Fear Conditioning

For contextual fear conditioning (Context A), animals were handled for two days with dummy connectors to acclimate them to the experimenter and being plugged into the fiber optic system. On day 3, contextual fear conditioning was conducted in Context A: a metal/black Plexiglass chamber (25cm x 31cm x 32cm) containing metal grid floors (19 stainless steel bars, 0.5 cm in diameter, and 1.25 cm apart). All CRF-ArchT and CRF-EGFP rats received bilateral illumination of either the CeA or BNST during training (~10 mW/mm2).

Each animal was placed in the chamber for 180s (baseline freezing measurement), followed by five 1s 0.6mA shocks spaced 180s apart. 24 hours later, animals were returned to Context A and tested for freezing to the context for 1080s (retention test measurement). The retention test was divided into the first half (seconds 0-539; defined as phasic) and second half (540-1080; defined as sustained). A few days later animals were brought to Context B (clear Plexiglas chamber that differed in lighting and odor) and given bilateral illumination for 3 minutes to evaluate if any light given off during training served as a discrete cue during training. Freezing was scored by FreezeFrame Software (Actimetrics, Wilmette, IL) with freezing defined as bouts of inactivity ≥ 0.75s as previously described (Asok et al., 2013).

4.2.6.2 Shock Sensitivity Training

Shock Sensitivity training was conducted (in Context A) to examine any changes in US processing driven by optogenetic inhibition. Thus, the same animals from contextual fear conditioning were tested for responses to varying levels of shock intensity (0 mA, 0.1mA, 0.3mA, 0.6mA, 1.0mA, 1.5mA, and 2.1mA) during light illumination. Responses were videotaped and scored offline by two experimenters blind to the experimental conditions or shock intensity levels. Responses were categorized on an ordinal scale of 0-4, similar to previous reports (Nielsen & Crnic, 2002a, 2002b). Responses were scored as follows: 0=no response, 1=flinch, 2=hop, 3=horizontal jump, and 4=vertical jump.

4.2.7 Cannula and Viral Expression confirmation

Given that our pilot data suggested that the EGFP expressed in our constructs was stable without immunohistochemical labeling, we verified expression and cannula placements post-mortem without fixation. Brain collection was identical to that used for RNAscope. However, slices were cut at 40 uM in the dark to preserve fluorescence. Slices were stored desiccated at -20ºC until they were mounted with Prolong gold antifade reagent containing DAPI. Imaging was conducted similarly to other experiments.

4.2.8 Statistical Analyses

We were interested in understanding the change in freezing from fear learning (i.e., acquisition) to phasic or sustained fear expression. Thus, we conducted a 2 (group) x 2 (freezing phase) repeated measures MANOVA to examine main effects

and interactions for ctx+shock vs. phasic fear expression and ctx.+shock vs. sustained fear expression. Following significant interactions we computed a difference score for each animal at phasic fear (post-shock – phasic fear) and sustained fear (post-shock – sustained fear) to examine the overall change in freezing similar to Wallace and Rosen (Wallace and Rosen, 2000). This approach allowed us to account for changes in freezing for each subject rather than evaluating gross differences between groups. We independently analyzed phasic and sustained fear. One animal (CRF-ArchT) in the $_{L}$CeA → $_{L}$BNST CRF pathway experiment showed a substantial increase in freezing between post-shock and retention tests and was removed from analysis. Independent samples t-test's was used to examine differences in the alternate context. Mann-Whitney U tests were used to examine ordinal shock sensitivity data.

4.3 Results

4.3.1 CRF-ArchT-EGFP selectively targets CRF+ cells in the LCeA

Immunohistochemical co-labeling (n=3) showed that the protein expression pattern of CRF-ArchT-EGFP cells and axons (Figure 4.2D) in the $_{L}$CeA highly resembled that of CRF mRNA found with a validated radiolabeled in situ hybridization assay (Figure 4.2C). $_{L}$CeA GFP+ neurons were found to express CRF (Figure 4.3B). Additionally, single cell in situ hybridization analyses showed that only $_{L}$CeA cells that synthesized CRF mRNA synthesized GFP mRNA and manufactured GFP protein (Figure 4.3C). However, adjacent cells that did not express CRF mRNA, did not express GFP mRNA or GFP protein (Figure 4.3C). Additionally, CRF-ArchT

injected into the PVN, an area the expresses significant amounts of CRF, revealed that GFP+ cells were co-localized with CRF+ cells (Figure 4.4C).

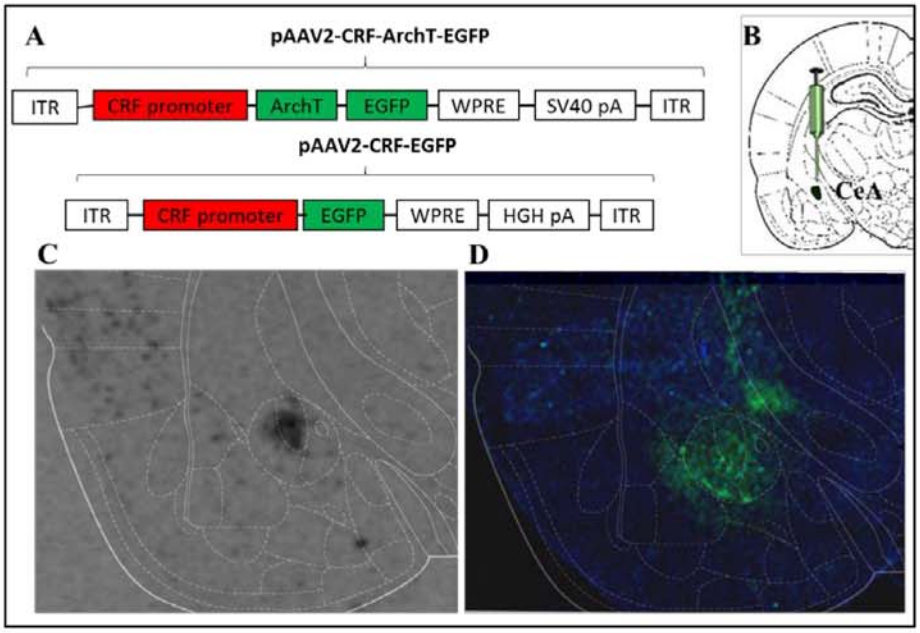


Figure 4.2. CRF-ArchT-EGFP is expressed in the LCeA. (A) Schematic representation of the CRF-ArchT-EGFP and CRF-EGFP control construct. (B) Schematic rat brain atlas slice of viral injection site. (C) Expression pattern of CRF mRNA using radiolabeled in situ hybridization. (D) Immunohistochemical section showing GFP labeling is restricted to the LCeA. Note the high similarity between GFP labeling and CRF mRNA in the LCeA.

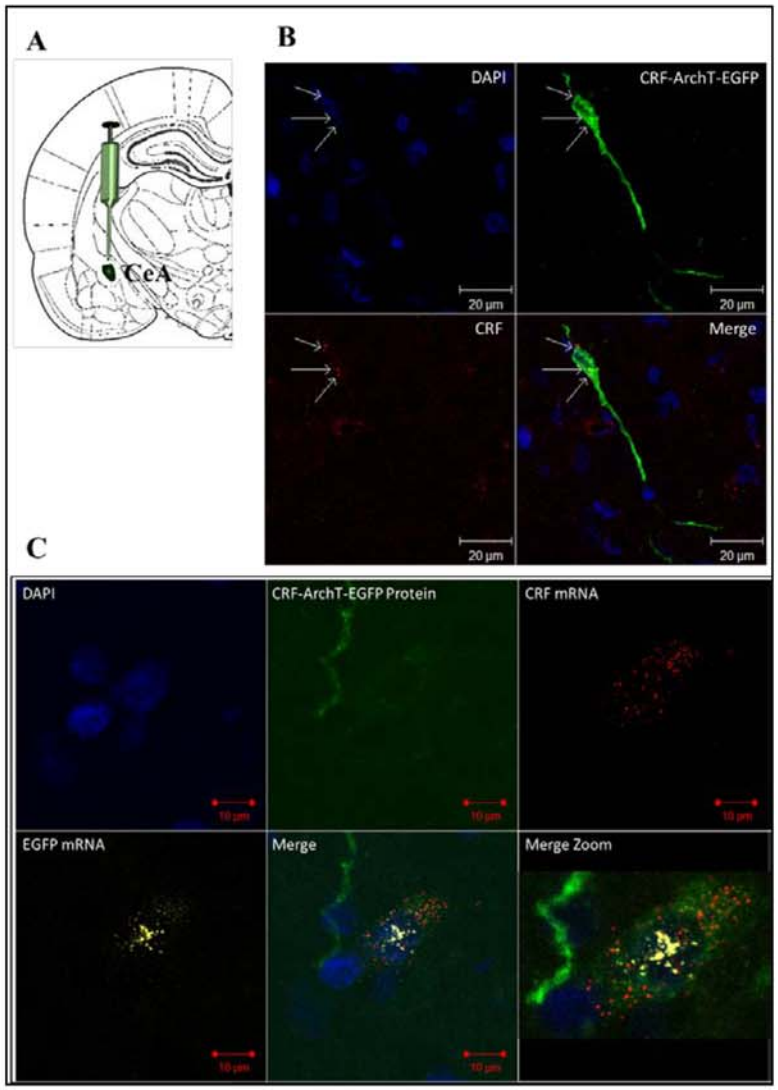


Figure 4.3. CRF-ArchT-EGFP is only produced in cells that synthesize CRF mRNA. (A) Schematic of CRF-ArchT injection site. (B) Immunohistochemical co-labeling showing the CRF-ArchT-EGFP neurons (green) are clustered around cell bodies (blue) and express CRF (red). (C) RNAscope showing how only cells (blue) that synthesize CRF (red) also synthesize EGFP mRNA (yellow-white) and also express EGFP protein (green). Note that the two adjacent cells (blue) that do not produce CRF mRNA (red) do not produce CRF-ArchT mRNA (yellow-white) or protein (green), despite CRF-ArchT axonal projections (green).

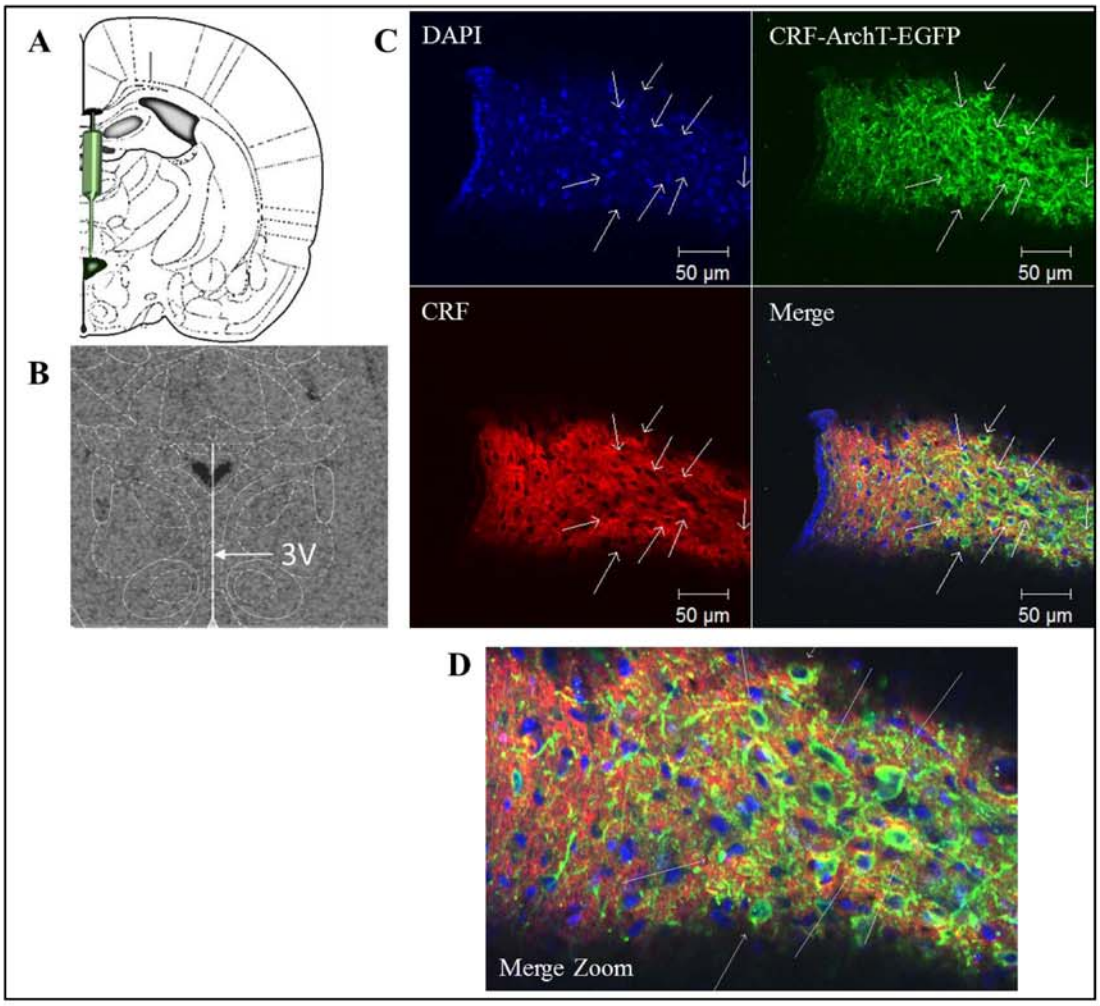


Figure 4.4. CRF-ArchT-EGFP immunohistochemical co-labeling in the PVN. (A) Schematic representation of viral injection site. (B) Expression pattern of CRF mRNA in the PVN with in situ hybridization. (C) CRF-ArchT (green) is co-localized with CRF (red) in the PVN.

4.3.2 CRF-ArchT-EGFP axonal projections are visible in the LBNST and optogenetic stimulation modulates activity in target cells

Evaluation of axonal projections in the LBNST following CRF-ArchT-EGFP injections in the LCeA (n=2) detected GFP+ expression along with CRF (Figure 4.5C). Additionally, axonal projections were clearly visible in the LBNST (Figure 4.5D).

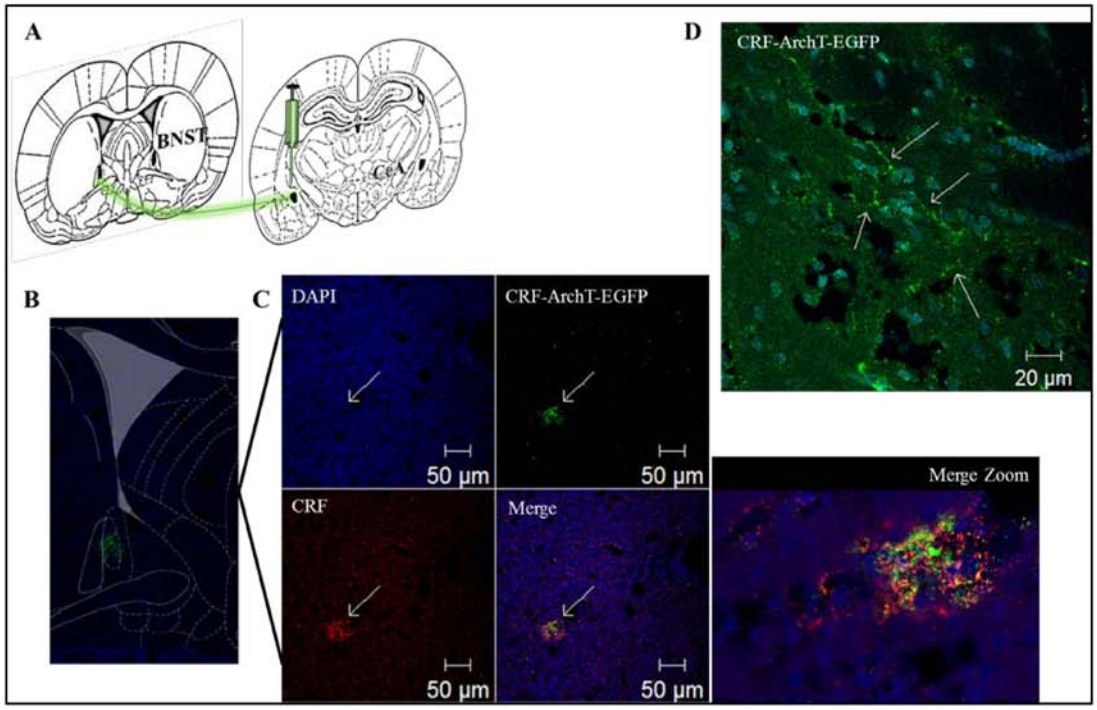


Figure 4.5. Axonal projections from the LCeA to the LBNST. (A) Schematic of viral injections site and target area for staining. (B) GFP overlay with a rat brain subsection. (C) Magnified view showing how GFP (green) was expressed in the same BNST area that expresses CRF (red). (D) Magnified view showing axonal processes within the LBNST.

4.3.3 Optogenetic inhibition of CRF+ $_{L}$CeA cells disrupts the phasic and sustained components of contextual fear expression

Optogenetic inhibition of CRF+ $_{L}$CeA cells (Figure 4.6A) during training had no effect on ctx. only freezing or context fear acquisition, but disrupted phasic and sustained fear expression 24 hours later. Light stimulation did not act as a CS or affect shock responsivity. These results were confirmed statistically below.

Groups (CRF-ArchT$_{n=13}$ vs. CRF-EGFP$_{n=11}$) did not differ at the ctx. only phase ($t(22)= -.528$, $p> .05$) or during the ctx. + shock phase ($t(22)= -.007$, $p> .05$).

For phasic fear, a 2 (group) x 2 (phase: ctx. + shock vs. phasic fear) repeated MANOVA revealed a main effect phase ($F_{1,22} = 28.747$, $p <.001$), no main effect of group ($F_{1,22} = 1.650$, $p > .05$), a group by phase interaction ($F_{1,22} = 5.309$, $p < .05$; Figure 4.6B). For sustained fear a 2 (group) x 2 (phase: ctx. + shock vs. sustained fear) repeated MANOVA revealed a main effect phase ($F_{1,22} = 39.274$, $p < .001$), a main effect of group ($F_{1,22} = 4.671$, $p < .05$), and a significant group by phase interaction ($F_{1,22} = 4.762$, $p < .05$; Figure 4.6B). An Independent samples t-test on difference scores revealed that animals in the CRF-ArchT group showed a greater reduction in freezing at both phasic ($t(22) = -2.304$, $p < .05$) and sustained ($t(22) = -2.182$, $p < .05$) fear measures relative to CRF-EGFP controls.

Optogenetic inhibition after contextual fear training did not alter freezing in another context ($t(17)=.607$, $p > .05$; Figure 4.6C), suggesting that the light did not act as a CS. Mann-Whitney U tests confirmed that optogenetic inhibition of CRF-ArchT $_{L}$CeA cells did not change sensitivity to the footshock US (p's > .05; Figure 4.6D).

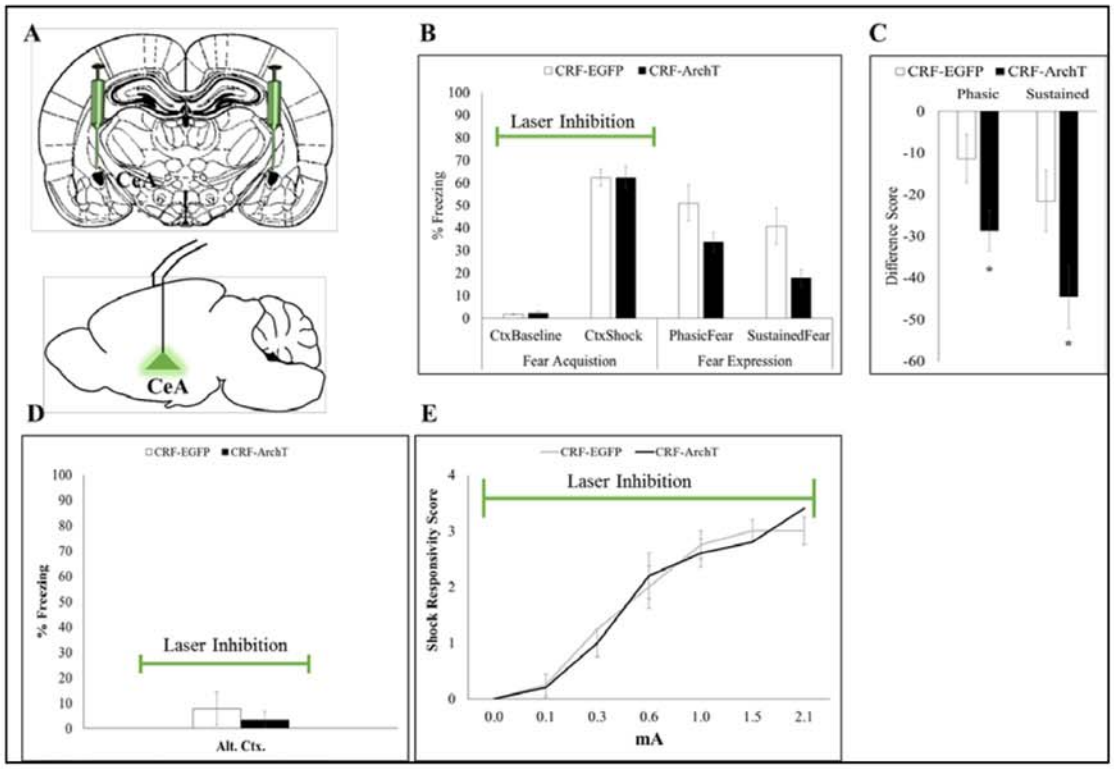


Figure 4.6. Optogenetic inhibition of LCeA CRF cell bodies disrupts phasic and sustained fear. (A) Schematic of viral injection site and fiber optic implantation. (B) Laser inhibition during training did not affect context only baseline freezing or freezing during context shock pairings, but disrupted phasic and sustained fear at the retention test. (C) CRF-ArchT rats showed a greater decrease at both phasic and sustained phases of the fear expression test relative to CRF-EGFP controls. (C) Laser inhibition in an alternate context did not affect freezing after fear learning. (D) Laser inhibition of CRF cells did not affect responsivity to varying foot-shock intensities. *p < .05, Error bars are ± S.E.M.

4.3.4 Optogenetic inhibition of $_{L}$CeA → $_{L}$BNST CRF pathway only disrupts the sustained component of contextual fear expression

Optogenetic inhibition of $_{L}$CeA → $_{L}$BNST CRF pathway (Figure 4.7A) during training had no effect on ctx. only freezing or context fear acquisition, but disrupted sustained fear 24 hours later. Light stimulation did not act as a CS or affect shock responsivity. These results were confirmed statistically below.

Groups (CRF-ArchT$_{n=8}$ vs. CRF-EGFP$_{n=10}$) did not differ at the ctx. only phase ($t(16)= -.656$, $p> .05$) or during the ctx. + shock phase ($t(16)= -.756$, $p> .05$).

For phasic fear, a 2 (group: CRF-ArchT and CRF-EGFP) x 2 (phase: ctx.+shock vs. phasic fear) repeated MANOVA revealed a marginal main effect phase ($F_{1,16} = 3.605$, $p = .076$), no main effect of group ($F_{1,16} = .022$, $p > .05$), no group by phase interaction ($F_{1,16} = .742$, $p >.05$; Figure 4.6B). However, for sustained fear, a 2 (group) x 2 (phase: ctx. + shock vs. sustained fear) repeated MANOVA revealed a main effect phase ($F_{1,16} = 33.835$, $p < .001$), no main effect of group ($F_{1,16} = .022$, $p > .05$), but a significant group by phase interaction ($F_{1,16} = 7.668$, $p < .05$). Independent samples t-test on difference scores revealed that animals in the CRF-ArchT group showed a greater reduction in freezing at the sustained ($t(16) = -2.769$, $p < .05$) fear measure relative to CRF-EGFP controls (Figure 4.6B).

Optogenetic inhibition after contextual fear training did not alter freezing in another context ($t(8)=-1.108$, $p > .05$; Figure 4.7C), suggesting that the light did not act as a CS. Mann-Whitney U tests confirmed that optogenetic inhibition of the $_{L}$CeA

→ LBNST CRF pathway did not change sensitivity to the foot-shock US (p's > .05; Figure 4.7D).

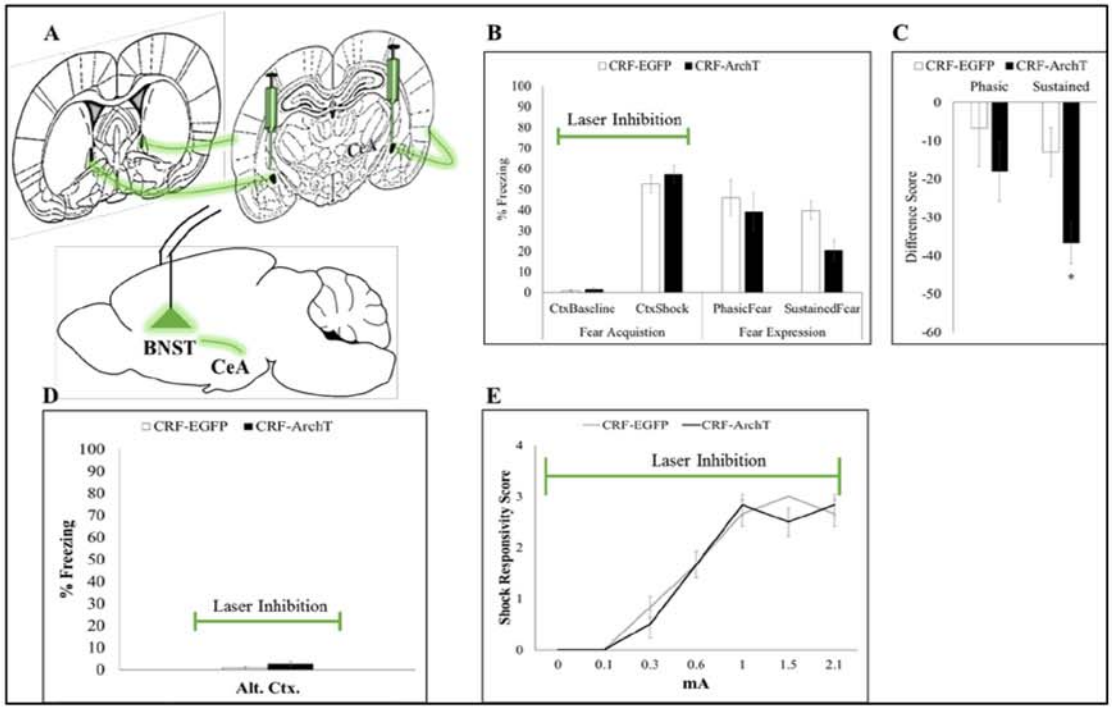


Figure 4.7. Optogenetic inhibition of LCeA CRF projections to the LBNST disrupts sustained fear. (A) Schematic of viral injection site and fiber optic implantation. (B) Laser inhibition during training did not affect context only baseline freezing or freezing during context shock pairings, but disrupted sustained fear at the retention test. (C) CRF-ArchT rats showed a greater decrease at the sustained, but not phasic, phase of the fear expression test relative to CRF-EGFP controls. (D) Laser inhibition in an alternate context did not affect freezing after fear learning. (E) Laser inhibition of CRF projections to the LBNST did not affect responsivity to varying foot-shock intensities. *p < .05, Error bars are ± S.E.M.

4.4 Discussion

The present study showed that an AAV2 CRF promoter driven archaerhodopsin construct (CRF-ArchT-EGFP) was capable of selectively targeting CRF+ cells. This was evident in two neurochemically distinct CRF cell populations: the CeA and the PVN. CRF axonal projections from the LCeA were clearly visible in the dorsal LBNST. mRNA analysis confirmed that only cells that synthesized CRF (1) synthesized CRF-ArchT-EGFP and (2) co-localized with CRF-ArchT-EGFP protein product, whereas cells that did not synthesize CRF mRNA showed neither. Our major finding was that optogenetic inhibition of CRF cells in the LCeA during acquisition of contextual fear disrupted phasic and sustained fear at the retention test. However, optogenetic inhibition of LCeA → LBNST CRF projections only disrupted sustained fear. Optogenetic inhibition of CRF+ cells in both the LCeA and LBNST did not influence baseline freezing to the conditioning context (CS) prior to shock, freezing to an alternate context after fear learning, or responsivity to foot-shocks (US). Our data show that an LCeA → LBNST CRF pathway is important for modulating fear expression to sustained, but not phasic, contextual threats.

The primary goal of our study was to evaluate if the LCeA → LBNST CRF pathway was important for fear learning, as has been hypothesized for nearly two decades (Davis, et al., 2010). First, we were able to confirm at the transcriptional level that only CRF synthesizing cells produced CRF-ArchT-EGFP. However, we did not immunohistochemically or functionally evaluate all LCeA CRF projections (e.g., to ventrolateral BNST, parabrachial nucleus, lateral hypothalamus; (Sakanaka, et al.,

1986)) in our study – an important future direction for understanding how these CRF pathways may regulate various physiological and behavioral components of fear. Additionally, we were unable to obtain robust CRF labeling with our CRF antibody in the CeA relative to the PVN, which may be due to a number of issues including: the antibody itself (Baker, 2015), a lack of physiological/psychological stress prior to staining, or more likely the low basal levels of CRF within the CeA (Palkovits, et al., 1985; Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002). Indeed, other studies have used colchicine to block axonal transport of CRF and overcome issues with visualizing CRF protein in the CeA (Pomrenze, Millan, et al., 2015; L. Wang et al., 2011). Regardless, we were able to show selectivity of our construct at the transcriptional level.

Optogenetic inhibition of $_{L}$CeA CRF+ cells and select projections to the $_{L}$BNST had no effect on the rate of acquisition relative to controls. Optical inhibition (1) only disrupted freezing 24 hours later and (2) only during specific phases (phasic vs. sustained) of the retention test. An alternative explanation of our data is that animals extinguished fear to the environment faster. It is possible that the $_{L}$CeA → $_{L}$BNST CRF inhibition could have facilitated extinction to the sustained phase of retention testing. A future experiment restricting optical inhibition to either the phasic or sustained parts of the retention test would help to tease apart the precise function of this pathway during fear expression. Additionally, laser stimulation alone (without context-US pairings) did not induce freezing to a context before or after fear learning. Thus, it is unlikely that laser-stimulation itself acted as a cue. However, it is unclear if

fear-related information was encoded in other pathways (i.e., state-dependent learning) that could have been active during fear learning.

While our study provides an important pathway and mechanism for expression of phasic and sustained fear, it is still unclear how LCeA CRF cells and projections to the LBNST globally modulate contextual fear learning. One possible explanation is through a corticosterone-related mechanism. Studies have found direct CRF projections from the CeA to the PVN (Marcilhac & Siaud, 1997) - although it is generally believed that CeA regulation of the PVN occurs through non-direct multi-synaptic pathways (i.e., via the BNST (Jankord & Herman, 2008; Prewitt & Herman, 1998)). Although, these LCeA CRF multisynaptic pathways have not been completely mapped to support this view.

Given that we did not measure corticosterone or CRF expression in the PVN following training it is difficult to say for sure. Previous work from our lab has shown that chronic corticosterone supplementation enhances the retention of contextual fear and increases CRF in the CeA - providing support for this possibility (Thompson, et al., 2004). Importantly, the amygdala is thought to provide feed-forward excitation of the PVN, whereas the hippocampus is thought to provide inhibition (Jankord & Herman, 2008; Schulkin, et al., 2005). CRF from the PVN regulates corticosterone secretion, and corticosterone depletion (through adrenalectomy) produces a similar effect (no effect on acquisition, but disruption of long-term freezing) to what we detected in the present study (Pugh, et al., 1997). In fact, corticosterone is known to increase CRF in the two regions we studied: the LCeA and the LBNST (Shinya

Makino, et al., 1994a, 1994b). However, given that $_{L}$CeA CRF cells are primarily GABAergic, it is also possible that GABA release plays an important role. Other studies have found that deletion of GABA-Aα1 in CRF+ neurons disrupts fear extinction, not acquisition, but in an auditory fear conditioning paradigm (Gafford et al., 2012; Gafford & Ressler, 2015). Thus, future studies should examine the (1) effect of optogenetic inhibition of CeA CRF cells on corticosterone secretion during contextual fear learning and retention, in addition to (2) the effects of selective deletion of GABA in CRF neurons (e.g., via an antisense tail in the CRF-ArchT-EGFP construct to knockdown GABA).

In summary, we show that an extended amygdala CRF pathway is critical for acquiring fear that is expressed in a sustained, but not phasic manner. We provide a simple AAV tool for the optogenetic manipulation of CRF cells. We show that CRF cells in the $_{L}$CeA and CRF projections to the $_{L}$BNST regulate discrete components of behavior in fear retention. This study is the first to identify how cellular activity in an extended amygdala pathway at the time of acquisition is crucial for learning to fear a context.

Chapter 5

CRF, CONTEXT FEAR, AND PTSD

5.1 Summary of Experiments

In chapter 3, we found that pre and post-training infusions of the highly selective CRFr1 antagonist antalarmin into the dorsal LBNST disrupted the retention of contextually conditioned fear. Additionally, administration of antalarmin prior to the retention test (during fear expression) appeared to reduce freezing relative to controls – suggesting that antalarmin may have also affected retrieval of the contextual fear memory. These results were not due to deficits in performance or to state-dependent learning effects, and suggest that CRFr1s in the LBNST, and consequently CRF itself, have an important role in the storage and retrieval of contextual fear. The experiments in chapter 3 provided the groundwork for the optogenetic experiments in Chapter 4.

In Chapter 4, we found that optogenetic inhibition of LCeA CRF cells during contextual fear training disrupted both phasic and sustained fear at the retention test. Importantly, selective inhibition of the LCeA → LBNST CRF pathway during contextual fear training only disrupted sustained fear at the retention test. These optogenetic effects were not due to deficits in performance (i.e., responding to the foot-shock).

Broadly, my dissertation points to an important mechanism that may differentiate fear learning in a manner that influences fear expression to phasic vs. sustained environmental threats. First, CRFr1s in the LBNST are important for the

consolidation and possibly expression of contextual fear – suggesting that they may play a role in the storage of contextual fear information. Second, activity in a CRF pathway from the LCeA to the LBNST at the time of learning is necessary for expressing fear to sustained, but not phasic threats. Speculatively, with some important caveats below, our results offer the possibility that LCeA → LBNST CRF projections to CRFr1s in the LBNST regulate the consolidation of fear to sustained, but not phasic, contextual threats. A graphical representation of this model is shown in Figure 5.1. This speculation fits well with what has been long hypothesized by Davis and colleagues (see figure in (Walker, et al., 2003)).

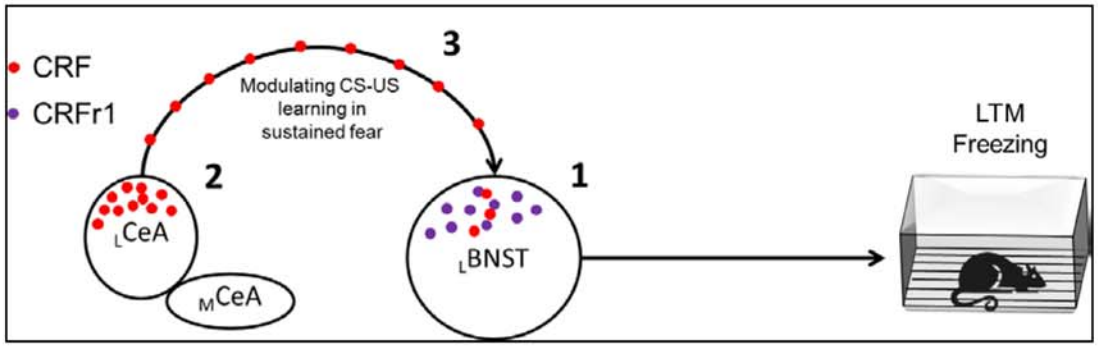


Figure 5.1 Summary of Findings. (1) CRFr1s in the LBNST are important for learning contextual fear. (2) CRF cells in the LCeA are important for modulating CS-US learning to both phasic and sustained threats. (3) LCeA → LBNST CRF projections modulate CS-US learning to sustained threats.

5.2 Important Caveats and Criticisms of the Present Experiments

Despite these exciting and novel findings, there are a number of important future experiments that would help to tease apart the behavioral, neuroanatomical, and neurochemical constraints of phasic and sustained contextual fear. The following sections are not an exhaustive analysis, but are intended to highlight some general

theoretical considerations (and challenges) that could be tested by future experiments (summarized in Table 5.1).

First, it is important to note the primary theoretical limitation with my interpretation of the data. That is, although we found that LCeA CRF cells and LCeA → LBNST CRF projections regulate fear learning that influences distinct components (phasic vs. sustained, respectively) of contextual fear expression, it is unclear how CRFr1s fit in to this story. CRFr1 antagonism within the LBNST disrupted fear at a five minute retention test – suggesting that CRFr1s in the LBNST are somehow important for the consolidation of, according to my definition (see Chapter 4), phasic fear during context conditioning. A number of factors could explain this disparity including differences in foot-shock intensity (1.5 mA vs. 0.6 mA) coupled with the overall length of the training session (~8 min.; see discussion below). Thus, it is unclear as to what temporal and qualitative factors fully govern the involvement of CRFr1s in the BNST during contextual fear learning. Furthermore, it is possible that overall BNST activity is regulated via the BLA during different types of threat. That is, the BLA is known to provide glutamatergic input to the LBNST (Walker, et al., 2003) and this pathway (or other unexplored pathways) may somehow regulate the involvement of CRFr1s in the LBNST during the expression of contextual fear at the retention test.

Second, we did not perform a "gain of function" experiment to clarify the importance of the LCeA → LBNST CRF pathway in modulating sustained contextual fear. That is, *if* this pathway is critical for modulating aspects of contextual fear memory storage, *then* increasing activity in these cells (i.e., using a channelrhodopsin targeted to CRF cells) during training with sub-threshold fear conditioning (e.g., 0.3

mA) parameters should enhance freezing relative to controls during the sustained component of contextual fear retention.

Third, (as an extension of points one and two above) we used single-trial conditioning (a single 1.5 mA shock) with our CRFr1 antagonist studies and "multi-trial" (5 shock 0.6 mA) conditioning with our optogenetic studies (see theoretical considerations of "multi-trial" described below). Thus, it is unclear if CRFr1 antagonism during our five shock .6 mA training would produce a similar effect. It would not be surprising if CRFr1 antagonism during a "multi-trial" contextual fear conditioning paradigm would reduce (but not abolish) freezing relative to controls. More importantly, whether our channelrhodopsin "gain of function" experiment proposed above could be blocked by CRFr1 antagonism in the LBNST is a critical future experiment for our overall hypothesis about CRF function in this pathway. Additionally, overexpressing CRF in the LBNST during optogenetic inhibition of the LCeA CRF → LBNST pathway would be important for elucidating how CRF may modulate CRFr1s in this pathway.

Fourth, we did not *abolish* all "fear" at the retention test. Following inhibition of LCeA CRF cells and projections to the LBNST during training, animals still exhibited elevated levels of freezing (~10-20%) at the retention test, although they were significantly less than controls. Although, we did not include an unpaired control group (5-shock training in an alternate context with light inhibition in the optogenetic context). However, it is possible that we are simply reducing the intensity, or strength, of phasic or sustained fear memories – a very important consideration in and of itself.

Fifth, as stated in the discussion of Chapter 4, we did not evaluate if selectively inhibiting LCeA CRF cells or projections to the LBNST during shock presentations (~

1 sec. in length) was capable of disrupting the expression (or "strength") of contextual fear memories. It is *critical to note* that we chose to alter our training parameters in Chapter 4 in order to extend the length of our training session. This was done for theoretical assumptions about the function of this pathway during "sustained threat," or "fear" that is long-lasting, as described by Michael Davis (Davis, et al., 2010; Walker & Davis, 2008; Walker, et al., 2009). This was especially important given the recent findings from Mark Bouton's lab that retention of contextual fear during long exposure to a context (~ 10 min.), but not short (~ 1 min.), is disrupted by lesions of the BNST (Hammack, et al., 2015). However, with contextual fear, contrasted against the startle studies of Michael Davis, it is unclear as to which specific features of the environment are being sampled during each "trial" of fear-learning (the context-CS is not discrete, although the shocks are). Furthermore, it is still unclear of when the switch from "short"-duration to "long"-duration fear occurs.

Theoretical assumptions gleaned from single-trial contextual fear conditioning (i.e., 1 shock presentation) studies suggest that a "strong" unitary contextual representation is formed in a time-dependent fashion before the shock is administered (Fanselow, 1986, 1990). However, see (O'Reilly & Rudy, 2000) for a computational account of the neocortex vs. hippocampal perspective. Given that rodents increase their freezing with each shock presentation (which I refer to here as a "multi-trial" contextual fear paradigm) during fear-acquisition, it is difficult to conclude (but easily assumed) that each US presentation is simply updating the contextual representation that was formed during the first few minutes of non-reinforced context exposure.

Recent computational models suggest that the environment is resampled during the retention test to guide behavior (Krasne, Cushman, & Fanselow, 2015), although

this is after fear learning has already occurred. However, it is quite plausible that during a multi-trial paradigm, the context is continually being re-sampled prior to each shock presentation (see theoretical support from (Fanselow, et al., 2014) countering equipotentiality and hippocampal function in addition to points from (Estes, 1950) on stimulus sampling theory) and certain salient features are more strongly associated with the fear representation than others.

Although my dissertation did not examine the hippocampus, it is possible that the unexplored ventral hippocampal CA1 projections to the LBNST (mentioned in Chapter 1) are important for modulating aspects of contextual fear learning, but under sustained threat conditions (not a single shock present with minimal context pre-exposure or testing). However, *if* CRF in the LCeA → LBNST pathway is crucial for modulating the strength of the consolidated fear memory (a valid speculation given the data), *then* inhibiting activity during select "CS-US" trials along the acquisition curve (i.e., approximately the first 3 of the 5 shocks) should "dose"-dependently disrupt retention test freezing. While this is a simple study, it is best left for future experiments given that theoretical implications differ a bit from the goal of my dissertation. Studies should also optogenetically manipulate glutamatergic activity in hippocampal and basolateral amygdala projections to the LBNST to (1) understand the true targets of these projections and (2) the functional role of these pathways in contextual fear learning.

Sixth, as mentioned in the discussion section of Chapter 4, it is possible that corticosterone signaling is altered with optogenetic inhibition of LCeA CRF cells and projections. While we identified an important function for a subpopulation of LCeA cells and a long-range CRF pathway (to the LBNST) in contextual fear learning, we

did not measure corticosterone levels. Measuring corticosterone following training and retention testing would be a valuable future experiment. Specifically, this experiment would help to elucidate if (1) we are manipulating a brain-body feedback loop (which would possibly point to hippocampal glucocorticoid involvement), and/or (2) if the $_{L}$CeA → $_{L}$BNST CRF pathway plays a critical role in this process.

Seventh, we did not evaluate other CRF pathways from the $_{L}$CeA. I did not conduct whole-brain mapping to evaluate all $_{L}$CeA CRF projections – a laborious, but relatively simple, study using our viral construct (see (Sakanaka, et al., 1986). Given the multisynaptic modulatory role of CRF proposed in the discussion of Chapter 4, this would be a valuable experiment. The $_{L}$CeA also projects to (1) the parabrachial nucleus (involved in respiration and also important for regulating fear in the BNST), ventrolateral BNST (which receives adrenergic inputs from the locus coeruleus), the periaqueductal grey itself (very recent findings that contrast the long-held belief that the mCeA is the prime output to regulate freezing behavior (Penzo, et al., 2014)), medial and lateral nuclei of the amygdala, ventromedial hypothalamus, trigeminal nucleus, and others (Swanson, et al., 1983). It is possible the CRF released in these other pathways (e.g., the parabrachial nucleus) could indirectly modulate the CRF pathway we investigated.

Eighth, we did not evaluate the molecular (i.e., intracellular and transcriptional) mechanisms that regulate this reduction in freezing. As mentioned in Chapter 2, CRFr1s are coupled to adenylate cyclase which can regulate cAMP to direct PKA activity to remove CREB2 repression from CREB1 to facilitate gene transcription (Kandel, 2001). Thus, it is unclear as to what is happening within these target CRFr1 cells (or in the polysynaptic network assumed from our whole-cell patch

clamp data – not shown) at the cellular and molecular levels during contextual fear-learning. This is an important direction with regard to my testable speculation that CRF released within the LCeA → LBNST pathway is acting at CRFr1s to regulate the "strength" of aversive memories.

A more interesting (and translationally relevant) question emerges from the experiments in this dissertation – how are contextual fear memories of varying strength represented within cells?! It has long been thought, and shown with much data, that changes in synaptic strength and connectivity are critical for memory (M. C. Lee, Yasuda, & Ehlers, 2010; Yiu et al., 2014). A number of studies (including those from our own lab) also support a role for specific genetic transcriptional changes (i.e., occurring at the level of the nucleus) that are necessary for contextual fear memory storage (Malkani, et al., 2004). *If* CRF released from the LCeA acting at CRFr1s in the LBNST is critical for modulating memory strength, *then* varying the intensity of training (and of the fear memory) should be evident in either (1) excitability at CRFr1 dendritic compartments, (2) CRFr1 nuclear compartments, or (3), and more likely, both. CRF knockdown in the CeA does increase CRFr1 receptors in the BNST – suggesting sensitization occurs with decreased peptidergic input (Resstel, et al., 2008). A first step towards understanding global activity within this network would be with genetically targeted real-time calcium imaging (i.e., using a CRFr1 promoter driven fast acting genetically encoded calcium indicator). This approach may provide insight into changes in (1) neuronal activity within CRFr1 cells of the LBNST during phasic and sustained components of contextual fear.

Critical Future Experiments	
1.	Whole brain evaluation of CRF projections from the LCeA using our novel viral mediated approach
2.	Channelrhodopsin driven excitation of LCeA CRF projections to the LBNST during sub-threshold fear conditioning coupled with CRFr1 antagonism in the LBNST.
3.	Optogenetic inhibition of LCeA → LBNST CRF projections with CRF supplementation in the LBNST.
4.	CRFr1 antagonism under strong and weak multi-trial contextual fear conditioning parameters.
5.	Optogenetic inhibition of LCeA → LBNST CRF projections during different context-shock pairings in multi-trial conditioning under strong and weak fear conditioning parameters.
6.	Measurement of corticosterone following training and testing with optogenetic inhibition of LCeA CRF cell bodies and LCeA → LBNST projections.
7.	Optogenetic inhibition of other LCeA CRF pathways coupled with measurement of other features of anxiogenesis (e.g., respiratory activity) during training and retention testing.
8.	LBNST CRFr1 calcium imaging following strong and weak fear conditioning and optogenetic inhibition of LCeA → LBNST CRF projections.

Table 5.1 Outline of critical future experiments needed to expand on the findings of my dissertation.

5.3 PTSD, CRF, and Memory – A New Approach for Preventative Medicine

While the previous section focused on future experiments that would tease apart the function of the LCeA → LBNST CRF pathway and LBNST CRFr1s during phasic and sustained contextual threats, it is important to place my findings and research goals into a more translationally-relevant "context." In Chapter 1, I mentioned that the primary focus of my research was to understand the neural basis of

fear. However, I also alluded to the fact that the findings of my dissertation may have a particular relevance to anxiety and trauma-related disorders. In particular, my dissertation findings are the prelude to future grants and experiments aimed at understanding the neural pathways and molecular substrates of post-traumatic stress disorder (PTSD). Most animal work has focused on trying to parallel the "stress-related" symptomology in PTSD. Relatively little work has focused on understanding how the acquisition and *consolidation* of the traumatic memory itself may be of critical importance for novel treatments.

Post-traumatic stress disorder (PTSD) is a debilitating disorder that affects almost 5-10% of adults in their lifetime (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995), despite the fact that almost 90% of people will experience a traumatic event in their life (Breslau et al., 1998). PTSD is characterized by a number of symptoms including flashbacks, hyperarousal, hypervigilance, rumination, and others (for the current DSM-V PCL-5 see (Weathers et al., 2013)). However, what may be at the core of PTSD is the formation of a traumatic memory following the experience of a highly aversive event (e.g., war, disaster, rape, etc.; for a nice review see (Elzinga & Bremner, 2002)). Beyond the psychological symptomology, individuals with PTSD also exhibit alterations in HPA axis activity (Yehuda, Golier, Halligan, Meaney, & Bierer, 2014), questionable changes in episodic memory (Isaac, Cushway, & Jones, 2006), and the dysregulation of glucocorticoid receptor factors (e.g., FKBP5 (Hauger et al., 2012)).

What is particularly interesting is that dysfunction of CRF systems in the brain may also contribute to PTSD (Risbrough & Stein, 2006). In fact, elevated levels of CRF in the cerebrospinal fluid have been found in individuals with PTSD (Bremner et al., 1997). In human populations, specific genetic predispositions (i.e., polymorphisms in CRFr1s - specifically rs242924)) have been associated with resilience against HPA-axis dysregulation following severe stress in childhood – that is, stressful experiences that occurred decades prior to assessment (Tyrka et al., 2009). In fact we, in collaboration with Mary Dozier's lab some years ago, had proposed to evaluate these specific CRFr1 polymorphisms in maltreated children. Hyper-excitation (or signaling) of CRF acting at CRFr1s is thought to be both maladaptive and possibly contribute to the changes seen with PTSD (Hauger, et al., 2012). This "hyperexcitability hypothesis" is very intriguing with regard to the focus of my dissertation and the newly developed CRF tool I have created.

While associative (e.g., fear incubation, fear extinction deficits, increased fear excitation, etc.) and non-associative (e.g., stress sensitization, habituation deficits, kindling, etc.) learning accounts have been considered for the development of PTSD (see Table 1 in (Lissek & van Meurs, 2014), changes in associative fear-learning are especially relevant (for a great review from Kerry Ressler's group see (Parsons & Ressler, 2013). It has been theorized (Rosen & Schulkin, 1998) and is quite possible that hyperexcitability within CRF systems induced during traumatic fear learning contribute to the future changes seen in PTSD. It is here, with PTSD, where I think my current and future work will be of particular importance. It is quite possible that

hyperexcitability in the $_{L}$CeA CRF → $_{L}$BNST CRFr1 pathway emerges during the formation of traumatic fear memories. In fact, the BNST does show hyperactivity in individuals with anxiety disorders (Yassa, Hazlett, Stark, & Hoehn-Saric, 2012). I believe understanding how dysregulation of the $_{L}$CeA CRF → $_{L}$BNST CRFr1 pathway occurs is a valuable focus. The first step towards this would be using newer approaches such as genetically encoded calcium indicators (GECIs) to visual this pathway during fear-learning and expression. Most importantly, these techniques in calcium imaging may provide the ability to visualize hyperexcitability in phenotypically distinct cell populations – a hypothesis that I am currently developing the tools to experimentally test.

5.4 Summary and Final Conclusions

My dissertation lays the foundation for future grants and experiments that will aim to evaluate how the $_{L}$CeA CRF → $_{L}$BNST CRFr1 pathway may function under different types of fear. This research may provide important insights into the underpinnings of disorders like PTSD and help focus on novel ways (e.g., genetically encoded biodegradable nanoparticles) to treat these disorders. That is, not years after the trauma has occurred, but during their formation (on the order of hours) and before the traumatic events take hold to produce hyperexcitability and psychological dysfunction. Rodent models have and will continue to be an integral component for this future work.

Research in the coming decades using newer techniques (experiments and techniques I will have the privilege of conducting and developing) will help to

evaluate if, in fact, the speculations and hypotheses I have asserted and started to test in my dissertation are true. As Rafael Yuste, the Columbia University neuroscientist, stated in his recent review titled *From the Neuron Doctrine to Neural Networks*, "…the history of neuroscience is the history of its methods" (Yuste, 2015). As newer methods and technologies are incorporated into examining neural function associated with vetted behavioral paradigms (such as fear conditioning), our understanding of normal and abnormal aberrations of fear will dramatically improve. I am excited and hopeful at the promise basic neuroscience research holds for treating psychological dysfunction in the coming century.

REFERENCES

Abel, T., & Lattal, K. M. (2001). Molecular mechanisms of memory acquisition, consolidation and retrieval. *Current opinion in neurobiology, 11*(2), 180-187.

Adhikari, A., Lerner, T. N., Finkelstein, J., Pak, S., Jennings, J. H., Davidson, T. J., . . . Ye, L. (2015). Basomedial amygdala mediates top-down control of anxiety and fear. *Nature*.

Adolphs, R. (2013). The biology of fear. *Current Biology, 23*(2), R79-R93.

Aggleton, E. J., Everitt, B. J., Cardinal, R. N., & Hall, J. (2000). The amygdala: a functional analysis.

Aguilera, G., & Liu, Y. (2012). The molecular physiology of CRH neurons. *Frontiers in neuroendocrinology, 33*(1), 67-84.

Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H., & Deisseroth, K. (2009). Temporally precise in vivo control of intracellular signalling. *Nature, 458*(7241), 1025-1029.

Alheid, G., De Olmos, J., & Beltramino, C. (1995). Amygdala and extended amygdala. *The rat nervous system, 2*, 495-578.

Antoniadis, E. A., & McDonald, R. J. (2001). Amygdala, hippocampus, and unconditioned fear. *Experimental Brain Research, 138*(2), 200-209.

Asok, A., Ayers, L. W., Awoyemi, B., Schulkin, J., & Rosen, J. B. (2013). Immediate early gene and neuropeptide expression following exposure to the predator

odor 2, 5-dihydro-2, 4, 5-trimethylthiazoline (TMT). *Behavioural brain research, 248*, 85-93.

Asok, A., Schreiber, W. B., Jablonski, S. A., Rosen, J. B., & Stanton, M. E. (2013). Egr-1 increases in the prefrontal cortex following training in the context preexposure facilitation effect (CPFE) paradigm. *Neurobiology of learning and memory, 106*, 145-153.

Ax, A. F. (1953). The Physiological Differentiation between Fear and Anger in Humans. *Psychosomatic Medicine, 15*(5), 433-442.

Baker, M. (2015). Antibody anarchy: A call to order. *Nature, 527*(7579), 545-551.

Bale, T. L., & Vale, W. W. (2004). CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol., 44*, 525-557.

Bayer, S. A. (1987). Neurogenetic and morphogenetic heterogeneity in the bed nucleus of the stria terminalis. *The Journal of comparative neurology, 265*(1), 47-64.

Beckerman, M. A., Van Kempen, T. A., Justice, N. J., Milner, T. A., & Glass, M. J. (2013). Corticotropin-releasing factor in the mouse central nucleus of the amygdala: ultrastructural distribution in NMDA-NR1 receptor subunit expressing neurons as well as projection neurons to the bed nucleus of the stria terminalis. *Experimental neurology, 239*, 120-132.

Bileviciute, I., Ahmed, M., Bergström, J., Ericsson-Dahlstrand, A., Kreicbergs, A., & Lundeberg, T. (1997). Expression of corticotropin-releasing factor in the peripheral nervous system of the rat. *Neuroreport, 8*(14), 3127-3130.

Blanchard, R. J., & Blanchard, D. C. (1969). Crouching as an index of fear. *Journal of comparative and physiological psychology, 67*(3), 370.

Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature, 361*(6407), 31-39.

Bolles, R. C. (1970). Species-specific defense reactions and avoidance learning. *Psychological review, 77*(1), 32.

Botta, P., Demmou, L., Kasugai, Y., Markovic, M., Xu, C., Fadok, J. P., . . . Cook, J. M. (2015). Regulating anxiety with extrasynaptic inhibition. *Nature neuroscience, 18*(10), 1493-1500.

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., & Kandel, E. R. (1998). Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learning & Memory, 5*(4), 365-374.

Bremner, J. D., Licinio, J., Darnell, A., Krystal, J. H., Owens, M. J., Southwick, S. M., . . . Charney, D. S. (1997). Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *American Journal of Psychiatry, 154*, 624-629.

Breslau, N., Kessler, R. C., Chilcoat, H. D., Schultz, L. R., Davis, G. C., & Andreski, P. (1998). Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. *Archives of general psychiatry, 55*(7), 626-632.

Campeau, S., Hayward, M. D., Hope, B. T., Rosen, J. B., Nestler, E. J., & Davis, M. (1991). Induction of the c-fos proto-oncogene in rat amygdala during unconditioned and conditioned fear. *Brain research, 565*(2), 349-352.

Canteras, N. S. (2002). The medial hypothalamic defensive system: hodological organization and functional implications. *Pharmacology Biochemistry and Behavior, 71*(3), 481-491.

Chow, B. Y., Han, X., & Boyden, E. S. (2012). Genetically encoded molecular tools for light-driven silencing of targeted neurons. *Progress in brain research, 196*, 49.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B., Letzkus, J. J., Vlachos, I., . . . Stadler, M. B. (2010). Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature, 468*(7321), 277-282.

Cordero, M. I., Merino, J. J., & Sandi, C. (1998). Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning. *Behavioral neuroscience, 112*(4), 885.

Cowansage, K. K., Shuman, T., Dillingham, B. C., Chang, A., Golshani, P., & Mayford, M. (2014). Direct reactivation of a coherent neocortical memory of context. *Neuron, 84*(2), 432-441.

Dabrowska, J., Hazra, R., Guo, J.-D., DeWitt, S., & Rainnie, D. G. (2013). Central CRF neurons are not created equal: phenotypic differences in CRF-containing

neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. *Frontiers in neuroscience, 7*.

Darwin, C. (1872). The expression of the emotions in man and animals. *London, UK: John Marry*.

Davis, M. (1992). The role of the amygdala in fear and anxiety. *Annual review of neuroscience, 15*(1), 353-375.

Davis, M., Walker, D. L., Miles, L., & Grillon, C. (2010). Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology, 35*(1), 105-135.

Day, H. E., Masini, C. V., & Campeau, S. (2004). The pattern of brain c-fos mRNA induced by a component of fox odor, 2, 5-dihydro-2, 4, 5-trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain research, 1025*(1), 139-151.

Deak, T., Nguyen, K. T., Ehrlich, A. L., Watkins, L. R., Spencer, R. L., Maier, S. F., . . . Webster, E. (1999). The Impact of the Nonpeptide Corticotropin-Releasing Hormone Antagonist Antalarmin on Behavioral and Endocrine Responses to Stress 1. *Endocrinology, 140*(1), 79-86.

Do-Monte, F. H., Quiñones-Laracuente, K., & Quirk, G. J. (2015). A temporal shift in the circuits mediating retrieval of fear memory. *Nature*.

Dong, H.-W., Petrovich, G. D., & Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Research Reviews, 38*(1), 192-246.

Dumont, É. C. (2009). What is the bed nucleus of the stria terminalis? *Progress in neuro-psychopharmacology & biological psychiatry, 33*(8), 1289.

Einarsson, E. Ö., & Nader, K. (2012). Involvement of the anterior cingulate cortex in formation, consolidation, and reconsolidation of recent and remote contextual fear memory. *Learning & Memory, 19*(10), 449-452.

Elzinga, B. M., & Bremner, J. D. (2002). Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? *Journal of affective disorders, 70*(1), 1-17.

Estes, W. K. (1950). Toward a statistical theory of learning. *Psychological review, 57*(2), 94.

Fanselow, M. S. (1980). Conditional and unconditional components of post-shock freezing. *The Pavlovian Journal of Biological Science: Official Journal of the Pavlovian, 15*(4), 177-182.

Fanselow, M. S. (1986). Associative vs topographical accounts of the immediate shock-freezing deficit in rats: implications for the response selection rules governing species-specific defensive reactions. *Learning and Motivation, 17*(1), 16-39.

Fanselow, M. S. (1990). Factors governing one-trial contextual conditioning. *Animal Learning & Behavior, 18*(3), 264-270.

Fanselow, M. S. (2000). Contextual fear, gestalt memories, and the hippocampus. *Behavioural brain research, 110*(1), 73-81.

Fanselow, M. S., Zelikowsky, M., Perusini, J., Barrera, V. R., & Hersman, S. (2014). Isomorphisms between psychological processes and neural mechanisms: From stimulus elements to genetic markers of activity. *Neurobiology of learning and memory, 108*, 5-13.

Felix-Ortiz, A. C., Beyeler, A., Seo, C., Leppla, C. A., Wildes, C. P., & Tye, K. M. (2013). BLA to vHPC inputs modulate anxiety-related behaviors. *Neuron, 79*(4), 658-664.

Fendt, M., Endres, T., & Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *The Journal of neuroscience, 23*(1), 23-28.

Forray, M. I., & Gysling, K. (2004). Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic–pituitary–adrenal axis. *Brain Research Reviews, 47*(1), 145-160.

Freud, S. (1895). Project for a scientific psychology.

Freud, S. (1920). A General Introduction to Psychoanalysis. *The Journal of Nervous and Mental Disease, 52*(6), 548-549.

Gafford, G., Jasnow, A. M., & Ressler, K. J. (2014). Grin1 receptor deletion within CRF neurons enhances fear memory.

Gafford, G. M., Guo, J.-D., Flandreau, E. I., Hazra, R., Rainnie, D. G., & Ressler, K. J. (2012). Cell-type specific deletion of GABA (A) α1 in corticotropin-releasing factor-containing neurons enhances anxiety and disrupts fear

extinction. *Proceedings of the National Academy of Sciences, 109*(40), 16330-16335.

Gafford, G. M., & Ressler, K. J. (2015). GABA and NMDA receptors in CRF neurons have opposing effects in fear acquisition and anxiety in central amygdala vs. bed nucleus of the stria terminalis. *Hormones and behavior*.

Gale, G. D., Anagnostaras, S. G., Godsil, B. P., Mitchell, S., Nozawa, T., Sage, J. R., . . . Fanselow, M. S. (2004). Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *The Journal of Neuroscience, 24*(15), 3810-3815.

Garfinkel, S. N., & Critchley, H. D. (2015). Threat and the Body: How the Heart Supports Fear Processing. *Trends in cognitive sciences*.

Goddard, G. V. (1964). Amygdaloid stimulation and learning in the rat. *Journal of comparative and physiological psychology, 58*(1), 23.

Goosens, K. A., & Maren, S. (2001). Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. *Learning & Memory, 8*(3), 148-155.

Grossman, S. P., Grossman, L., & Walsh, L. (1975). Functional organization of the rat amygdala with respect to avoidance behavior. *Journal of comparative and physiological psychology, 88*(2), 829.

Gungor, N. Z., Yamamoto, R., & Pare, D. (2015). Optogenetic study of the projections from the bed nucleus of the stria terminalis to the central amygdala. *Journal of Neurophysiology, 114*(5), 2903-2911.

Hammack, S. E., Todd, T. P., Kocho-Schellenberg, M., & Bouton, M. E. (2015). Role of the bed nucleus of the stria terminalis in the acquisition of contextual fear at long or short context-shock intervals. *Behavioral neuroscience, 129*(5), 673.

Hashikawa, K., Naka, M., Nakayama, D., Matsumoto, N., Neve, R., & Matsuki, N. (2013). Blockade of stimulus convergence in amygdala neurons disrupts taste associative learning. *The Journal of neuroscience, 33*(11), 4958-4963.

Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., . . . Callaway, E. M. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. *Nature, 468*(7321), 270-276.

Haufler, D., Nagy, F. Z., & Pare, D. (2013). Neuronal correlates of fear conditioning in the bed nucleus of the stria terminalis. *Learning & Memory, 20*(11), 633-641.

Hauger, R. L., Olivares-Reyes, J. A., Dautzenberg, F. M., Lohr, J. B., Braun, S., & Oakley, R. H. (2012). Molecular and cell signaling targets for PTSD pathophysiology and pharmacotherapy. *Neuropharmacology, 62*(2), 705-714.

Herman, J. P., & Cullinan, W. E. (1997). Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis. *Trends in neurosciences, 20*(2), 78-84.

Herman, J. P., Ostrander, M. M., Mueller, N. K., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 29*(8), 1201-1213.

Hernandez-Rabaza, V., Hontecillas-Prieto, L., Velazquez-Sanchez, C., Ferragud, A., Perez-Villaba, A., Arcusa, A., . . . Canales, J. (2008). The hippocampal dentate gyrus is essential for generating contextual memories of fear and drug-induced reward. *Neurobiology of learning and memory, 90*(3), 553-559.

Holland, P. C., & Bouton, M. E. (1999). Hippocampus and context in classical conditioning. *Current opinion in neurobiology, 9*(2), 195-202.

Hollenstein, K., de Graaf, C., Bortolato, A., Wang, M.-W., Marshall, F. H., & Stevens, R. C. (2014). Insights into the structure of class B GPCRs. *Trends in pharmacological sciences, 35*(1), 12-22.

Huff, M. L., Emmons, E. B., Narayanan, N. S., & LaLumiere, R. T. (2016). Basolateral amygdala projections to ventral hippocampus modulate the consolidation of footshock, but not contextual, learning in rats. *Learning & Memory, 23*(2), 51-60.

Huff, M. L., Miller, R. L., Deisseroth, K., Moorman, D. E., & LaLumiere, R. T. (2013). Posttraining optogenetic manipulations of basolateral amygdala activity modulate consolidation of inhibitory avoidance memory in rats. *Proceedings of the National Academy of Sciences, 110*(9), 3597-3602.

Isaac, C. L., Cushway, D., & Jones, G. V. (2006). Is posttraumatic stress disorder associated with specific deficits in episodic memory? *Clinical psychology review, 26*(8), 939-955.

Janak, P. H., & Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature, 517*(7534), 284-292.

Jankord, R., & Herman, J. P. (2008). Limbic regulation of Hypothalamo-Pituitary-Adrenocortical function during acute and chronic stress. *Annals of the New York Academy of Sciences, 1148*(1), 64-73.

Johansen, J. P., Cain, C. K., Ostroff, L. E., & LeDoux, J. E. (2011). Molecular mechanisms of fear learning and memory. *Cell, 147*(3), 509-524.

Josselyn, S. A. (2010). Continuing the search for the engram: examining the mechanism of fear memories. *Journal of psychiatry & neuroscience: JPN, 35*(4), 221.

Kalin, N. H., & Takahashi, L. K. (1990). Fear-motivated behavior induced by prior shock experience is mediated by corticotropin-releasing hormone systems. *Brain research, 509*(1), 80-84.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *science, 294*(5544), 1030-1038.

Kandel, E. R. (2005). *Psychiatry, psychoanalysis, and the new biology of mind*: American Psychiatric Pub.

Keene, C. S., & Bucci, D. J. (2008). Neurotoxic lesions of retrosplenial cortex disrupt signaled and unsignaled contextual fear conditioning. *Behavioral neuroscience, 122*(5), 1070.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Archives of general psychiatry, 62*(6), 593-602.

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of general psychiatry, 52*(12), 1048-1060.

Kim, J., Kwon, J.-T., Kim, H.-S., Josselyn, S. A., & Han, J.-H. (2014). Memory recall and modifications by activating neurons with elevated CREB. *Nature neuroscience, 17*(1), 65-72.

Kim, J. J., Rison, R. A., & Fanselow, M. S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short-and long-term contextual fear. *Behavioral neuroscience, 107*(6), 1093.

Kim, M., & Davis, M. (1993). Electrolytic lesions of the amygdala block acquisition and expression of fear-potentiated startle even with extensive training but do not prevent reacquisition. *Behavioral neuroscience, 107*(4), 580.

Kim, S.-Y., Adhikari, A., Lee, S. Y., Marshel, J. H., Kim, C. K., Mallory, C. S., . . . Lim, B. K. (2013). Diverging neural pathways assemble a behavioural state from separable features in anxiety. *Nature, 496*(7444), 219-223.

Klampfl, S. M., Brunton, P. J., Bayerl, D. S., & Bosch, O. J. (2015). CRF-R1 activation in the anterior-dorsal BNST induces maternal neglect in lactating rats via an HPA axis-independent central mechanism. *Psychoneuroendocrinology, 64*, 89-98.

Klüver, H., & Bucy, P. C. (1939). Preliminary analysis of functions of the temporal lobes in monkeys. *Archives of Neurology & Psychiatry, 42*(6), 979-1000. doi: 10.1001/archneurpsyc.1939.02270240017001

Korte, S. (2001). Corticosteroids in relation to fear, anxiety and psychopathology. *Neuroscience & Biobehavioral Reviews, 25*(2), 117-142.

Kovács, K. J. (2013). CRH: the link between hormonal-, metabolic-and behavioral responses to stress. *Journal of chemical neuroanatomy, 54*, 25-33.

Krasne, F. B., Cushman, J. D., & Fanselow, M. S. (2015). A Bayesian context fear learning algorithm/automaton. *Frontiers in Behavioral Neuroscience, 9*, 112.

Kwon, O.-B., Lee, J. H., Kim, H. J., Lee, S., Lee, S., Jeong, M.-J., . . . Chang, S. (2015). Dopamine Regulation of Amygdala Inhibitory Circuits for Expression of Learned Fear. *Neuron, 88*(2), 378-389.

LaBar, K. S., Gatenby, J. C., Gore, J. C., LeDoux, J. E., & Phelps, E. A. (1998). Human Amygdala Activation during Conditioned Fear Acquisition and Extinction: a Mixed-Trial fMRI Study. *Neuron, 20*(5), 937-945. doi: http://dx.doi.org/10.1016/S0896-6273(00)80475-4

Lanuza, E., Nader, K., & Ledoux, J. (2004). Unconditioned stimulus pathways to the amygdala: effects of posterior thalamic and cortical lesions on fear conditioning. *Neuroscience, 125*(2), 305-315.

Lebow, M., & Chen, A. (2016). Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Molecular Psychiatry*.

LeDoux, J. (2003). The emotional brain, fear, and the amygdala. *Cellular and molecular neurobiology, 23*(4-5), 727-738.

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *The Journal of neuroscience, 8*(7), 2517-2529.

Lee, M.-C., Yasuda, R., & Ehlers, M. D. (2010). Metaplasticity at single glutamatergic synapses. *Neuron, 66*(6), 859-870.

Lee, Y., & Davis, M. (1997). Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. *The Journal of neuroscience, 17*(16), 6434-6446.

Lehner, M., Taracha, E., Skórzewska, A., Turzyńska, D., Sobolewska, A., Maciejak, P., . . . Wisłowska-Stanek, A. (2008). Expression of c-Fos and CRF in the brains of rats differing in the strength of a fear response. *Behavioural brain research, 188*(1), 154-167.

Li, C.-I., Maglinao, T. L., & Takahashi, L. K. (2004). Medial amygdala modulation of predator odor-induced unconditioned fear in the rat. *Behavioral neuroscience, 118*(2), 324.

Li, H., Penzo, M. A., Taniguchi, H., Kopec, C. D., Huang, Z. J., & Li, B. (2013). Experience-dependent modification of a central amygdala fear circuit. *Nature neuroscience, 16*(3), 332-339.

Lissek, S., & van Meurs, B. (2014). Learning models of PTSD: Theoretical accounts and psychobiological evidence. *International journal of psychophysiology*.

Lovinger, D. M., & Kash, T. L. (2015). Mechanisms of Neuroplasticity and Ethanol's Effects on Plasticity in the Striatum and Bed Nucleus of the Stria Terminalis. *Alcohol Research, 31*(1), 109-124.

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews Neuroscience, 10*(6), 434-445.

Makino, S., Gold, P. W., & Schulkin, J. (1994a). 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 research, 640*(1), 105-112.

Makino, S., Gold, P. W., & Schulkin, J. (1994b). 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 research, 657*(1), 141-149.

Makino, S., Schulkin, J., Smith, M., Pacak, K., Palkovits, M., & Gold, P. (1995). Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and pituitary by glucocorticoids and stress. *Endocrinology, 136*(10), 4517-4525.

Malkani, S., Wallace, K. J., Donley, M. P., & Rosen, J. B. (2004). An egr-1 (zif268) antisense oligodeoxynucleotide infused into the amygdala disrupts fear conditioning. *Learning & Memory, 11*(5), 617-624.

Marcilhac, A., & Siaud, P. (1997). Identification of projections from the central nucleus of the amygdala to the paraventricular nucleus of the hypothalamus which are immunoreactive for corticotrophin-releasing hormone in the rat. *Experimental physiology, 82*(2), 273-281.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual review of neuroscience, 24*(1), 897-931.

Maren, S., & Fanselow, M. S. (1997). Electrolytic lesions of the fimbria/fornix, dorsal hippocampus, or entorhinal cortex produce anterograde deficits in contextual fear conditioning in rats. *Neurobiology of learning and memory, 67*(2), 142-149.

Marks, W., Fenton, E., Guskjolen, A., & Kalynchuk, L. (2015). The effect of chronic corticosterone on fear learning and memory depends on dose and the testing protocol. *Neuroscience, 289*, 324-333.

Matsuo, N. (2015). Irreplaceability of Neuronal Ensembles after Memory Allocation. *Cell reports, 11*(3), 351-357.

McGaugh, J. L. (2000). Memory--a century of consolidation. *science, 287*(5451), 248-251.

Murawski, N. J., & Asok, A. (2015). *Digital fear conditioning in rats: utilizing LCD-based visual context manipulations during conditioning*. Paper presented at the Society for Neuroscience Meeting Planner 355.20/CC37, Chicago, IL.

Nabavi, S., Fox, R., Proulx, C. D., Lin, J. Y., Tsien, R. Y., & Malinow, R. (2014). Engineering a memory with LTD and LTP. *Nature*.

Nagano, Y., Kaneda, K., Maruyama, C., Ide, S., Kato, F., & Minami, M. (2015). Corticotropin-releasing factor enhances inhibitory synaptic transmission to type III neurons in the bed nucleus of the stria terminalis. *Neuroscience letters, 600*, 56-61.

Namburi, P., Beyeler, A., Yorozu, S., Calhoon, G. G., Halbert, S. A., Wichmann, R., . . . Felix-Ortiz, A. C. (2015). A circuit mechanism for differentiating positive and negative associations. *Nature, 520*(7549), 675-678.

Nielsen, D. M., & Crnic, L. S. (2002a). Automated analysis of foot-shock sensitivity and concurrent freezing behavior in mice. *Journal of neuroscience methods, 115*(2), 199-209.

Nielsen, D. M., & Crnic, L. S. (2002b). Elevated plus maze behavior, auditory startle response, and shock sensitivity in predisease and in early stage autoimmune disease MRL/lpr mice. *Brain, behavior, and immunity, 16*(1), 46-61.

Nijsen, M. J., Croiset, G., Diamant, M., De Wied, D., & Wiegant, V. M. (2001). CRH signalling in the bed nucleus of the stria terminalis is involved in stress-induced cardiac vagal activation in conscious rats. *Neuropsychopharmacology, 24*(1), 1-10.

NIMH. (2013). Research Domain Criteria (RdOC): Negative Valence Systems Retrieved 1/8/2016, 2016, from http://www.nimh.nih.gov/research-priorities/rdoc/constructs/negative-valence-systems.shtml

O'Reilly, R. C., & Rudy, J. W. (2000). Computational principles of learning in the neocortex and hippocampus. *Hippocampus, 10*(4), 389-397.

Olschowka, J. A., O'Donohue, T. L., Mueller, G. P., & Jacobowitz, D. M. (1982). The distribution of corticotropin releasing factor-like immunoreactive neurons in rat brain. *Peptides, 3*(6), 995-1015.

Orsini, C. A., Kim, J. H., Knapska, E., & Maren, S. (2011). Hippocampal and prefrontal projections to the basal amygdala mediate contextual regulation of fear after extinction. *The Journal of neuroscience, 31*(47), 17269-17277.

Owens, M. J., & Nemeroff, C. B. (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev, 43*(4), 425-473.

Palkovits, M., Brownstein, M. J., & Vale, W. (1985). *Distribution of corticotropin-releasing factor in rat brain.* Paper presented at the Federation proceedings.

Pape, H.-C., & Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological reviews, 90*(2), 419-463.

Parsons, R. G., & Ressler, K. J. (2013). Implications of memory modulation for post-traumatic stress and fear disorders. *Nature neuroscience, 16*(2), 146-153.

Paxinos, G., & Watson, C. (1982). The rat brain in stereotaxic coordinates (2nd ed.). New York: Academic Press/Elsevier.

Paxinos, G., & Watson, C. (2007). The rat brain in stereotaxic coordinates (6th ed.). Boston: Academic Press.

Penzo, M. A., Robert, V., & Li, B. (2014). Fear conditioning potentiates synaptic transmission onto long-range projection neurons in the lateral subdivision of central amygdala. *The Journal of Neuroscience, 34*(7), 2432-2437.

Perrin, M., Donaldson, C., Chen, R., Blount, A., Berggren, T., Bilezikjian, L., . . . Vale, W. (1995). Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proceedings of the National Academy of Sciences, 92*(7), 2969-2973.

Perusini, J. N., & Fanselow, M. S. (2015). Neurobehavioral perspectives on the distinction between fear and anxiety. *Learning & Memory, 22*(9), 417-425.

Petrovich, G., & Swanson, L. (1997). Projections from the lateral part of the central amygdalar nucleus to the postulated fear conditioning circuit. *Brain research, 763*(2), 247-254.

Phillips, R., & LeDoux, J. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral neuroscience, 106*(2), 274.

Pitts, M. W., & Takahashi, L. K. (2011). The central amygdala nucleus via corticotropin-releasing factor is necessary for time-limited consolidation processing but not storage of contextual fear memory. *Neurobiology of learning and memory, 95*(1), 86-91.

Pitts, M. W., Todorovic, C., Blank, T., & Takahashi, L. K. (2009). The central nucleus of the amygdala and corticotropin-releasing factor: insights into contextual fear memory. *The Journal of neuroscience, 29*(22), 7379-7388.

Pomrenze, M. B., Maiya, R., Blasio, A., Hopf, F. W., Gordon, G., Dadgar, J., . . . Messing, R. O. (2015). *Central amygdala CRF neurons regulate both anxiety*

and fear behaviors in rats Paper presented at the Society for Neuroscience Meeting Planner 433.05/X28, Chicago, IL.

Pomrenze, M. B., Millan, E. Z., Hopf, F. W., Keiflin, R., Maiya, R., Blasio, A., . . . Crawford, E. (2015). A transgenic rat for investigating the anatomy and function of corticotrophin releasing factor circuits. *Frontiers in neuroscience, 9*, 487.

Ponnusamy, R., Poulos, A. M., & Fanselow, M. S. (2007). Amygdala-dependent and amygdala-independent pathways for contextual fear conditioning. *Neuroscience, 147*(4), 919-927.

Poulos, A. M., Ponnusamy, R., Dong, H.-W., & Fanselow, M. S. (2010). Compensation in the neural circuitry of fear conditioning awakens learning circuits in the bed nuclei of the stria terminalis. *Proceedings of the National Academy of Sciences, 107*(33), 14881-14886.

Poulos, A. M., Ponnusamy, R., Dong, H. W., & Fanselow, M. S. (2010). Compensation in the neural circuitry of fear conditioning awakens learning circuits in the bed nuclei of the stria terminalis. *Proc Natl Acad Sci U S A, 107*(33), 14881-14886. doi: 1005754107 [pii]

10.1073/pnas.1005754107

Prewitt, C. M., & Herman, J. P. (1998). Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. *Journal of chemical neuroanatomy, 15*(3), 173-186.

Pugh, C. R., Tremblay, D., Fleshner, M., & Rudy, J. W. (1997). A selective role for corticosterone in contextual-fear conditioning. *Behavioral neuroscience, 111*(3), 503.

Radke, A. K. (2009). The role of the bed nucleus of the stria terminalis in learning to fear. *The Journal of neuroscience, 29*(49), 15351-15352.

Radley, J. J., Gosselink, K. L., & Sawchenko, P. E. (2009). A discrete GABAergic relay mediates medial prefrontal cortical inhibition of the neuroendocrine stress response. *The Journal of neuroscience, 29*(22), 7330-7340.

Radulovic, J., Rühmann, A., Liepold, T., & Spiess, J. (1999). Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *The Journal of neuroscience, 19*(12), 5016-5025.

Radulovic, J., Sydow, S., & Spiess, J. (1998). Characterization of native corticotropin-releasing factor receptor type 1 (cRFR1) in the rat and mouse central nervous system. *Journal of neuroscience research, 54*(4), 507-521.

Rajasethupathy, P., Sankaran, S., Marshel, J. H., Kim, C. K., Ferenczi, E., Lee, S. Y., . . . Lo, M. (2015). Projections from neocortex mediate top-down control of memory retrieval. *Nature*.

Redondo, R. L., Kim, J., Arons, A. L., Ramirez, S., Liu, X., & Tonegawa, S. (2014). Bidirectional switch of the valence associated with a hippocampal contextual memory engram. *Nature*.

Regev, L., Tsoory, M., Gil, S., & Chen, A. (2012). Site-specific genetic manipulation of amygdala corticotropin-releasing factor reveals its imperative role in mediating behavioral response to challenge. *Biological psychiatry, 71*(4), 317-326.

Resstel, L., Alves, F., Reis, D., Crestani, C., Corrêa, F., & Guimaraes, F. (2008). Anxiolytic-like effects induced by acute reversible inactivation of the bed nucleus of stria terminalis. *Neuroscience, 154*(3), 869-876.

Risbrough, V. B., & Stein, M. B. (2006). Role of corticotropin releasing factor in anxiety disorders: a translational research perspective. *Hormones and behavior, 50*(4), 550-561.

Rodrigues, S. M., LeDoux, J. E., & Sapolsky, R. M. (2009). The influence of stress hormones on fear circuitry. *Annual review of neuroscience, 32*, 289-313.

Root, C. M., Denny, C. A., Hen, R., & Axel, R. (2014). The participation of cortical amygdala in innate, odour-driven behaviour. *Nature, 515*(7526), 269-273.

Roozendaal, B., Brunson, K. L., Holloway, B. L., McGaugh, J. L., & Baram, T. Z. (2002). Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. *Proceedings of the National Academy of Sciences, 99*(21), 13908-13913.

Rosen, J. B. (2004). The neurobiology of conditioned and unconditioned fear: a neurobehavioral system analysis of the amygdala. *Behavioral and Cognitive Neuroscience Reviews, 3*(1), 23-41.

Rosen, J. B., Asok, A., & Chakraborty, T. (2015). The smell of fear: innate threat of 2, 5-dihydro-2, 4, 5-trimethylthiazoline, a single molecule component of a predator odor. *Frontiers in Neuroscience, 9*, 292.

Rosen, J. B., Donley, M. P., Gray, D., West, E. A., Morgan, M. A., & Schulkin, J. (2008). Chronic corticosterone administration does not potentiate unconditioned freezing to the predator odor, trimethylthiazoline. *Behavioural brain research, 194*(1), 32-38.

Rosen, J. B., & Schulkin, J. (1998). From normal fear to pathological anxiety. *Psychological review, 105*(2), 325.

Rudy, J. W. (2009). Context representations, context functions, and the parahippocampal–hippocampal system. *Learning & Memory, 16*(10), 573-585.

Rudy, J. W., & O'Reilly, R. C. (2001). Conjunctive representations, the hippocampus, and contextual fear conditioning. *Cognitive, Affective, & Behavioral Neuroscience, 1*(1), 66-82.

Sakanaka, M., Shibasaki, T., & Lederis, K. (1986). Distribution and efferent projections of corticotropin-releasing factor-like immunoreactivity in the rat amygdaloid complex. *Brain research, 382*(2), 213-238.

Salzman, C. D., & Fusi, S. (2010). Emotion, cognition, and mental state representation in amygdala and prefrontal cortex. *Annual review of neuroscience, 33*, 173.

Sanghvi, R., Mogalian, E., Machatha, S. G., Narazaki, R., Karlage, K. L., Jain, P., . . . Yalkowsky, S. H. (2009). Preformulation and pharmacokinetic studies on

antalarmin: a novel stress inhibitor. *Journal of pharmaceutical sciences, 98*(1), 205-214.

Sapolsky, R. M. (2015). Stress and the brain: individual variability and the inverted-U. *Nature neuroscience, 18*(10), 1344-1346.

Schafe, G. E., Nadel, N. V., Sullivan, G. M., Harris, A., & LeDoux, J. E. (1999). Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase. *Learning & Memory, 6*(2), 97-110.

Schulkin, J., Morgan, M. A., & Rosen, J. B. (2005). A neuroendocrine mechanism for sustaining fear. *Trends in neurosciences, 28*(12), 629-635.

Selye, H. (1950). Stress and the general adaptation syndrome. *British medical journal, 1*(4667), 1383.

Seo, D.-o., & Monfils, M.-H. (2012). Linking Fear Learning to Memory Consolidation *Encyclopedia of the Sciences of Learning* (pp. 2057-2058): Springer.

Sharp, P. B., Miller, G. A., & Heller, W. (2015). Transdiagnostic dimensions of anxiety: Neural mechanisms, executive functions, and new directions. *International journal of psychophysiology, 98*(2), 365-377.

Skórzewska, A., Lehner, M., Wisłowska-Stanek, A., Krząścik, P., Ziemba, A., & Płaźnik, A. (2014). The effect of chronic administration of corticosterone on anxiety-and depression-like behavior and the expression of GABA-A receptor alpha-2 subunits in brain structures of low-and high-anxiety rats. *Hormones and behavior, 65*(1), 6-13.

Smagin, G. N., Heinrichs, S. C., & Dunn, A. J. (2001). The role of CRH in behavioral responses to stress. *Peptides, 22*(5), 713-724.

Sugar, J., Witter, M. P., van Strien, N. M., & Cappaert, N. L. (2011). The retrosplenial cortex: intrinsic connectivity and connections with the (para) hippocampal region in the rat. An interactive connectome. *Frontiers in neuroinformatics, 5*.

Sullivan, G., Apergis, J., Bush, D., Johnson, L. R., Hou, M., & Ledoux, J. (2004). Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus. *Neuroscience, 128*(1), 7-14.

Sun, N., & Cassell, M. D. (1993). Intrinsic GABAergic neurons in the rat central extended amygdala. *Journal of Comparative Neurology, 330*(3), 381-404.

Swanson, L. W., Sawchenko, P. E., Rivier, J., & Vale, W. W. (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology, 36*(3), 165-186.

Takahashi, L. K. (2001). Role of CRF 1 and CRF 2 receptors in fear and anxiety. *Neuroscience & Biobehavioral Reviews, 25*(7), 627-636.

Thompson, B. L., Erickson, K., Schulkin, J., & Rosen, J. B. (2004). Corticosterone facilitates retention of contextually conditioned fear and increases CRH mRNA expression in the amygdala. *Behavioural brain research, 149*(2), 209-215.

Tonegawa, S., Liu, X., Ramirez, S., & Redondo, R. (2015). Memory engram cells have come of age. *Neuron, 87*(5), 918-931.

Tovote, P., Fadok, J. P., & Lüthi, A. (2015). Neuronal circuits for fear and anxiety. *Nature Reviews Neuroscience, 16*(6), 317-331.

Tye, K. M., Prakash, R., Kim, S.-Y., Fenno, L. E., Grosenick, L., Zarabi, H., . . . Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature, 471*(7338), 358-362.

Tyrka, A. R., Price, L. H., Gelernter, J., Schepker, C., Anderson, G. M., & Carpenter, L. L. (2009). Interaction of childhood maltreatment with the corticotropin-releasing hormone receptor gene: effects on hypothalamic-pituitary-adrenal axis reactivity. *Biological psychiatry, 66*(7), 681-685.

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.

Van Diest, I., Bradley, M. M., Guerra, P., Van den Bergh, O., & Lang, P. J. (2009). Fear-conditioned respiration and its association to cardiac reactivity. *Biological psychology, 80*(2), 212-217.

Vicentini, J. E., Céspedes, I. C., Nascimento, J. O., Bittencourt, J. C., & Viana, M. B. (2014). CRF type 1 receptors of the medial amygdala modulate inhibitory avoidance responses in the elevated T-maze. *Hormones and behavior, 65*(3), 195-202.

Waddell, J., Bouton, M. E., & Falls, W. A. (2008). Central CRF receptor antagonist α-helical CRF9-41 blocks reinstatement of extinguished fear: The role of the bed nucleus of the stria terminalis. *Behavioral neuroscience, 122*(5), 1061.

Walker, D. L., & Davis, M. (1997). Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *The Journal of neuroscience, 17*(23), 9375-9383.

Walker, D. L., & Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Struct Funct, 213*(1-2), 29-42. doi: 10.1007/s00429-008-0183-3

Walker, D. L., & Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Structure and Function, 213*(1-2), 29-42.

Walker, D. L., Miles, L. A., & Davis, M. (2009). Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 33*(8), 1291-1308.

Walker, D. L., Toufexis, D. J., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European journal of pharmacology, 463*(1), 199-216.

Wallace, K. J., & Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behavioral neuroscience, 114*(5), 912.

Wallace, K. J., & Rosen, J. B. (2001). Neurotoxic lesions of the lateral nucleus of the amygdala decrease conditioned fear but not unconditioned fear of a predator

odor: comparison with electrolytic lesions. *The Journal of neuroscience, 21*(10), 3619-3627.

Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., . . . Luo, Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of Molecular Diagnostics, 14*(1), 22-29.

Wang, L., Goebel-Stengel, M., Stengel, A., Wu, S. V., Ohning, G., & Taché, Y. (2011). Comparison of CRF-immunoreactive neurons distribution in mouse and rat brains and selective induction of Fos in rat hypothalamic CRF neurons by abdominal surgery. *Brain research, 1415*, 34-46.

Weathers, F., Litz, B., Keane, T., Palmieri, P., Marx, B., & Schnurr, P. (2013). The PTSD checklist for DSM-5 (PCL-5). Scale available from the National Center for PTSD.

Wellman, L. L., Yang, L., Ambrozewicz, M. A., Machida, M., & Sanford, L. D. (2013). Basolateral amygdala and the regulation of fear-conditioned changes in sleep: role of corticotropin-releasing factor. *Sleep, 36*(4), 471-480.

Whalen, P. J., & Phelps, E. A. (2009). *The human amygdala*: Guilford Press.

Wilensky, A. E., Schafe, G. E., Kristensen, M. P., & LeDoux, J. E. (2006). Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. *The Journal of neuroscience, 26*(48), 12387-12396.

Wong, M., Licinio, J., Pasternak, K., & Gold, P. (1994). Localization of corticotropin-releasing hormone (CRH) receptor mRNA in adult rat brain by in situ hybridization histochemistry. *Endocrinology, 135*(5), 2275-2278.

Wood, S. C., & Anagnostaras, S. G. (2011). Interdependence of measures in Pavlovian conditioned freezing. *Neuroscience letters, 505*(2), 134-139.

Wootten, D., Christopoulos, A., & Sexton, P. M. (2013). Emerging paradigms in GPCR allostery: implications for drug discovery. *Nature Reviews Drug Discovery, 12*(8), 630-644.

Xu, W., & Südhof, T. C. (2013). A neural circuit for memory specificity and generalization. *science, 339*(6125), 1290-1295.

Yassa, M. A., Hazlett, R. L., Stark, C. E., & Hoehn-Saric, R. (2012). Functional MRI of the amygdala and bed nucleus of the stria terminalis during conditions of uncertainty in generalized anxiety disorder. *Journal of psychiatric research, 46*(8), 1045-1052.

Yehuda, R., Golier, J. A., Halligan, S. L., Meaney, M., & Bierer, L. M. (2014). The ACTH response to dexamethasone in PTSD. *American Journal of Psychiatry*.

Yiu, A. P., Mercaldo, V., Yan, C., Richards, B., Rashid, A. J., Hsiang, H.-L. L., . . . Kushner, S. A. (2014). Neurons are recruited to a memory trace based on relative neuronal excitability immediately before training. *Neuron, 83*(3), 722-735.

Yuste, R. (2015). From the neuron doctrine to neural networks. *Nature Reviews Neuroscience, 16*(8), 487-497.

Zelikowsky, M., Bissiere, S., & Fanselow, M. S. (2012). Contextual fear memories formed in the absence of the dorsal hippocampus decay across time. *The Journal of neuroscience, 32*(10), 3393-3397.

Zelikowsky, M., Bissiere, S., Hast, T. A., Bennett, R. Z., Abdipranoto, A., Vissel, B., & Fanselow, M. S. (2013). Prefrontal microcircuit underlies contextual learning after hippocampal loss. *Proceedings of the National Academy of Sciences, 110*(24), 9938-9943.

Zimmerman, J. M., & Maren, S. (2011). The bed nucleus of the stria terminalis is required for the expression of contextual but not auditory freezing in rats with basolateral amygdala lesions. *Neurobiology of learning and memory, 95*(2), 199-205.

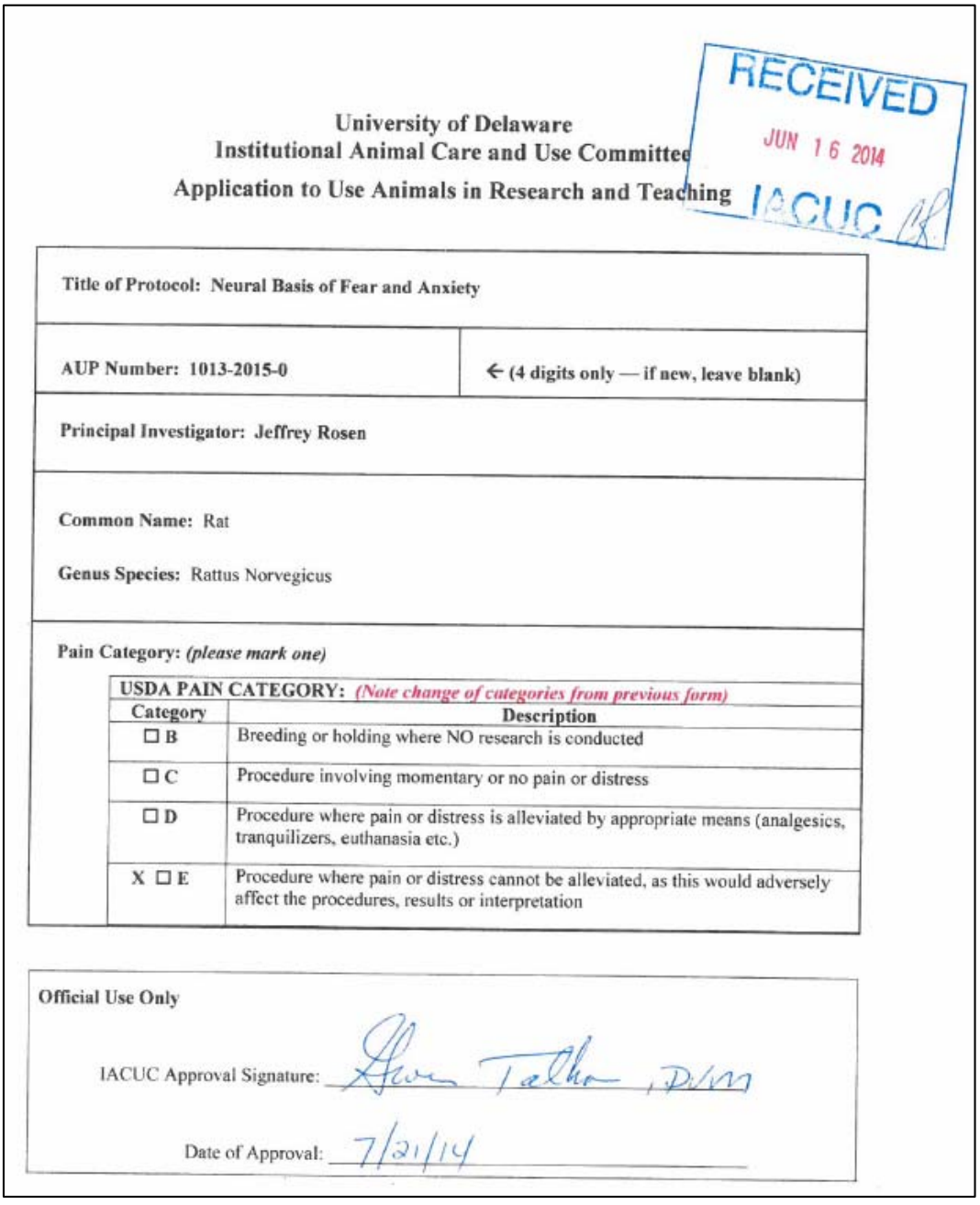
Zinebi, F., Xie, J., Liu, J., Russell, R. T., Gallagher, J. P., McKernan, M. G., & Shinnick-Gallagher, P. (2003). NMDA currents and receptor protein are downregulated in the amygdala during maintenance of fear memory. *The Journal of neuroscience, 23*(32), 10283-10291.

Zorrilla, E. P., Schulteis, G., Ormsby, A., Klaassen, A., Ling, N., McCarthy, J. R., . . . De Souza, E. B. (2002). Urocortin shares the memory modulating effects of corticotropin-releasing factor (CRF): mediation by CRF 1 receptors. *Brain research, 952*(2), 200-210.

Zorrilla, E. P., Valdez, G. R., Nozulak, J., Koob, G. F., & Markou, A. (2002). Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. *Brain research, 952*(2), 188-199.

Appendix A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS



RECEIVED
JUN 16 2014
IACUC

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

Title of Protocol: Neural Basis of Fear and Anxiety

AUP Number: 1013-2015-0 ← (4 digits only — if new, leave blank)

Principal Investigator: Jeffrey Rosen

Common Name: Rat

Genus Species: Rattus Norvegicus

Pain Category: *(please mark one)*

USDA PAIN CATEGORY: *(Note change of categories from previous form)*

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
☐ D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
X ☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glenn Tatha, DVM

Date of Approval: 7/21/14

RECEIVED
JAN 20 2015
IACUC JH

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

Title of Protocol: Neural basis of fear and anxiety	
AUP Number: 1013-2015-A	**← (4 digits only)**
Principal Investigator: Jeffrey Rosen	

Requested Changes

I am requesting a change to: ***(Check all that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☐ Animal Numbers *(Complete Section 2)*
- ☐ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

(Complete Section 7)

Changes MUST NOT be initiated until IACUC approval is granted

Official Use Only

IACUC Approval Signature: Gwen Tallman, DVM

Date of Approval: 2/9/15

Appendix B

PERMISSIONS

RightsLink | Copyright Clearance Center

Thank You For Your Order!

Dear Mr. Arun Asok,

Thank you for placing your order through Copyright Clearance Center's RightsLink service. Elsevier has partnered with RightsLink to license its content. This notice is a confirmation that your order was successful.

Your order details and publisher terms and conditions are available by clicking the link below:
http://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=29ca24b4-c0d8-40a4-965a-016b2c531573

Order Details
Licensee: Arun Asok
License Date: Mar 19, 2016
License Number: 3832551241912
Publication: Elsevier Books
Title: The Rat Brain in Stereotaxic Coordinates
Type Of Use: reuse in a thesis/dissertation
Total: 0.00 USD

To access your account, please visit https://myaccount.copyright.com.

Please note: Online payments are charged immediately after order confirmation; invoices are issued daily and are payable immediately upon receipt.

To ensure that we are continuously improving our services, please take a moment to complete our customer satisfaction survey.

B.1:v4.2

+1-855-239-3415 / Tel: +1-978-646-2777
customercare@copyright.com
http://www.copyright.com