The Impact of Repeated Stress on Neuropeptidergic Regulation of Monoamine Systems

Julia Cristine Lemos

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2012

Reading Committee:

Charles Chavkin, Co-Chair

Paul Phillips, Co-Chair

David Perkel

John Neumaier

Program Authorized to Offer Degree:

Neurobiology and Behavior

University of Washington

Abstract

The Impact of Repeated Stress on Neuropeptidergic Regulation of Monoamine Systems

Julia Cristine Lemos

Co-Chairs of Supervisory Committee:

Professor Charles Chavkin, Department of Pharmacology

Professor Paul Phillips, Department of Psychiatry and Behavioral Science

To promote an organism's evolutionary fitness, neurocircuitry has developed that encodes and responds to stressors. Stress motivates appropriate responding to environmental challenges through a diverse array of mechanisms that integrate cognitive, emotional and motor functions. Neuropeptides are released in an activity-dependent fashion throughout the brain in response to acute stressors and other arousing environmental stimuli and impinge on monoaminergic systems to allow processing of stimuli and produce appropriate behavioral responses. Here I show that corticotropin releasing factor (CRF) causes the release of dynorphin, the endogenous ligand for kappa opioid receptors, in limbic brain regions including the basolateral amygdala (BLA) and dorsal raphe nucleus (DRN) to produce a negative affective state. The DRN is the major serotonergic projection nucleus in the brain. We demonstrate that the dynorphin-KOR system has net inhibitory regulation of serotonergic DRN neuronal excitability. While CRF acting in some limbic regions produces negative affect, when CRF acts specifically in the nucleus accumbens, a subcortical region that is critical for interfacing cognitive, emotional and motor inputs, it promotes appetitive behaviors. I found that intra-nucleus accumbens infusions of CRF produce conditioned

place preference and promotes exploration of a novel stimulus. CRF does this by potentiating dopamine release within the nucleus accumbens through co-activation of CRF R1 and R2.

Severe or chronic stress produces an affective shift in responses to subsequent stressors from engagement to withdrawal, a hallmark symptom of Major Depressive Disorder. Stressful trauma may lead to this shift by amplifying the negative affective component of stress and ablating the motivation component. Repeated stress leads to several neuroadaptations, many of which target the functionality of stress-related neuropeptides themselves. I discovered that repeated swim stress dysregulates both CRF and dynorphin-KOR regulation of dopamine and serotonin systems respectively. Stress exposure causes a net reduction in KOR-mediated inhibitory regulation of serotonergic neuronal excitability through a p38α MAPK-dependent mechanism. While stress-induced alterations in KOR regulation of serotonin cells was relatively transient, stress-induced ablation of CRF's ability to potentiate dopamine release was remarkably long-lasting, not recovering for at least 90 days. The behavioral consequence of this long-term dysregulation of CRF-dopamine interactions was a switch in the subjective perception of CRF actions in the nucleus accumbens such that CRF was now experienced as aversive. This dysregulation of CRF signaling in the nucleus accumbens is dependent on stress-induced glucocorticoid activity. The following body of work characterizes how neuropeptides impinge on monoamine systems to produce affect and motivated behaviors in responses to arousing environmental stimuli. Importantly, offers biological substrates that underlie the etiology of stress-induced psychopathologies such as Major Depressive Disorder.

Table of Contents

List of Figures....iii

List of Tables.... v

Chapter 1: Introduction.... 1

Depression and Anxiety Disorders: Etiology and evolution.... 1

Early conceptual framework of Depression.... 2

Stress-induced Depression.... 4

Models of stress-induced depression.... 7

Molecules and systems that underlie stress and depression.... 10

The HPA axis.... 10

Norepinephrine, Serotonin and Dopamine.... 12

Stress-related neuropeptides.... 23

Chapter 2: CRF receptor activation engages the Dynorphin-KOR system to produce a negative affective state.... 38

Introduction.... 38

Methods.... 40

Results.... 42

Discussion.... 43

Chapter 3: Stress exposure produces a switch from appetitive to aversive signaling by CRF in the nucleus accumbens.... 49

Introduction.... 49

Results and Discussion.... 50

Methods.... 61

Chapter 4: Repeated stress dysregulates kappa opioid receptor signaling in the dorsal raphe through a p38 MAPK dependent mechanism.... 90

Introduction.... 90

Methods.... 92

Results.... 98

Discussion.... 106

Chapter 5: Bi-directional alteration of 5-HT1A autoreceptor function following different stress-exposure paradigms requires activation of the kappa opioid system.... 122

Introduction 122
Methods 123
Results 126
Discussion 128
Chapter 6: Dissertation Conclusions 133
References 145

List of Figures

Figure 2. 1. CRF-induced KORp-ir in limbic brain regions is blocked by norBNI pre-treatment. 45
Figure 2.2. CRF-induced KORp-ir is absent in animals lacking the gene for preprodynorphin (Dyn -/-). 46
Figure 2.3. CRF-induced KORp-ir can be prevented by blockade of either CRF R1 or CRF R2. 47

Figure 3.1.Cellular localization of CRF peptide, CRF R1 and CRF R2 in the nucleus accumbens. 67
Figure 3.2. CRF peptide is localized cholinergic interneurons throughout the nucleus accumbens. 68
Figure 3.3. CRF R1 co-localization to TH positive fibers. 69
Figure 3.4. CRF R2 antibody validation and localization in the nucleus accumbens of WT littermate and R2 KO mice. 70
Figure 3.5. CRF increases dopamine release in the nucleus accumbens through co-activation of CRF R1 and R2. 71
Figure 3.6. Evoked electrical currents detected by carbon fiber electrodes placed in the nucleus accumbens core are solely attributable to dopamine release. 72
Figure 3.7. Time course of CRF or vehicle effect on evoked dopamine release. 73
Figure 3.8. Cannula placements for naïve used in the conditioned place preference assay. 74
Figure 3.9. Intra-nucleus accumbens infusion of CRF produces a conditioned place preference. 75
Figure 3.10. Pre- and post-test times for CRF (5 ng) bilateral injections and CRF (500 ng) unilateral injections. 76
Figure 3.11. Intra-accumbens dopamine depletion with 6-OHDA blocks conditioned place preference for Intra-accumbens CRF microinfusion. 77
Figure 3.12. CRF is endogenously released in the nucleus accumbens to mediate exploration of a novel object. 78
Figure 3.13. Stress exposure abolishes the CRF mediated increase in evoked dopamine release without recovery for at least 90 days. 79
Figure 3.14. CRF is abolished following one- or two-day swim stress exposure. 80
Figure 3.15. Kappa opioid regulation of dopamine release in the nucleus accumbens is unaffected in mice exposed to swim stress. 81
Figure 3.16. Animals displayed enhanced depression-like behavior compared to naïve animals even up to 90 days following initial stressor exposure. 82
Figure 3.17. Basal evoked dopamine release was not affected by stress exposure. 83
Figure 3.18. Loss of CRF response following stress exposure is not age related. 84

Figure 3.19. Pre-treatment with glucocorticoid receptor antagonist mifepristone prior to swim stress session protects CRF response. 85
Figure 3.20. Stress exposure switches CRF action in the nucleus accumbens from appetitive to aversive in a conditioned place preference paradigm. 86
Figure 3.21. Pre- and post-test times for CRF conditioned place preference in mice exposed to two-day FSS and allowed to recover for 90 days. 87
Figure 3.22. Stress exposure abolishes CRF-dependent component of novel object exploration. 88

Figure 4.1. KOR activation by U69,593 depresses evoked glutamatergic EPSCs recorded in 5-HT neurons of the DRN. 112
Figure 4.2. U69,593 produces a norBNI-sensitive decrease in mEPSC frequency and amplitude. 113
Figure 4.3. KOR activation by U69,593 has no effect of evoked GABAergic IPSCs or mIPSCs. 114
Figure 4.4. KOR activation increases GIRK currents post-synaptically. 115
Figure 4.5. Repeated forced swim stress causes the release of dynorphin and KOR activation. 116
Figure 4.6. Repeated forced swim stress causes a reduction of KOR-activated GIRK current. 117
Figure 4.7. Repeated stress exposure does not alter KOR mediated depression of glutamatergic synaptic transmission. 118
Figure 4.8. p38MAPk mediates the stress-induced reduction in KOR-activated GIRK current. 119
Figure 4.9. Repeated stressor exposure produces a KOR-dependent increase in phosphorylation of the tyrosine 12 residue of KIR 3.1 in the DRN. 120
Figure 4.10. Excision of p38α from 5-HT neurons blocks stress-induced phosphorylation of GIRK, but not KOR. 121

Figure 5.1. Activation of 5-HT_{1A} by 5-CT produces a concentration dependent increase in GIRK current in 5-HT neurons of the DRN. 130
Figure 5.2. Two-day and seven-day stress exposure causes bi-directional adaptations to 5-HT_{1A} receptor function. 131
Figure 5.3. NorBNI pre-treatment prior to stress-exposure blocks stress-induced alterations in 5-HT1A function. 132

List of Tables

Table 2.1. Relative CRF induced KORp-ir across limbic brain regions. 48

Table 3.1. Quantification of EM labeling in 100-nm sections through the rostro-caudal axis of the nucleus accumbens to assess co-localization of CRF receptors and TH immunoreactivity. 89

Acknowledgements

I would like to thank Dr. Charles Chavkin and Dr. Paul Phillips for their outstanding mentorship and support. Charley, you have inspired me to always demand rigor from my data, to not just be descriptive, but mechanistic in my observations and fight for the experiments I believe in and get them funded. Paul, you have taught me to never undersell my data or myself, to be at the cusp of innovation and in fact, to lead the charge and to always put my initial scientific observations to task. I appreciate that both of you have always advocated for me throughout my graduate career. I would also like to thank my supervisory committee, especially my reading committee, for their helpful comments and advice. I have great admiration for the post-docs and grad students I have worked with over the years; I have learned so much from all of you. I would like to thank Dr. Michael Bruchas and Dr. Ben Land for not only being great scientific colleagues, but also great friends and making me laugh all day long. I would also like to thank the entire Chavkin and Phillips lab members past and present, especially those who contributed to my studies, for their scientific input and friendship. Thank you to my electrophysiology pals, Karina Leal, Marta Soden, Frank Kalume, John Meitzen and Sam Gale for their technical help and honest input, especially at the start of my graduate school career. Thank you to my former mentors at University of Pennsylvania for their encouragement to pursue this career. Finally, I would like to extend a particularly warm thank you to my friends and family for their support during the graduate school process.

Dedication

To the memory of

David Alan Stahly

1969-1994

Chapter 1: Introduction

Depression and Anxiety Disorders: Etiology and evolution

Anxiety-related (i.e. Generalized anxiety disorder, PTSD, phobias, OCD, panic disorders) and mood-related (i.e. Major Depressive Disorder, bipolar disorder, dysthymia) disorders have a life-time prevalence of 28.8% and 20.6% respectively[1]. As these disorders are often co-morbid, it is likely that approximately 35-40% of individuals will experience some anxiety or mood related disorder at some point in his or her life. Major Depressive Disorder (MDD) has the single largest lifetime prevalence (17%) of any mood or anxiety-related disease (21% of women and 13% of men) with an onset of 23-35 years of age[1]. Following initial diagnosis and treatment, 80% of patients will have a recurrence of the disease or experience “subthreshold” depressive symptoms (i.e. dysthymia), and 20-25% of patients will have a chronic unremitting course that requires continued intervention throughout their lifetime[2,3]. These statistical realities signal an urgency to better understand and treat MDD, especially since at present there are still some major pitfalls in both our current diagnosis and treatment of MDD. While diagnosis of neurological diseases has become increasingly more objective and quantitative, our diagnostic criteria for MDD (as well as other psychiatric diseases) still remains inherently subjective and qualitative being heavily reliant on self-report. For example, out the nine symptoms in the Diagnostic and Statistical Manual of Mental Disorders (DSM IV-TR)[4], the two principal symptoms are “depressed mood nearly every day for a period of two weeks” and “loss or diminished interest or pleasure.”

While pharmaceutical treatment has proved more efficacious than placebo, typical SSRIs for example, achieve remission in only approximately 33% of the patients[2]. Moreover, a more recent review and critique of clinical trials asserted that typical antidepressant treatment had only a 16% efficacy in patients with severe depression, 12% efficacy for moderate depression and had no efficacy for mild depression[5]. In contrast to neurological disorders, there is still a public stigmatization of mental disorders like MDD. In order to improve both diagnosis and treatment, there needs to be a deeper understanding of the biological basis of mood disorders. As neuroscientists interested in studying mood and anxiety disorders, we are charged with rooting psychological descriptions of these disorders in biology. To do this we have turned to animal models in which a mood disorder such as MDD has been highly compartmentalized such that one small component of the disease can specifically be linked to a biological adaptation or abnormality.

Early conceptual framework of Depression

Cognitive behavioral theory and therapy as well as pharmaceutical therapies have guided pre-clinical animal modeling of mood disorders. One of the most pervasive forms of non-pharmaceutical therapy for mood disorders is Cognitive-Behavioral Therapy (CBT) made famous by Aaron T. Beck (c.1963). Cognitive theory is rooted in four components of human interaction with the environment: cognition (perception of a stimulus), affect (fear, joy, euphoria, dysphoria), motivation (to approach/avoid the stimulus) and behavior (action taken in response to the stimulus) [6,7]. Dysfunction in any one of these systems, Beck believed, affected all other systems and was a principal cause of psychopathology. Beck described individuals with depression as having a

“shift to a negative perceptual bias” [8]. From its initial inception, Beck developed his cognitive theory further to describe schemas. Schemas are essentially internal categorizations of cues that inform a set of interpretations and actions that may first develop during early childhood.[8,9] If the schemas become negatively biased or rigid, Beck believed that could develop into mental pathology. Importantly, Beck posited that maladaptive schemas developed in childhood could lay dormant until activated by specific triggers (stressful life events)[8,9]. Cognitive therapy relies on the individual to recognize cues or triggers that lead to a depressive state and intercept them cognitively and behaviorally to break habitual patterns of stimulus and response.

Another major theory of depression in modern psychology is the theory of learned helplessness or hopelessness first proposed and studied by Martin Seligman[10]. The theory of learned helplessness posits that individuals exposed to inescapable aversive situations will learn that their behavioral responding does not produce any desired outcome. In essence, there is a learned dissociation between response and outcome such that in subsequent situations in which responding could influence outcome (i.e. escapable situations) the subject does not respond or becomes increasingly passive in coping with the environment stimulus. In other words, learned helplessness is characterized by an undermining of motivation to actively respond to stimuli in the environment (particularly negative or aversive stimuli)[11-13]. Our animal models of “depression-like” behavior either directly strive to mirror one of these theories (the most prominent being learned helplessness) or try to reduce these theories into individual components.

Perhaps most influential in guiding our modeling and hypotheses of mood disorders has been the clinical efficacy of antidepressants and antianxiety medications. Nearly every clinically efficacious drug for treating MDD targets one or multiple monoaminergic systems: serotonin, norepinephrine and dopamine systems; all of these antidepressants elevate extracellular levels of one or two of these neurochemicals by blocking their respective transporters or by inhibiting monoamine oxidase (MAO) [14-16]. Clinicians treating tuberculosis patients with iproniazid, a MAO inhibitor (which increases monoamine tone), found that patients reported elevated mood[17]. In contrast, in the 1950s, patients being given reserpine (which depletes monoamine stores) for hypertension reported depressed mood[17]. The efficacy of these pharmaceutical therapies has provided evidence that dysregulation of these three neurochemical systems is critical to the etiology of mood disorders. However, while both the pre-clinical and clinical communities both agree and recognize that these three monoaminergic systems are involved in the etiology of MDD, how they each contribute, what dysfunctions occur in the systems, how they occur and what the biological consequences are of elevating monoamine levels in the brain remains unclear. As such, the vast majority of pre-clinical focus on the etiology of MDD and other related mood disorders has focused on monoaminergic systems and has given rise to the "monoamine depletion" hypothesis of depression[14,18-22].

Stress-induced Depression

A core principle of all psychological theories of MDD and related mood disorders is that a particularly stressful life event or a stressful period of one's life pushes the individual into a chronic pathological state[8,23]. In the mid-1930s, Hans Selye, a

pioneering stress neuroendocrinologist, defined stress in the following manner: "*Stress* refers to a condition and *stressor* to the stimulus causing it. It covers a wide range of phenomena, from mild irritation to drastic dysfunction that may cause *severe health breakdown*." In the 1950s, Selye developed the idea of the "General Adaptation Syndrome" in which an organism undergoes three stages: "alarm reaction", "stage of resistance" and "stage of exhaustion". Selye asserted that the adaptive ability of the organism was finite[24,25]. Finally in the 1970s, Selye coined the terms eustress and distress that first defined the basic concepts of adaptive and pathological stress respectively[26-28]. McEwen and Sapolsky (1995) suggested that stress operated on an inverted U shaped curve, in particular, with regards to cognition. Their work clearly demonstrated that mild to moderate levels of stress positively enhance cognitive ability to a point; passed that point increasing levels of stress were correlated with poor cognitive performance [29]. Selye[26] and later McEwen and Sapolsky[29] eluded to this continuum of stress, to put it more simply as a range of "good" stress to "bad" stress. Korte and colleagues (2005) wrote "Indeed, the functional aspects of stress have been neglected too often"[30]. This is an important point because probably one of the most elusive questions with the largest number of inconsistent answers is: Where does "good" stress stop and "bad" stress begin? For example, are all neuroadaptations caused by even a single stress exposure pathological? Biologically, how do you distinguish "good" neuroadaptations (i.e. compensatory) from "bad" neuroadaptations (i.e. lead to vulnerability)?

Evolutionary biologists and behavioral ecologists have studied this question through the conceptual development and investigation of *allostasis* as a framework for

understanding stress-induced neuroadaptations [30]. While it is possibly true that MDD as a syndrome is uniquely human, elements of depression have evolved over time as a strategy for coping with periods of high environmental turmoil. *Allostasis* refers to the mechanism by which an organism maintains stability in a changing environment through physiological adaption (in other words, changing its own set point). The fundamental principle of allostasis suggests that not all stress-induced adaptions are pathological, in fact, most are adaptive [30,31]. The “Hawk-Dove” theory of coping strategies suggests that even within a species there are animals that cope with the environment using a “Hawk” strategy which includes active/pro-active engagement with the environment, “fight-flight” reaction to threat and greater risk taking. In contrast, a “Dove” strategy entails a passive withdrawal from the environment, “freeze-hide” reactions and thorough and cautious investigation of the world. Importantly these two different strategies can be observed within the same species as evidenced by experimental studies in rodent species. The social defeat stress model (see below) capitalizes on displays of aggression and submission from different individual mice or rats. Furthermore, individuals can switch their strategies in stressful situations from active to passive coping (i.e. Hawk to Dove). Interestingly, there are physiological correlates in animals displaying “dove” or passive coping strategies that are consistent with biomarkers of depression in humans (see below for further detail). One hypothesis of how depression-like behavior may have evolved is that repeated or severe stress may cause individuals to allostatically shift into a more permanent “dove” or “prey” mode within their environment. In contrast, individuals that have been continually reinforced when they displayed “Hawkish” strategies may engage in riskier behaviors. It is likely adaptive to

exploit both coping strategies in a context-dependent fashion. However, in higher order mammals, being forced into chronic “Dove” or “Hawk” mode may present as diagnosable pathologies such as depression or mania respectively.

Models of stress-induced depression

Below are descriptions and evaluations of four commonly used animal models of stress-induced depression-like behavior: learned helplessness (LH), forced swim stress (FSS), chronic mild stress (CMS) and social defeat stress (SDS). It should be noted that this is not a complete list of assays and particularly leaves out early life stress assays such as maternal separation and prenatal stress. These models have been developed and are continually assessed based on three dimensions of validity: predictive, face and construct validity.

Following Seligman’s initial development of the learned helplessness model in dogs and later rodents[10], Maier and Watkins extended this model greatly by adding yoked and unyoked conditions and in doing so illuminated the importance of stress predictability and controllability[32]. Here the animals are given inescapable foot shocks in rodent shuttle boxes. In this design, sometimes there are pairs of rodents that are yoked. One animal is given a shock that it can either avoid or escape, while the yoked counterpart receives shocks randomly based on the behavior displayed by the first subject. Assessment of learned helplessness is carried out after a certain period of time (e.g. 24 hours) and includes assessment of avoidance and escape behavior when given a “controllable” foot shock [11], with failure to display these behaviors as a marker of LH. In both straightforward inescapable shock (IS) as well as in the yoked animal condition there is evidence of depression-like behaviors and in fact alterations in behavior are

evident in contexts that are vastly different than that associated with the stress administration [13]. LH is sensitive to antidepressant treatment and thus has predictive validity.

Forced swim stress (FSS) is considered another type of learned helplessness. FSS was first developed by Porsolt (1977) and has since been modified in several ways[33]. Generally, rats or mice are placed in a large bucket of water without any possible method of escape for a period of time. Porsolt et al. (1977) observed that given a certain period of time animals transition from active escape behaviors (e.g. swimming, climbing) to passive coping behaviors (e.g. immobility). Moreover, after one or many exposures, the transition from active to passive coping behaviors is faster such that animals with a prior history of FSS are overall more immobile than naïve animals [34]. Additionally, animals that have been exposed to FSS also show depression-like behaviors in other tasks (e.g. hypophagia). This assay is the most commonly used model of stress-induced depression both in academic and industry settings largely due to the fact that it has a very strong predictive validity. That is, antidepressants have robust enhancement of active behaviors despite prior swim exposure[34,35]. Moreover, from a practical standpoint it is a relatively easy and inexpensive assay that can be conducted very quickly. A major weakness of this assay is that it is prone to false positives. Other agents that are not clinically used as antidepressants such as the stimulants like cocaine or caffeine can also enhance active behaviors and decrease immobility[35].

CMS is an experimental procedure in which an animal is subjected to mild, variable stress over a period of several days to weeks that consists of parameters such

as constant light, change of cage-mate and cage rotation. Following the end of the CMS protocol, depression-like behaviors are assessed using various endpoints including: Immobility in a forced swim test (FST), LH potentiation, sexual behavior, aggression, grooming and REM sleep[36,37]. The greatest strength of this assay is its face validity, meaning that it “looks like” the human experience at face value. Also, it provides a way of studying the effects of unpredictable stress, similar to that in the yoked foot shock experiments described above, yet without the same physical distress. Moreover, one can assess the effects of chronic antidepressant treatment in this assay[35-38]. The most prominent weakness in this assay is the variability in results between labs. Since all the stressors used are relatively mild stressors, one can imagine that this assay is more sensitive to extraneous environmental stressors or other influences. However, in a recent review, Willner describes a series of recent studies in which CMS produced similar effects on both behavioral and physiological endpoints across several different groups of researchers [37].

In the Social Defeat Stress (SDS) model, a smaller “intruder” male is placed in the home cage of an aggressive, larger resident male that has been conditioned to attack intruders (housed with receptive females) [39,40]. The intruder male will often display defensive postures. Sometimes experimenters will allow the intruder male to be continually attacked. In other situations, as soon as the intruder displays defensive posture, he is placed in a mesh enclosure within the resident home cage for a certain period of time. Using similar behavioral endpoints as described in the “CMS” section, depression-like behaviors can be assessed and are present following this protocol. The major strength of this assay is its clear face validity [39]. Moreover, the construction of

dominant and subordinate interactions is clearly ecologically relevant to rodent behavior. While rats and mice are often used, hamsters, voles and non-human primates could also be used in modified versions of this assay to assess the generality of these behavioral manipulations in producing depression across species. All of these models in addition to the ones not fully described have obvious advantages and disadvantages. In this body of work we chose to use one mode of stress exposure, forced swim stress, in one species to assess stress-induced neuroadaptations.

Molecules and systems that underlie stress and depression

The HPA axis

Activation of the hypothalamic-pituitary-adrenal (HPA) axis results in the release of corticosterone (rodents) or cortisol (primates) from the adrenal cortex [41]. HPA axis activation occurs in a pulsatile and circadian fashion, preceding periods of activity. Corticosterone or cortisol is also released in response to stressful or arousing stimuli including predator encounter, but also food anticipation [41-43]. Corticotropin-releasing factor (CRF; also known as corticotropin-releasing hormone) is a 41 amino acid neuropeptide [44] that is highly concentrated in parvocellular of the neurons paraventricular nucleus of the hypothalamus and in concert with arginine vasopressin (AVP) is released into the anterior pituitary to cause the release of adrenocorticotropic hormone (ACTH), which in turn causes the release of corticosterone/cortisol from the adrenal glands [41]. Corticosterone negatively feeds back on the brain by crossing the blood-brain barrier, activating glucocorticoid receptors, which inhibits hormone secretion from the hypothalamus and pituitary gland, reduces CRF and AVP secretion and reduces the cleavage of proopiomelanocortin (POMC) into ACTH and β-endorphin,

effectively returning the HPA system back to homeostatic levels. Glucocorticoids can have extra-hypothalamic actions as well. Sapolsky and McEwen have shown that the actions of corticosterone in the brain lie on an inverted U shaped curve where increasing levels of corticosterone can enhance cognitive performance, primarily through its actions in the hippocampus. However, accumulating or chronically elevated levels of corticosterone can lead to hippocampal/pyramidal cell atrophy and inhibition of neurogenesis [29,45-47].

Dysregulation of the HPA axis is now recognized as a biomarker of depressive disorders and may be specifically responsible for the cognitive impairments typical in depression. In animal models of stress-induced psychopathology, HPA axis responsivity is either exaggerated or prolonged and in some models of chronic stress, glucocorticoid negative feedback is disrupted[48 45,49-54]. The effects of glucocorticoid receptor (GRs) activation in the brain have been investigated for several decades; however, the molecular actions of glucocorticoids, particularly during chronic stress exposure, remain poorly understood. Upon binding corticosterone, GRs dimerize, then translocate to the nucleus and directly bind to DNA at sequences called glucocorticoid response elements producing transcriptional changes in protein expression. GRs can also have protein-protein interactions with other transcription factors that can also lead to *de novo* transcriptional changes[55,56]. In fact the majority of GR's repressive actions come from these protein-protein interactions rather than direct DNA binding. It has been demonstrated that one way in which GR-chaperone complexes exert their repressive actions is by blocking the ability of other transcription factors, notably factors such as NF-κB, from having their transcriptional effects [56]. GRs can also have non-genomic

actions that are independent of transcription. For example, GRs can reduce the production of arachidonic acid by inhibiting the EGF signaling pathway[56]. This diversity in downstream signaling mechanisms positions the glucocorticoid receptor complex in a way in which it can cause vast and long-term adaptations in response to chronic stressor exposure.

Norepinephrine, Serotonin and Dopamine

While perhaps an oversimplification of our current understanding of the disparate roles of these three transmitters, generally it is thought that changes in tone and release properties of these three transmitters translate to bi-directional changes in arousal, affect and motivation respectively. Importantly, it is the disruption in homeostatic balance of any or all these three monoaminergic neurotransmitters in either direction that has been associated with depression and anxiety.

Norepinephrine

Norepinephrine and epinephrine (also known as noradrenaline and adrenaline respectively) have been studied extensively regarding their role in stress-responding and are thought to act as acute first responders to cause immediate behavioral action and heighten cognitive arousal and attention[57-62]. Norepinephrine (NE) has two receptor classes, α- and β-receptors and is released peripherally in response to acute stressors as part of the "fight or flight" response to cause vasoconstriction and increased heart rate primarily through β-receptor coupling to calcium channels[63-66]. Centrally NE is released throughout the forebrain from two major NE containing nuclei located in the hindbrain, the locus coeruleus (LC) and nucleus tractus solitarius (NTS).

These nuclei are typically identified neuroantomically as being positive for tyrosine hydroxylase, the enzyme responsible for converting tyrosine to DOPA (also used a marker for dopamine). Generally the LC is thought to have a broad noradrenergic innervation throughout the forebrain, particularly to the hippocampus and prefrontal cortex [67-69]. Aston-Jones and colleagues described two distinct types of electrophysiological modes of LC neurons; one in which the tonic activity is shifted to a burst-pause pattern that focuses attention and one in which there is low level sustained tonic activity that is hypothesized to enable scanning of the environment and flexibility [70]. It is therefore thought that acute elevation in norepinephrine throughout both the central and peripheral nervous is critical for acute responding to acute stressors. However, in pathological states such as depression there appears to be a hypo-arousal that may be due to basally reduced levels of synaptic norepinephrine. Indeed, there are disparate pharmacological treatments for acute anxiety and depression that target the noradrenergic system. Patients are often prescribed β-blockers ($\beta_{1,2,3}$-adrenergic receptor antagonist) for acute anxiety; however, the first generation of tricyclic antidepressants (i.e. desipramine, imipramine) inhibited the noradrenergic (as well as serotoninergic and dopaminergic) transporter to effectively elevate noradrengeric tone in the brain [19,65,66]. Moreover, some of the newer antidepressant compounds, including venlafaxine and duloxetine are serotonin-norepinephrine reuptake inhibitors. This dichotomy in noradrenergic tone in instances of acute stress compared to a depression-like state presumably brought on by chronic or severe stress is a feature also shared by the serotonin and dopamine systems and in all three cases is poorly understood in terms of mechanism.

Serotonin

Serotonergic innervation originates from the B1-B9 areas of the midbrain, with the largest number of serotonergic projections to the forebrain originating from the dorsal and median raphe nuclei. These areas are typically identified neuroantomically for being positive for tryptophan hydroxylase, the enzyme that converts tryptophan to 5-hydroxytryptophan (5-HTP), which then gets converted to serotonin otherwise known as 5-hydroxytryptamine (5-HT). Understanding the actions of serotonin in the brain under normal conditions has been challenging due to the ever-growing number of cloned receptors and wide distribution of serotonergic innervation. Unlike both catecholaminergic systems, the serotonin system has 14 cloned receptors: 5-$HT_{1A,B,D,E,F}$ (coupled to $G_{i\alpha}$ class of G-protein), 5-$HT_{2A,B,C}$ (G_q), 5-HT_3 (non-selective cation channel), 5-HT_4 (G_s), 5-$HT_{5A,B}$ (G_s), 5-HT_6 (G_s), 5-HT_7 (G_s)[71]. Of the monoamines, the serotonergic system has arguably the most ubiquitous innervation in the central nervous system and has receptors and/or transporters on virtually every cell type within the brain (i.e. neurons, astrocytes, microglia, myelin)[71]. As such, serotonin has been demonstrated to play a role in affective state, cognition, circadian rhythm/sleep-wake cycle, temperature regulation, appetite, gut motility, blood pressure, cerebrovascular perfusion, nociception, sexual behavior and reproduction, social aggression, sensorimotor gating and responses to stress [72,73].

There are two key questions that remain elusive with regards to the serotonin system:

1. What is the function of serotonin in mediating behavioral actions?

2. What are the cellular and molecular mechanisms that underlie chronically lower serotonin levels in depressed patients?

There have been two leading theories of serotonin's function in motivated behaviors that have emerged over the last few decades. First is the hypothesis that reduction in serotonin tone/release below normal levels enhances the perception of threat or punishment and promotes a negative affective state or avoidance behavior[72,74]. This hypothesis has been supported experimentally through serotonin depletion experiments in rats, monkeys and humans[18,20] and is also supported by clinical evidence that enhancement of serotonin through selective serotonin reuptake inhibitors (SSRIs) ameliorates depression [15,72]. The second theory, also supported by preclinical evidence, posits that there is a direct relationship between serotonin levels and behavioral or response inhibition (i.e. increase in serotonin would cause an increase in behavioral inhibition) [73]. These two processes could translate to the relative impulsivity of the individual and how much the individual weights certain operant costs. Serotonin depletion in the forebrain produces an increase in premature actions (impulsivity) as well as greater delay discounting (enhanced perception of punishment)[75,76]. It is likely that serotonin has a role in both behavioral inhibition and negative affect. However it is also possible that these two processes of negative affect encoding and response inhibition are supported by serotonin acting in disparate regions. For example, acute stress exposure causes elevation in serotonin in the dorsolateral striatum, a region involved in action selection and habit formation (behavioral inhibition) and simultaneously decreases serotonin in the lateral septum and the amygdala, regions involved in negative affect and fear learning (aversive learning) [77]. Importantly, dysregulation of either or both of these behavioral processes can lead to stress

vulnerability and depression by producing behavioral hyperresponsivity/enhanced passive coping and enhanced perception of punishment or negativity.

The role of serotonin in depression was serendipitously discovered when patients with “unrelated” chronic diseases such as hypertension were treated with agents that manipulated monoamine stores and levels. Since then, the efficacy of second and third generation SSRIs in alleviating symptoms of depression has provided strong evidence that somehow the serotonin system is critically involved in the etiology of depression. Throughout the 70s and 80s it was shown that depressed patients had lower plasma tryptophan levels, lower CSF 5-HTIAA (5-HT metabolite) and decreased platelet 5-HT uptake[78]. It has been demonstrated that at least a subset of depressed/suicidal patients (~40%) had low serotonin content in post-mortem tissue and that tryptophan depletion can precipitate depression in normal adults and cause rapid recurrance in depressed patients being treated with SSRIs[14,15,17,18,20,79-81].

Importantly, both the studies conducted on serotonin content in depressed patients as well as the efficacy of SSRIs do not provide concrete proof of a causal relationship (as opposed to compensatory) between serotonin tone and depression. The studies examining serotonin and tryptophan content only provide a correlation with depression. Furthermore, an important caveat of SSRIs is that they take approximately 28 days to take effect despite immediate elevation of serotonin, suggesting that chronic elevation of serotonin is necessary and may rely on distal as well as proximal signaling effects. It has been posited that the ameliorative effects of SSRIs come from their ability to stimulate neurogenesis in the hippocampus [82,83]; this treatment may compensate for glucocorticoid hippocampal atrophy described by Sapolsky, McEwen

and others[29,45]. Indeed, while inhibiting adult neurogenesis in the hippocampus is not sufficient to cause a depression-like phenotype, it does seem to be required, in part, for achieving maximal efficacy of SSRIs[82,83]. Nevertheless, it seems plausible that chronically lower levels of 5-HT in the forebrain may lead to increased susceptibility to stress insult.

As such, a large amount of research effort has focused on mechanisms underlying the depression in 5-HT tone and release in depressed patients. Specifically, research has focused on 5-HT neuronal excitability and firing, 5-HT clearance and turnover by the 5-HT transporter and TPH2 production. (There are two isoforms of TPH, 1 and 2, TPH2 is the isoform that is most strongly expressed in the brain.) There is significant evidence to suggest that the reduction is serotonin levels may be due to an increased number of 5-HT_{1A} autoreceptors at the cell membrane of 5-HT containing neurons and therefore, increased suppression of tonic firing of 5-HT neurons. In both depressed patients and animal models of depression, there is evidence that 5-HT_{1A} autoreceptors, but not post-synaptic 5-HT_{1A} heteroreceptors are upregulated [84-86]. Moreover, the C(−1019)G (rs6295) promoter polymorphism in the 5-HT_{1A} receptor that confers increased binding potential is associated with higher vulnerability to depression and anxiety [87]. In a transgenic mouse model of this polymorphism in which animals had high or low expression of 5-HT_{1A} autoreceptors, with unaltered heteroreceptors, the investigators demonstrated that high 5-HT_{1A} autoreceptor activity conferred increased depression-like behavior, and the transgenic mice were also less responsive to antidepressant treatment. While the role of the 5-HT_{1A} autoreceptor in mood regulation is compelling, it has also been demonstrated that 5-HT neurons in the dorsal raphe of

stress-hyperresponsive Wistar Kyoto rats have a significantly hyperpolarized basal resting membrane potential and are therefore, less excitable[88]. These animals also have significantly reduced TPH2 present within the dorsal raphe consistent with what has been shown in clinically depressed humans [88]. For obvious reasons, experimenters have focused on 5-HT transporter function (5-HTT) in depressed patients. Notably, there was evidence to suggest that variations in a polymorphic 44 base pair insertion/deletion region of the *SLC6A4* gene that encodes 5-HTT allele, specifically the short homozygous form of the transporter, can lead to anxiety-related behaviors and susceptibility to depression [89]. Interestingly, a recent study demonstrated that this transporter polymorphism was significantly predictive of stress perception and depressed mood in women, but not for men [90].

An important question that remains is what exactly causes the transition from normal serotonin functioning to a pathological state in which multiple loci of serotonergic regulation are altered? It is generally accepted that given a certain level of intrinsic vulnerability of an individual, the individual must go through some traumatic or chronic life event that precipitates pathological neuroadaptations, but what are the biological mechanisms at work during these life events? Moreover, of the many neuroadaptations that occur during periods of chronic or traumatic stress, which adaptations are causal and which are compensatory?

Dopamine

The dopamine system has recently been given more attention for its potential role in mood disorders[91] and has been a more recent target of antidepressant pharmaceutical treatment. Since the late 90s, learning theory, economics and neuroscience have

converged on the dopamine system. In terms of neuroeconomic theory, it has become the single most referenced neurochemical system in the brain[92]. The dopaminergic system is quite different from the noradrenergic and serotonergic systems in that its projections to the forebrain are relatively restricted to a few target regions. Dopamine containing neurons are located in A9 and 10 of the midbrain in the ventral tegmental area (VTA) and substantia nigra *pars compacta* (SNc). The densest dopaminergic innervation in the brain is in the ventral and dorsal striatum of the basal ganglia. Dopaminergic neurons also innervate the prefrontal cortex, basolateral amygdala, hippocampus and hypothalamus, with some reciprocal connections with dorsal raphe. The dopamine system is also a tractable system to study since, similarly to the noradrenergic system, but to a greater extent, it has only two classes of receptors, D1-like receptors (D1 and D5, G_s coupled) and D2-like receptors (D2,D3 and D4, G_i-coupled).

In the late 70s, the pioneering work of Roy Wise and others was the first to suggest that dopamine was critical in reward learning. A seminal study demonstrated that the dopamine blocking agent pimozide prevented operant lever pressing for amphetamine, which was not blocked by norepinephrine blocking agents [93]. This was followed by a subsequent study in 1978 that demonstrated that neuroleptics blocked the rewarding qualities of food[94]. These two studies spawned the next three decades of work on the role of the dopamine system in behavior, much of which supported the concept that dopamine was somehow involved in reward learning. In doing so, they also sparked a lot of controversy and competing theories regarding the exact role of dopamine in reward learning. Initially Wise and others posited that dopamine caused

an increase in hedonic state and "euphoria-like" sensation[95]. This assertion was supported by some of the early work done on cocaine and opiate self-administration and intracranial self-stimulation (ICSS)[96]. Cocaine, though a non-selective monoamine transporter blocker (and local anesthetic), robustly enhances dopamine levels systemically, and animals will perform operant tasks to receive cocaine infusions. Likewise, during ICSS, a stimulating electrode is targeted at the medial forebrain bundle, the dopaminergic fiber tract from the midbrain to the striatum, and an animal will robustly press a lever for stimulation [96].

Since these initial observations, several other theories have been posited that have moved away from the idea that dopamine is a hedonic signal *per se*, but rather, suggest that dopamine is critical for learning about reward value, approaching reward and obtaining reward. One theory that has received a huge amount of attention since the seminal publication by Wolfram Schulz, Peter Dayan and P. Reed Montague is that dopamine acts as a prediction error signal [97], a component of both the Rescorla-Wagner model of associative learning and the temporal difference reinforcement model of instrumental learning. Schulz and colleagues (1997) observed during a basic Pavlovian task, presentation of a positive unconditioned stimulus (US) (fruit juice) to a non-human primate elicited an elevation in VTA dopamine cell firing that was time-locked to the presentation of the reward. When the US was paired with a conditioned stimulus (CS), two things happened. First, once the reward was expected or fully predicted, VTA neurons no longer elevated their firing to the presentation of the reward. Additionally, the value of the reward had transferred to the CS, such that dopamine cells would now fire to presentation of the CS. If a reward was given that was greater than predicted or

unexpected (i.e. given at another time interval), that would cause greater firing of dopamine cells consistent with a positive prediction error. In contrast, when a reward was predicted, but not delivered there was a suppression of firing rate indicative of a negative prediction rate [97]. These findings were also supported by more recent work using fast scan cyclic voltammetry with chronic microsensors, which demonstrated that dopamine release in the nucleus accumbens followed in similar pattern to changes in dopamine firing rate during Pavlovian conditioning described by Schultz[98]. In addition, it has been demonstrated that, during Pavlovian conditioning, both firing rates and subsequent dopamine release, scale with increasing reward value[99,100].

Paralleling this work the work of Schulz and others was the work of Salamone and colleagues that showed that dopamine not only encodes reward value, but it is also necessary for increasing effort associated with obtaining a reward[101]. In other words, elevation in dopamine allows individuals to overcome cost to obtain better rewards[101]. Therefore, if dopamine scales with both increasing cost and increasing value, one could surmise that dopamine acts to facilitate an individual working harder for greater reward[102]. Interestingly, dopamine does not appear to be particularly sensitive to temporal costs (i.e. delays prior to rewards). Again, using fast scan cyclic voltammetry in an operant conditioning task, it was shown that more dopamine is released when a reward requires an increasing amount of effort [99,103-105]. However, importantly, when presented the option of a high-cost or low-cost option to receive a fixed reward, there is more dopamine released to the low-cost option under some circumstances; this only occurred when contingencies were novel[99]. These data suggest that the encoding of

predicted outcome by dopamine is more complicated than the expected value, cost or even net utility (value - cost).

The subjective value or utility of an action or state can be modulated by the internal state of the individual. Internal states such as stress level or relative satiety can shift the utility curve. It is at this point where neuroendocrine systems tightly modulate the dopamine system to shift behavior. For example, it has been shown that both acute stress and glucocorticoid activity can increase dopamine levels in the striatum. Increases in the activity of dopamine neurons as well as an elevation in tone parallels the circadian activity of glucocorticoids as well as the rise in glucocorticoid levels immediately prior to food intake[104] and also occurs during acute stress exposure in a glucocorticoid-sensitive fashion. It has been shown that rats that demonstrate higher responses to novelty show greater changes in glucocorticoid dependent dopamine release[105].

From a neuroethological perspective one challenge has been to connect the work done with operant and Pavlovian conditioning in an artificial environment with how the rodent deals with a far more complex environmental landscape, and moreover, what dopamine does in “the real world.” One could imagine that within a complex environmental landscape there is one basic tenet, “everything you do should ensure your reproductive success,” meaning you need sufficient nutrition, sufficient mate acquisition and not to get eaten by predators. To do this with any success, particularly as a prey species, there should be a constant analysis of costs and benefits that can be shifted in times of high predation or poor food resources. However, it is necessary for the individual to explore and engage in its environment in order to acquire food or mates

despite some level of risk. Therefore, in line with the ideas of Salamone and colleagues, perhaps a critical neuroethological role of dopamine is to overcome certain costs or even more specifically certain fears in order to effectively engage in the environment[102].

What is particularly useful about the constructs that have been imposed on the dopamine system is that they offer more tractable predictions that can be experimentally tested compared to the serotonin and norepinephrine system. Indeed, for both catecholaminergic systems, despite their complexity, there is still a more simplified set of rules that can be ascribed to the behavior of the system upon presentation of and interactions with certain environmental stimuli. One disadvantage of the serotonin system is that its inherent complexity causes there to be an increasing number of exceptions to the predictions made by the two prevailing theories outlined above.

Stress-related neuropeptides

Neuropeptides are proteins that are stored in dense core vesicles and are released in an activity-dependent manner to act as modulatory transmitters throughout the brain. There are a large number of neuropeptides present both in the hypothalamus as part of the neuroendocrine system, but also present in every cognitive and limbic brain structure. However, study of neuropeptides is particularly difficult, primarily due to the lack of spatial and temporal resolution currently available in measuring their release. There are electrophysiological, electrochemical and microdialysis methods that can measure the release of small molecule neurotransmitters both directly and indirectly. There are far fewer instances in which endogenous neuropeptide release has been measured and most of these studies have been through indirect proxies of neuropeptide

release. There are even fewer instances in which the peptide itself has been measured through a microdialysis probe. Despite its lack of temporal resolution, direct measurements of extracellular outflow of neuropeptide have been carried out, but have presented challenges due to the size of the molecule. Indeed the release of larger neuropeptides such as CRF (with a total of 41 amino acids) have been especially challenging to measure due to technical constraints. Because of this, the vast majority of research on neuropeptides as neurotransmitters have relied on exogenous application of the peptide, since in this kind of experimental manipulation the experimenter can artificially control the temporal and spatial resolution of the actions of the neuropeptide.

Neuropeptide release

Neurotransmission of neuropeptides is remarkably different compared to the release and actions of small molecule neurotransmitters. Most small molecules are packaged in small clear vesicles that are approximately 50 nm in size; 5-HT being a notable exception. In contrast, neuropeptides are packaged in dense core vesicles that are 100-300 nm, and are manufactured in the Golgi and trafficked to release sites. The kinetics and requirements for dense core vesicle exocytosis are fundamentally different from release of small molecule neurotransmitters from small clear vesicles.

In the seminal work of Lily and Yuh Nung Jan, it was demonstrated using the bullfrog sympathetic ganglia that neuropeptides (i.e. LHRH) can co-exist and be co-released along with classic neurotransmitters (i.e. transmitters contained in small clear vesicles). They found that both neuropeptide release and the functional consequence

of that release required a greater stimulation (excitatory drive) and had a much slower onset and offset respectively [106-110]. It was later demonstrated in the hippocampus of guinea pigs that the neuropeptide dynorphin could be endogenously released from the hilus of the dentate gyrus using high frequency stimulation[111-113]. This was both in contrast to the much weaker stimulation required to evoke glutamate release from synaptic terminals and also produced direct inhibitory modulation (see below) of glutamatergic synaptic activity[111-115].

Behaviorally-induced stimulation of neuropeptide release coupled with pharmacology is the most common approach in determining the modulatory role of endogenously released neuropeptides. One of the first demonstrations of behaviorally evoked release of endogenous neuropeptides came from the seminal work of Huda Akil and colleagues. First, they demonstrated that analgesia produced by focal stimulation of the periaqueductal grey could be prevented by the non-selective opioid antagonist naloxone[116]. Akil and colleagues went on to show that stress-induced analgesia (the suppression of pain perception under stressful conditions) was mediated by the release of the endogenous opioid agonist β-endorphin[117]. These first studies laid the groundwork for all of the behavioral pharmacology that has been used to advance our understanding of neuropeptide regulation of the brain.

CRF as a neurotransmitter

CRF was first discovered by Vale and colleagues in 1981 to be the critical initiation factor of the HPA response[44]. CRF has two cloned receptors, CRF R1[118] and R2[119], both of which are promiscuous receptors in that they can couple to both $G_{S\alpha}$ and

$G_{q\alpha}$[120,121] and at high concentrations have even been shown to couple to $G_{i\alpha}$ *in vitro*[121]. R1 and R2 are approximately 80% homologous, with the major distinction between the two receptors occurring at the hydrophobic core. Very little is known about the downstream signaling cascades that are initiated following CRF-receptor binding. As is typical of GPCRs, agonist stimulation evokes ERK 1/2 activity and recruits β-Arrestin 2[120,121].

While CRF is expressed at high abundance in the hypothalamus, it has subsequently been shown that CRF is present in several extra-hypothalamic limbic nuclei including the central nucleus of the amygdala (CeA) and BNST[122]. Importantly, along with CRF, which binds preferentially to CRF R1 at lower concentrations, but can also bind to CRF R2 at higher concentrations, the urocortins (Urocortin I, II, III) are neuropeptides that belong to the CRF family of neuropeptides that preferentially activate CRF R2s[123]. Both CRF R1 and CRF R2 have a wide distribution through the CNS [124-126]. Activation of CRF R1s drives the initiation of the HPA axis response[41,123]. The first formulations of the relative roles CRF R1 and CRF R2 came from the development of mice in which the gene encoding for R1 or R2 had been deleted. The initial conclusions from a broad range of classic behavioral measures of depression and anxiety-like behavior suggested that CRF R1 mediated stress-induced anxiety and CRF R2 produced anxiolysis[123]. This led to the assertion that CRF R1 acts to initiate stress responding both by activating the HPA axis and anxiety-related limbic structures and CRF R2 acted to return the animal to behavioral homeostatsis. These findings were also corroborated and extended with behavioral pharmacology that has shown that both the activating and aversive qualities of stressor exposure can be blocked with CRF R1

antagonists[127,128] and recapitulated with CRF administration via the intracerebroventricular (i.c.v) route[129-131]. There is also an abundance of evidence suggesting that CRF has a critical role stress-induced reinstatement[132] and the aversive components of protracted withdrawal[133]. A caveat to these types of studies is that they presume that under physiological conditions CRF administered i.c.v. is released broadly throughout the brain, which may or may not be the case. They also presume that CRF infused i.c.v. is then made available to receptors relatively equally across brain regions, where clearly CRF i.c.v. will be more abundant in regions that are more proximal to the ventricular system. These studies were extremely influential and still remain, in large part, the accepted dogma in the field. However, with the rise of region-specific analysis of the role of CRF R1 and CRF R2 and the use of conditional knock-outs, it is clear that the actions of CRF R1 and R2 are far more complicated. Moreover, the behavioral consequences of CRF receptor activation are diverse and dependent on where and when this occurs.

Most of our understanding of how CRF acts in disparate brain regions to modulate activity and produce behavioral effects comes from a combination of electrophysiology in brain slices and micro-infusion of CRF into discrete nuclei. Using slice electrophysiology it has been demonstrated that CRF modulates both synaptic transmission and cell excitability. An interesting feature of CRF (and related peptides) is that the regulation of synaptic plasticity is often bidirectional. In some limbic brain regions including the CeA, lateral septum and dorsal raphe, CRF has a bidirectional effect on neuronal excitability which is either dependent on concentration/dose of ligand or on the specific CRF-like ligand used (i.e. CRF vs. Urocortins) [134-136]. In both cases,

biased activation of either CRF R1 or CRF R2 is suggested as the mechanism of this bidirectionality. While generally CRF is an “activating” neurotransmitter insofar as it stimulates the release of neurotransmitter it can have a net inhibitory effect on cell excitability through increased release of inhibitory neurotransmitters such as GABA. The electrophysiological actions of CRF have been studied in several brain regions. Importantly, CRF impinges on all three major monoaminergic nuclei to regulate cell excitability. CRF increases LC neuronal firing through activation of CRF R1 receptors. More specifically, CRF transitions LC firing from a phasic to tonic labile mode[62,137-140]. This has been shown to lead to elevated cortical activation[62]. It has been suggested that CRF regulation of LC noradrenergic neurons enhances cognitive arousal and flexibility during periods of acute stress. Interestingly, following i.c.v. administration of CRF, animals display an impairment in an attentional set shifting task (an assay of cognitive flexibility), however, when CRF is administered directly into the LC, animals displayed an improvement in set shifting suggesting an enhancement of cognitive flexibility[141].

In the serotonergic DRN, CRF inhibits neuronal firing at lower concentrations/doses via activation of CRF R1 receptors and produces its effects primarily through enhanced release of GABA from pre-synaptic terminals synapsing onto 5-HT neurons[135]. It has also been shown that CRF R1 activation increases inward current on non 5-HT (presumed GABAergic) neurons. However, at higher concentrations, CRF increases 5-HT neuronal firing via CRF R2 activation through direct increase in inward current post-synaptically[135]. The behavioral consequences of CRF regulation of serotonin neurons is not well understood. Direct infusions of CRF

into the dorsal raphe produce an inhibition of exploratory behavior of novel objects[142] and potentiates acquisition and expression of social defeat behavior (i.e. submissive posture and escape/fleeing)[143]. Thus CRF modulates serotonin neuron activity to encode both the perception and reaction of the aversive component of a stressor. By inhibiting serotonin release in discrete brain regions CRF can produce a negative affective state that can be associated with a given stimulus and simultaneously cause behavioral activation enabling the organism to escape or avoid the stimulus.

The actions of CRF in the dopaminergic VTA have been the most enigmatic due to potentially conflicting results from various studies. It has been demonstrated that CRF R1 activation can both enhance tonic firing rates through coupling to HCN channels[144], but also can enhance slow D2-IPSCs (GIRK currents) thought to depress tonic firing[145]. Similarly confusing, activation of CRF R2 enhances NMDA receptor-mediated excitatory currents known to facilitate bursting[146], and also mobilizes calcium post-synaptically to open small calcium activated potassium currents shown to depress bursting[147]. It is unclear if these finding are in opposition or if these CRF mediated effects occur in sequence. Behaviorally, intra-VTA infusions of CRF stimulate locomotor activity.[130] However, it has also been shown that CRF administered i.c.v can stimulate locomotor activity independent of dopamine[129].

Dynamic regulation of CRF receptors

CRF and CRF receptor levels and function are dynamically regulated by prior stress exposure as well as drug exposure. Interestingly, it has been shown in heterologous systems that CRF R1 is more readily internalized by agonist stimulation

than R2, whereas agonist stimulation causes R2 to rapidly diffuse and redistribute[121]. It has also been demonstrated in heterologous systems that glucocorticoid receptor activation suppresses synthesis for both CRF R1 and R2 mRNAs [148,149]. *In vivo*, CRF treatment to the whole animal as well as swim stress exposure causes R1 internalization in the LC.[150,151] Functionally, stress exposure has a biphasic effect on CRF receptor sensitivity: CRF R1 function at lower doses (30-300 ng) is sensitized, while CRF R1 function at higher doses (1 µg) is desensitized[152]. Consistent with what has been reported in heterologous systems, swim stress exposure causes a redistribution of CRF R1 and R2 receptors in 5-HT neurons of the dorsal raphe such that R1 is internalized and R2 present in the cytoplasm redistributes to the cell surface[153]. In several different models of stress-hyperresponsivity and depression (WKY rat, swim exposure), R1 inhibition of 5-HT neuronal excitability are abolished, yet the excitatory effects of R2 are retained[88,154,155]. Interestingly, chronic cocaine exposure has been shown to redistribute CRF receptors to alter the functionality of CRF at the synapse[156-158]. In the VTA, chronic cocaine potentiates the excitatory effects of the CRF[159]. Notably, chronic morphine treatment has similar sensitizing effects on CRF's ability to increase LC discharge as swim stress[160]. CRF receptor distribution could be one molecular target of stress-drug cross-sensitization.

Dynorphin-KOR

The dynorphins are a family of neuropeptides sharing common structural features that confer kappa opioid receptor selectivity and that are derived from a common precursor (preprodynorphin). The principal biologically active forms include: dynorphin A(1-17), dynorphin A(1-8), dynorphin B(1-13), alpha-neo endorphin, beta-

neo-endorphin and big-dynorphin[161-164]. It has recently been demonstrated that the dynorphin-KOR system is a critical mediator of the aversive and analgesic consequences of stress. While CRF has primarily been studied for its role in stress, the dynorphin-KOR system plays a role in several other processes including learning and memory and pain perception and has been implicated in neurological diseases such as epilepsy and neuropathic pain as well as addiction, anxiety and depression. These pathologies share the common feature of disruption of normal synaptic neurotransmission and disruption of the induction of neuroplasticity. There is compelling evidence to suggest that dynorphin, the endogenous ligand for KOR [161], is released in response to stressor exposure as well as during pathological hyperexcitability typical of seizure, neural injury and CNS inflammation. What appears as a common theme in these different diseases is disruption of dynorphin homeostasis. In both psychiatric and neurological disease models it is the imbalance of dynorphin-KOR function in either direction that appears to be critical to the etiology of these diseases.

The specific relationships between stress exposure, dynorphin release, and subsequent changes in synaptic functioning remain unclear. Pfeiffer et al. (1986) reported that in male human subjects, KOR agonists produced feelings of dysphoria, aversion and anxiety. Some subjects also report psychotomimetic actions such as racing thoughts and feelings of body distortion as well, while others report pseudohallucinations (hallucinations that are recognized by the individual as a hallucination and not something that is really occurring), loss of self-control and inability to focus attention, and these responses were blocked by naloxone [165]. Salvinorin A (Sal A), the active component of the hallucinogen *salvia divinorum*, is a potent and selective

KOR agonist, and cognitive-emotional alterations have been reported following human recreational use of *salvia divinorum* (*for review see* [166]). In pre-clinical models of anxiety, depression and addiction like behavior it has been shown that KOR antagonism blocks anxiety-like behavior on the elevated plus maze, immobility in the forced swim stress assay and both stress-induced reinstatement and potentiation of cocaine seeking[167-173].

KOR is a member of the opioid family of receptors which also include the mu and delta opioid receptors. All three opioid receptors are inhibitory in that they are all $G_{i\alpha}$ coupled and suppress neuronal excitability. Interestingly, while they have the same coupling, KOR and MOR often have opposite functional effects and behavioral effects. MORs are often present on GABAergic inhibitory interneurons and therefore, when activated produce a disinhibition which behaviorally results in locomotor activation and feelings of euphoria, not dysphoria.

The acute inhibitory actions of the KOR activation have been well-studied. Furthermore, there is more known about the functional consequences of downstream activation of MAP kinases. Generally, KOR activation causes negative regulation of calcium channels and positive regulation of potassium channels through Gβγ-gating mechanisms. More specifically, KORs are commonly localized to pre-synaptic terminals and upon activation reduce release probability. Indeed, at nearly every synapse examined, KOR activation has been shown to reduce glutamatergic release probability. Interestingly, in some regions it has also been shown simultaneously to inhibit GABAergic release probability[174]. In the hippocampus, KOR activation prevents the induction of LTP[113,175,176]. Post-synaptically, KOR activation couples to G-protein

activated inward rectifying potassium channels (GIRK)[177-181], known to suppress membrane excitability and in the hippocampus has been shown to positively couple to both M-channels (voltage-gate potassium channel Kv7 or KCNQ named for its coupling to muscarinic acetylcholine recptors)[182] and Shaker-type potassium channels. It has been demonstrated more recently that KOR has an acute inhibitory influence on all three monoamine systems and does so by directly inhibiting pre-synaptic release of monoamines through actions at the terminals, and suppressing the neuronal excitability of monoamine-containing neurons in the midbrain through both suppression of glutamatergic drive and direct post-synaptic inhibition via activation of GIRK channels.

Alterations in Dynorphin-KOR function in disease states

There is a body of evidence to suggest that dynorphin peptide and message expression is upregulated in both epileptic humans and animal models of epilepsy [183-187]. Normally, dynorphin immunoreactivity in the hippocampus is confined to the granule cell layer of the dentate gyrus and mossy fiber terminal region in the hilus and CA3 regions [185,188]. In contrast, hippocampi taken from epileptic humans show expanded dynorphin immunoreactivity in the inner molecular layer of the dentate gyrus, suggesting that dynorphin is expressed in sprouted recurrent collaterals that may underlie some of the hyperexcitability evident in this syndrome [185,186]. In a rodent pilocarpine-induced model of epilepsy, prior administration of the KOR agonist U50,488 attenuated pilocarpine induced seizure activity by increasing seizure latency, decreasing seizure duration, decreasing mossy fiber sprouting and increasing hilar neuronal survival [183]. There are also significant increases in dynorphin expression in the hippocampus following amygdala kindled seizures [189,190]. Along the same line, Dyn -/- animals have a

lower threshold for onset of seizure, which can be rescued by pretreatment with U50,488 in a GNTI-sensitive (KOR-dependent) manner [184]. This study is particularly interesting since there have been reports that polymorphisms in the preprodynorphin gene promoter are associated with temporal lobe epilepsy [191,192]. These studies suggest that in instances of pathological hyperexcitability dynorphin might be upregulated as a compensatory mechanism.

In humans, the brains of Alzheimer's disease patients have significant increases in dynorphin expression compared to age matched controls [193]. In more recent studies, using standard behavioral models of learning, intra-hippocampal injection of U50,488 impaired contextual freezing (contextual fear conditioning) and increased swim path length in the Morris water maze assay, both of which indicate that acute KOR activation in the hippocampus produces learning deficits [194].

With regard to the role of the dynorphin-KOR system in psychiatric disease the majority of attention has been very focused on the inhibitory effects of KOR activation on the dopamine system. The notion that was put forward by Wise in the late 70s suggests that if dopamine is the "euphoria" or reward molecule, then dynorphin's inhibitory effect on dopamine would underlie its dysphoric effects. Psychostimulants increase extracellular dopamine by inhibiting dopamine uptake (inhibition of DAT). Through activation of D1-like receptors, dopamine increases CREB activation, which in turn up-regulates dynorphin gene expression [91,195]. It has been proposed that this upregulation of dynorphin expression would result in increased dynorphin release, which would cause increased inhibition of the dopamine system[91,196]. As stated above,

stress exposure similarly increases dynorphin expression and could also exert its dysphoric effects by inhibiting the dopamine system.

However, there has been more recent evidence that the aversive component of stress exposure (assayed by conditioned avoidance experiments) as well as stress-induce analgesia is, in part, due to KOR actions in the dorsal raphe and regulation of serotonin tone. Moreover, many of these KOR-dependent stress-related behaviors, particularly the aversive aspects, are mediated by KOR-induced downstream activation of p38MAP kinase, not its acute ion channel coupling. KOR activates ERK 1/2, p38 MAPK and c-Jun kinase (JNK). KOR forms a complex with β-arrestin 2 that is dependent on GRK3 phosphorylation of the Ser369 residue of the C-terminal tail of KOR. While U50,488 induced p38 MAPK activity is dependent on GRK3, β-arrestin 2 and Ser369 phosphorylation, the early phase of increased ERK1/2 activity was not dependent on this complex. Members of the MAPK family have been shown to influence both long-term and short-term synaptic plasticity. Briefly, ERK can modulate the conductance of dendritic voltage-gated potassium channels, which in turn fine tunes membrane excitability. ERK regulates AMPA receptor insertion into the membrane and promotes dendritic spine growth and stabilization in CA1 pyramidal cells [197,198]. ERK also phosphorylates learning related transcription factors CREB and *elk* which go on to regulate the expression of several learning/memory related genes. Recent studies have suggested a distinct role of p38 MAPK in regulating neuroplasticity. ERK and p38 MAPK have opposing effects on AMPA-R surface expression and function in the same cell [199,200] suggesting divergent pathways for regulating plasticity. Additionally, p38 MAPK phosphorylates sodium channels (specifically Na_V 1.8) in the dorsal root ganglia,

increasing current density in these neurons [201]. Bruchas et al. (2007) demonstrated that repeated swim stress increases phospho-p38 MAPK expression in the hippocampus, striatum and cortex. Importantly, repeated swim stress is required to activate p38 MAPK; there is no appreciable increase in activity above basal levels following a single swim stress. This increase in phospho-p38 MAPK expression was blocked by norBNI, and dependent on β-Arrestin2 and GRK3 expression [202]. Inhibition of p38 MAPK by infusion of SB203580 attenuated U50,488 induced conditioned place aversion and decreased immobility in the FSS assay, but did not attenuate stress-induced analgesia. This study suggests that KOR activation of p38 MAPK in key brain regions plays a critical role in associative learning of context with aversive stimuli in specific behavioral assays.

The serotonergic DRN has recently been identified as a critical site for U50,488 induced aversion and analgesia. Blockade of KORs in the DRN via microinfusion of norBNI blocked U50,488-mediated aversion and analgesia. Likewise, virally rescued KORs specifically in the DRN of KOR -/- animals rescued both of these behaviors. Interestingly, when the Ser369 residue of KOR, necessary for GRK 3 recruitment was mutated to an alanine, the U50,488-induced conditioned aversion was absent, yet the U50,488 induced analgesia was retained. Combining these two lines of research, the investigators hypothesized that KOR-induced activation of p38α MAPK in serotonin neurons of the DRN mediates, at least in part, stress-induced aversion or dysphoria-like mood states. SERT-Cre, ePet-Cre and p38α-floxed transgenic mouse line were crossed to derive transgenic mice in which p38α was excised specifically from SERT or Pet-containing neurons (i.e. serotonin neurons). These animals showed significant

reduction in swim stress immobility, a blockade of conditioned place aversion to the KOR agonist and a blockade of stress-induced reinstatement of cocaine place preference. This study demonstrated that it is the downstream activation of MAPK signaling cascades, and not the acute Gβγ effects of KOR *per se*, that underlies KOR induced conditioned aversion. Moreover, acute KOR-mediated stress-related behaviors such as anxiety-like responding and analgesia may still be dependent of acute regulation of DRN excitability and not as dependent on downstream signaling.

Summary

We have come very far in identifying molecules involved in stress responding and psychiatric pathology and have taken strides to reduce certain aspects of behavior to individual molecules. While we understand a great deal about these discrete biological modules, we also recognize that the brain is not compartmentalized in this fashion. Rather, all of the molecules that have been described above interact with one another within the microcircuitry of limbic brain regions. One of the primary goals of this thesis work was to better understand how monoamines and neuropeptides interact with each other under different environmental conditions to produce behavioral outcomes.

Chapter 2: CRF receptor activation engages the Dynorphin-KOR system to produce a negative affective state

A subset of data and figures displayed here are adapted from:

Land BB, Bruchas MR**, Lemos JC**, Melief EJ, Xu M, Chavkin C. (2008) Stress-induced Dysphoria is Encoded by the Dynorphin-Kappa Opioid System. ***J Neurosci.*** 28:407-414.

and

Bruchas MR, Land BB, **Lemos JC,** Chavkin C. (2009). CRF1-R activation of the Dynorphin/Kappa Opioid system in the mouse basolateral amygdale mediates anxiety-like behavior. ***PLOS One.*** 4(12): 1-9.

Specific contributions:

J.C.L aided in cannulation surgeries, conducted immunohistochemistry experiments and collected fluorescent images using epifluorescent and confocal microscopes. M.R.B. and B.B.L. also conducted cannulation surgeries and performed the microinfusion.

Introduction

There are several neuropeptides that are released during periods of enhanced arousal such as exposure to an acute stressor. Corticotropin releasing factor (CRF) is a 41 a.a. neuropeptide that is initiates the HPA axis response following its release from hypothalamus, but also acts extra-hypothalamically to regulate the excitability of different limbic regions[41,125]. It is not well understand how CRF interacts with other neuropeptides also implicated in mediating stress and arousal responses. A recent set of studies demonstrated that the negative affective state produced by opiate withdrawal is absent or significantly reduced in mice deficient for the gene encoding either CRF R1

or CRF R2[203,204]. Moreover, the dysphoria-like effects of opiate withdrawal observed in mice require endogenous dynorphin release and activation of KORs. It is unclear if the CRF and the dynorphin systems act in parallel or sequentially. We hypothesized that CRF causes the release of dynorphin to produce a negative affective component of the stress response. To test this we conducted a series of behavioral studies using both pharmacological and genetic methods to elucidate the relationship between CRF and the dynorphin-KOR system in mediating the behavioral reaction to stress. Using the conditioned place aversion assay, we found that CRF administered intracerebroventricularly (i.c.v.) produced a conditioned place aversion previously described in rats[131], that could be prevented by pre-treatment with the KOR antagonist norBNI administered intraperitoneally (i.p.) and was absent in mice lacking the gene for preprodynorphin (see Land et al. 2008 for a full description of the results). CRF place aversion was mediated by CRF R2, but not CRF R1. Importantly conditioned place aversion produced by KOR agonism with U50,488 was not prevented by pre-treatment with CRF receptors antagonists suggesting that CRF is upstream of dynorphin [205]. The authors concluded that the aversive component of CRF acting throughout the brain requires the dynorphin-KOR system. In a subsequent set of behavioral experiments (see Bruchas et al. 2009 for a full description results), the authors also found that CRF i.c.v increased anxiety-like behavior assayed with the elevated plus maze, this time through activation of CRF R1 that also required the dynorphin-KOR system [167]. These studies provided strong behavioral evidence that CRF causes the release of dynorphin and activation of KORs, yet lack anatomical resolution. To characterize the anatomical substrates underlying KOR-mediated aversion and anxiety-like behavior we conducted

fluorescent immunohistochemistry and measured immunoreactivity for the phosphorylated Ser369 residue of KOR (KORp). This antibody is a good indication of activated KOR as it requires agonist binding and GRK3 recruitment [206]. Moreover, this antibody is a proxy endogenous dynorphin release since elevation of KORp-immunoreactivity (ir) produced by neuropathic pain is absent in Dynorphin -/- mice [207]. We assessed CRF-induced KORp-ir in several limbic brain regions and identified which CRF receptors were responsible for any observed effects.

Methods

Subjects

Male C57BL/6 mice, age >50 days, were maintained under a 12-h light–dark cycle (7a.m. to 7p.m. light) with access to standard food and water *ad libitum.* All procedures on animal subjects were approved by the University of Washington IACUC committee. Mice housed together (2 - 4 per cage) were subjected to the same behavioral treatment. Prodynorphin (Dyn -/-) knock-out mice and CRF R1 -/- mice were derived as described in [208] and [118] respectively and have been backcrossed to C57BL/6 background >10 generations.

Cannulation Surgeries

Isoflurane-anesthetized mice were mounted on a stereotaxic alignment system (David Kopf Instruments, Tujunga, CA), guide cannulas (26GA, 2.4 mm from pedestal) (Plastics One, Roanoke, VA) were placed in the lateral ventricle at 1.0 mm lateral, 0.4 mm posterior from bregma, and 3.0 mm below the skull. Cannulas were anchored with screws using dental cement. After surgery, mice recovered in their home cage for 6–8

d. Mice were injected intracerebroventricularly by placing a 33 gauge internal cannula (Plastics One) into the guide cannula and attached to a flared PE20 tube connected to a 10 µl Hamilton syringe. Injection volumes (1 or 5 µl) of saline or drug CRF (1 µg) were performed during a 2 min period. Some animals were pre-treated with norBNI or the selective CRF R1 antagonist, Antalarmin (10 mg/kg) i.p. or the selective CRF R2 antagonist anti-sauvagine 30 (1 nmol) i.c.v. 30 minutes following CRF injections the animals were intracardially perfused with 4%PFA in 0.1M PB.

Immunohistochemistry

Methods for immunohistochemistry experiments were similar to those reported previously (Bruchas et al. 2007). Mice were intracardially perfused with 4% paraformaldehyde in 0.1M PB. It has been our experience that phospho-protein immunoreactivity (ir) is of higher quality if PBS perfusion prior to 4% PFA is omitted. Brains were dissected and cryoprotected with 30% sucrose in 0.1M PB at 4°C overnight (or until the brains submerge), frozen and cut into 30 µm sections with a Leica SM200R microtome and placed in 0.1M PB with 0.1% sodium azide until processing. We had previously generated a rabbit polyclonal antibody against the phosphorylated Ser369 residue of KOR [206] that is now available commercially at Abcam. Briefly, floating sections are washed 3 x 10 min in PBS and then blocked for 60 min in 5% normal goat serum, 0.3% Triton-X in PBS. Importantly, we have found that for this phospho-antibody use of floating sections produces higher quality staining than thaw mounted sections. Sections were incubated in primary antibody (1:200) for 72 hr at 4°C. Often slices are co-labeled with mouse anti-GAD 67(Millipore) was used at 1:1000. Slices were then washed in PBS 10-15 min x 3 and incubated in goat Alexa Fluor antibody

(mouse or rabbit, respectively) at 1:500 to 1:750 for 2 hr at RT. Following secondary antibody incubation, slices were washed 3 x 10 min in PBS, then 2 x 10 min in 0.1M PB. Sections were then mounted on Superfrost plus slides and converslipped with Vectashield (Vector Laboratories).

Standard epifluorescent and confocal microscopy techniques (Nikon Eclipse E600 and Leica SL, respectively) were used to image sections for phospho-antibody staining. For comparisons of phospho-ir from different treatment groups, three animals from each group were perfused, sectioned and processed in parallel. Imaging of sections from animals exposed to different treatments were done at the same time with the same camera settings. As it is typical for different rounds of staining to yield different immunoreactive intensities, images from each treatment group displayed in this study came from the same round of staining.

Results

Brain tissue sections were co-labeled with KORp and GAD67. GAD67, the pre-cursor for GABA, was used as a regional marker and also to assess co-localization of KOR with GABA-containing neurons. CRF administration to the lateral ventricle produced a robust increase in KORp-ir compared to saline injected animals in several limbic brain regions (Figure 2.1 and Table 2.1). Notably, KORp labeling over baseline was highest in the basolateral amygdala (BLA), dorsal raphe nucleus (DRN) and CA3 region of the dorsal hippocampus. CRF-induced elevation of KORp-ir was blocked by pre-treatment with the KOR selective antagonist norBNI (10 mg/kg) given i.p. 1 hr prior to the CRF injection (Figure 2.1). CRF produces similar elevations in KORp-ir as U50,488 (20

mg/kg) i.p. injection compared to saline injected (either i.c.v. or i.p.) animals. In the BLA, KORp-ir was present on primary neurons as well as GAD67-positive GABAergic interneurons. Importantly, CRF does not increase KORp-ir in Dynorphin -/- animals indicating that CRF causes endogenous dynorphin release which in turn activates KORs. U50,488 does enhance KORp-ir in Dynorphin -/- demonstrating that KORs have the capacity to bind agonist and become phosphorylated in these animals (Figure 2.2). Interestingly, we found that CRF elicited increases in KORp-ir were blocked by pre-treatment with either the selective CRF R1 antagonist Antalarmin (Anta) or the selective CRF R2 antagonist anti-sauvagine 30 (ASVG 30) (Figure 2.3). Furthermore, as in wildtype animals, CRF increased KORp-ir in CRF R1 littermates compared to saline injected littermates, yet this increase was absent in CRF R1 -/- mice (Figure 2.3). Taken together, these data indicate that CRF can activate KORs by causing the release of dynorphin in several limbic brain regions implicated in emotional responding. Moreover, it can do so through either CRF R1 or CRF R2.

Discussion

The primary goal of these studies was to directly test behaviorally the sequential interaction of CRF and dynorphin systems and characterize this interaction in terms of anatomical substrates. CRF evokes dynorphin release and KOR activation to varying degrees in several limbic brain regions. CRF mediated release of dynorphin in some of these brain regions may encode an aversive component of stress, but it some regions may also serve as a way in which a region (i.e. the hippocampus) is returned back to homeostasis after periods of heightened excitability. Interestingly, this CRF-dynorphin interaction is common to both anxiety-like and dysphoria-like behavior despite utilizing

disparate CRF receptors to elicit dynorphin release. In addition to the initial characterization of CRF-dynorphin interactions in behavioral assays that model dysphoria-like and anxiety-like behavior, Bruchas et al. (2009) also characterized the role of KOR activation specifically in the BLA in CRF mediated aversion and anxiety. NorBNI injected directly into the BLA, blocked both CRF (icv)-mediated anxiety-like behavior[167] and CRF (icv) mediated aversion (unpublished observations). CRF acts through CRF R1 to produce anxiety-like behavior and R2 to produce dysphoria-like behavior, yet blockade of either CRF receptor blocks KOR activation in the BLA. These data suggest that CRF evoked release of dynorphin in the BLA through either CRF R1 or CRF R2 produces a negative affective state that can effect both measures of anxiety and dysphoria assayed by elevated plus maze and condition place aversion respectively.

Several studies have suggested that both CRF and dynorphin play a critical role in mediating behavioral responses to stress. However, this set of studies was the first to elucidate a direct sequential interaction of these two neuropeptide systems in which CRF causes the release of dynorphin to produce negative affect. Understanding how these systems are intertwined is critical to our understanding of how complex arousing stimuli are processed and in turn responded to by an organism.

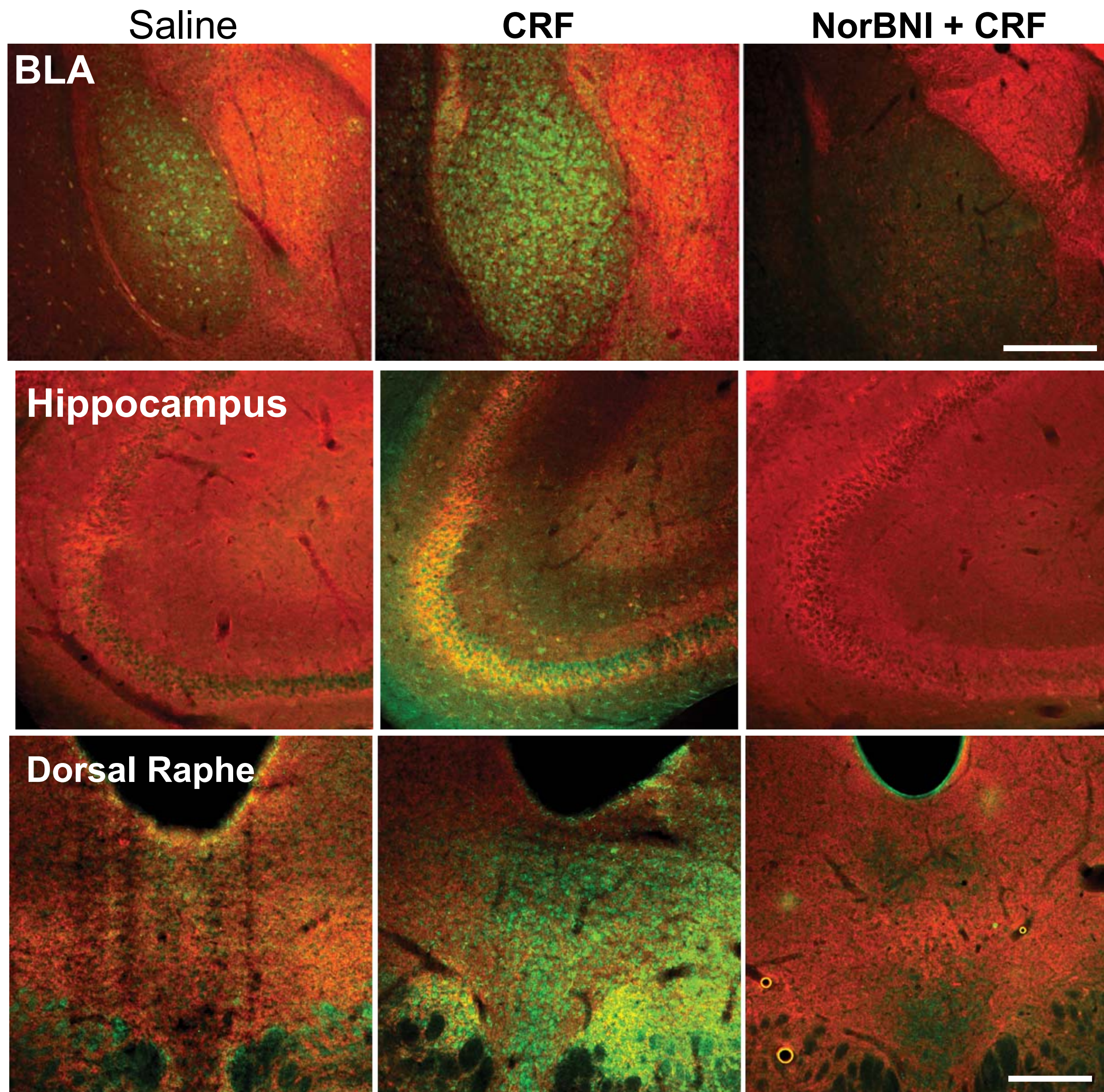


Figure 2. 1. CRF-induced KORp-ir in limbic brain regions is blocked by norBNI pre-treatment.

Fluorescent images of KORp-ir (green) and GAD 67 (red) co-localization in the BLA (scale bar = 200 µm), hippocampus and dorsal raphe (scale bar = 100 µm) of animals injected with vehicle (saline) or CRF i.c.v. Animals pre-treated with the KOR antagonist norBNI did not show CRF-induced elevation of KORp-ir over saline condition (right column).

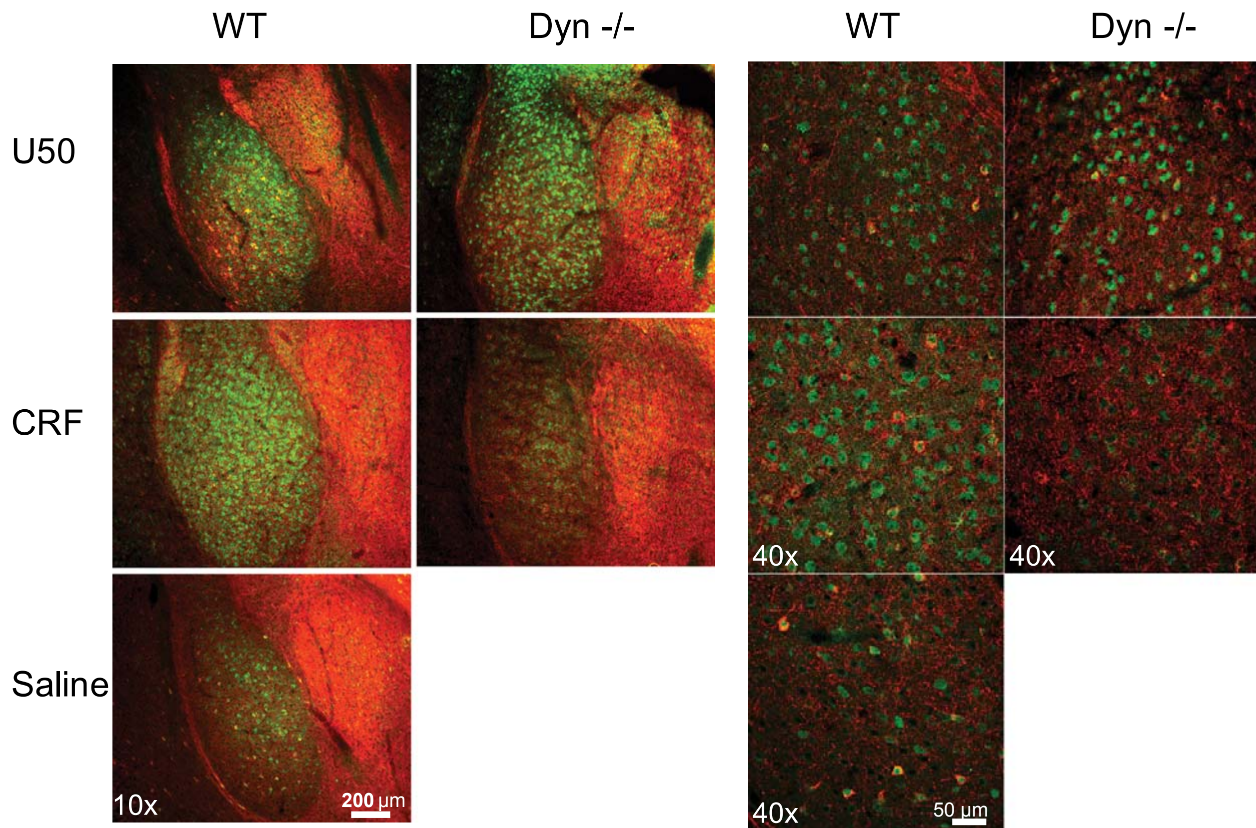


Figure 2.2. CRF-induced KORp-ir is absent in animals lacking the gene for preprodynorphin (Dyn -/-).

Fluorescent images of KORp-ir (green) and GAD 67 (red) co-localization in the BLA at 10x (left) and 40x (right) magnification in WT or Dyn -/- animals injected with U50,488 (20 mg/kg) i.p. or CRF i.c.v.

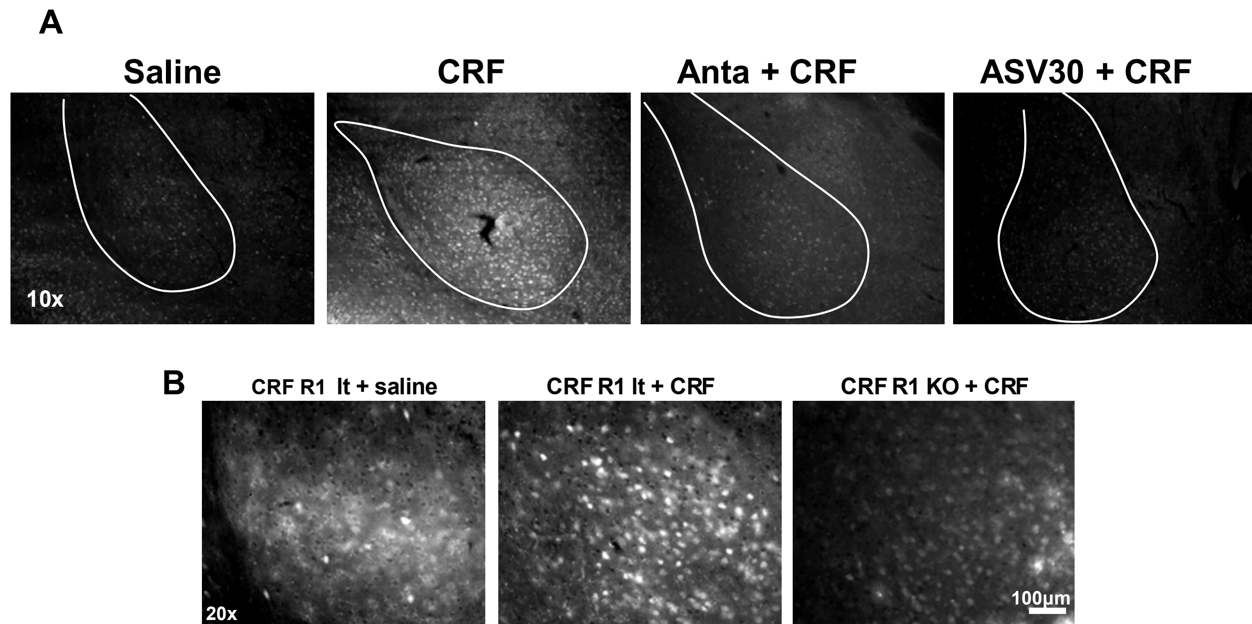


Figure 2.3. CRF-induced KORp-ir can be prevented by blockade of either CRF R1 or CRF R2.

A. 10x fluorescent images of KORp-ir in the BLA. The CRF elicited increases in KORp-ir over saline condition was blocked by pre-treatment with either the selective CRF R1 antagonist antalarmin (10 mg/kg) i.p. or the selective CRF R2 antagonist ASVG-30 (1 nmol) i.c.v. administered 15 minutes prior to CRF injection. B. 20x fluorescent images of KORp-ir in the BLA. The CRF elicited increase in KORp-ir over saline condition was absent in CRF R1 -/- animals.

Table 2.1. Relative CRF induced KORp-ir across limbic brain regions.

Region	CRF-induced KORp-ir
Dorsal raphe nucleus	+++
Basolateral amygdala	+++
Dorsal Hippocampus	+++
Ventral Pallidum	++
Ventral tegmental area	++
Nucleus accumbens	++
Bed nucleus of the Stria Terminalis	+
Caudate putamen	Not detected
Central nucleus of the amygdala	Not detected

Chapter 3: Stress exposure produces a switch from appetitive to aversive signaling by CRF in the nucleus accumbens

The following chapter has been adapted from:

Lemos JC, Wanat MJ, Smith JS, Reyes BAS, Hollon NG, Van Bockstaele EJ, Chavkin C, Phillips PEM. Stress exposure produces a switch from appetitive to aversive signaling by CRF in the nucleus accumbens . *Under review at Nature*

Specific contributions:

J.C.L. performed immunohistochemistry. J.C.L. and N.G.H. carried out fast-scan cyclic voltammetry experiments. J.C.L., M.J.W. and J.S.S. performed the behavioral experiments. B.A.S.R. and E.J.V. provided transmission electron microscopy data. J.C.L., M.J.W., C.C. and P.E.M.P. developed the conceptual and experimental framework.

Introduction

Stressors motivate an array of adaptive responses ranging from "fight or flight" to an internal urgency signal facilitating long-term goals[30,31]. However, traumatic or chronic uncontrollable stress promotes the onset of Major Depressive Disorder where acute stressors lose their motivational properties and are perceived as insurmountable impediments[8]. Consequently, stress-induced depression is a debilitating human condition characterized by an affective shift from engagement of the environment to withdrawal[209]. An emerging neurobiological substrate of depression and associated pathology is the nucleus accumbens, a region with the capacity to mediate a diverse range of stress responses by interfacing limbic, cognitive and motor circuitry[41]. Here we report

that corticotropin releasing factor (CRF), a neuropeptide released in response to acute stressors[210,211] and other arousing environmental stimuli[212,213], acts in the nucleus accumbens of naïve mice to increase dopamine release through co-activation of CRF R1 and R2 receptors. Remarkably, severe stress exposure that produces a depression-like phenotype[10] completely abolishes this effect without recovery for at least 90 days. This loss of CRF's capacity to regulate dopamine release in the nucleus accumbens is accompanied by a switch in the reaction to CRF from appetitive to aversive, indicating a diametric change in the emotional response to acute stressors. Thus, the current findings offer a biological substrate for the switch in affect which is central to stress-induced depressive disorders.

Results and Discussion

CRF activates the hypothalamic-pituitary-adrenal axis and regulates the excitability of limbic brain regions[214]. It signals via two receptor subtypes, CRF R1 and CRF R2, which are distributed widely throughout the brain[125,214]. In the nucleus accumbens, CRF facilitates appetitive processes including motivation for cued rewards[215] and social bonding behavior[216]. Since these processes are, at least in part, mediated by dopamine[217,218], we hypothesized that CRF acts within the nucleus accumbens to enhance dopamine transmission. To test the validity of this hypothesis, we investigated the proximity of CRF-containing axonal fibers to those containing dopamine using fluorescent immunohistochemistry. Dense CRF immunoreactivity was present throughout the rostro-caudal axis of the nucleus accumbens core and lateral shell and in the most rostral portion of the medial shell. It displayed dense punctal staining

characteristic of fiber terminals consistent with previous studies[122], as well as staining in sparsely located large cell bodies (cholinergic interneurons, Figure 3.1 and 3.2) Tyrosine hydroxylase (TH) immunoreactivity was used as a marker for dopamine-containing terminals which were interdigitated with CRF-positive fibers (Figure 3.1a). These findings indicate that the configuration of terminals in the nucleus accumbens is amenable for interactions between CRF and dopamine transmission.

To test the capacity for direct regulation of dopamine transmission by CRF, we used immunohistochemistry to co-label the CRF receptors, R1 or R2, with TH. CRF R1 immunoreactivity displayed punctate staining with co-localization of TH immunoreactivity on fiber segments in addition to localization on cell bodies within the nucleus accumbens (Figure 3.1b and 3.3). CRF R2 immunoreactivity had a more diffuse, but still, punctate pattern of staining similar to that in other regions[153], with some co-localization with TH-immunoreactivity (Figure 3.1c and Figure 3.4). Expression of CRF receptors on subcellular profiles in the nucleus accumbens, including TH-positive terminals, was confirmed at higher spatial resolution using transmission electron microscopy (Figure 3.1d; quantified in Table 3.1). These data demonstrate a subcellular localization of CRF receptors that is well suited for modulation of dopamine release, establishing anatomical evidence for CRF-dopamine interactions in the nucleus accumbens.

Next, we tested the functional effect of CRF on dopamine release in the nucleus accumbens in acute coronal brain slices. We used fast-scan cyclic voltammetry at carbon-fiber microelectrodes to monitor dopamine release evoked by a single biphasic

electrical pulse (2 ms/phase, 100-500 μA) delivered once per minute via a local bipolar stimulating electrode (Figure 3.5a). This stimulation produced a transient increase in the detectable current which was absent in mice lacking the gene encoding TH in dopamine-containing, but not other catecholamine-containing, neurons ($Th^{fs/fs}$; $Dbh^{Th/+}$ mice[219]) demonstrating that the signal is unequivocally attributable to dopamine transmission (Figure 3.6). Vehicle or CRF (10, 100 or 1000 nM) was applied to the slice for 15 minutes following five minutes of stable baseline and the resultant effect was quantified by averaging the evoked dopamine current in the last ten minutes. Following application of vehicle, there was a modest decrease (~7 %) in dopamine release (Figure 3.5b). In contrast, CRF increased dopamine release in a concentration-dependent manner with similar affinity to its electrophysiological actions in limbic brain regions[214]. This effect was significantly greater than vehicle at 100 and 1000 nM (27.8 ± 6.7 and 30.0 ± 8.4 % respectively, mean ± s.e.m.; $F_{3, 49} = 5.026$, $p < 0.01$, one-way ANOVA with Dunnett's post-hoc t-tests; Figure 3.5). At these concentrations of CRF, the increase in dopamine release was greatest in the first two minutes of application and thereafter the effect remained relatively stable (Figure 3.7a). A similar increase in dopamine release was observed following 100-nM CRF with a five-minute inter-stimulus interval that did not produce the decline in dopamine release during vehicle application (Figure 3.7b and c).

To characterize the receptor subtype(s) mediating this action of CRF on dopamine release, we applied the selective CRF R1 antagonist, antalarmin (1 μM), or the selective CRF R2 antagonist, anti-sauvagine 30 (ASVG 30; 250 nM) to the slice, beginning 20 minutes before CRF application. Interestingly, either antagonist alone was

sufficient to block the CRF-mediated increase in evoked dopamine release ($F_{2, 50}$ = 5.142, $p < 0.01$, one-way ANOVA with Dunnett's post-hoc t-tests; Figure 3.5c) indicating that co-activation of both receptors is required. In concordance with this pharmacological evidence, CRF (10, 100, 1000 nM) failed to increase dopamine release in the nucleus accumbens of mice with deletion of either the CRF R1[220] or R2[119] gene (Figure 3.5d and e). In further support of a mechanism requiring co-activation of both receptor subtypes, application of the selective CRF R1 agonist Stressin 1 (100 or 300 nM) or the selective CRF R2 agonist Urocortin 3 (100 or 300 nM) failed to significantly increase dopamine release when applied individually ($p > 0.05$ compared to respective vehicles; Figure 3.5f and g), but significantly increased dopamine release when co-applied ($F_{3,36}$ = 3.528, $p < 0.05$ vs vehicle, one-way ANOVA with Dunnett's post-hoc t-tests). The effect of the agonists together could be blocked by pre-treatment with Antalarmin and ASVG 30 (unpaired t-test, $p > 0.05$; Figure 3.5h). Together these data provide convergent evidence that the CRF-elicited increase in dopamine release is mediated through co-activation of CRF R1 and R2.

The ability for CRF to positively regulate dopamine in the nucleus accumbens is parsimonious with its capacity to enhance appetitive behaviors[215,216]. However, administration of CRF by the ICV route produces conditioned place aversion[131,205] which, on the surface, is at odds with our findings as most other agents that act in the nucleus accumbens to increase or mimic dopamine produce conditioned place preference[221]. Conversely, we would predict CRF would cause a conditioned place preference when restricted to the nucleus accumbens if the increase in dopamine by CRF in the nucleus accumbens brain slice has relevance to the behaving animal.

Therefore, we used a place-conditioning assay to assess the subjective qualities of CRF infused *directly* into the nucleus accumbens (cannula placement shown in Figure 3.8). The place-conditioning apparatus consisted of two visually distinct test chambers separated by a smaller neutral compartment. On day 1, mice were allowed to freely roam the apparatus and the time they spent in each chamber was recorded (Figure 3.9a). On days 2 and 3, mice received CRF bilaterally into the nucleus accumbens (500 ng per side in 200 nl artificial cerebrospinal fluid) or vehicle infusions and then were isolated in one of the test chambers for 30 minutes. Four hours later they received the alternative infusion and were isolated in the other test chamber for 30 minutes. On day 4, mice were again allowed free access to the apparatus. Mice exhibited a significant preference for the CRF-paired context (see representative trace in Figure 3.9b), demonstrating that intra-nucleus-accumbens CRF (500 ng) was an appetitive stimulus to these animals (conditioning by drug, $F_{1,12} = 6.435$, $p < 0.001$ two-way repeated-measures ANOVA, Figure 3.9c). This dose of CRF is within the range that produces selective effects *in vivo*[130,215,222-227], but it is difficult to ascertain the steady-state concentration at receptors as CRF undergoes both radial diffusion and active clearance[228]. Nonetheless, even at a lower dose of CRF (5 ng/ 200 nl) where the injected concentration prior to diffusion is only about twice the highest steady-state concentration applied to the brain slice, conditioned place preference was observed (conditioning by drug, $F_{1,14} = 5.415$, $p < 0.05$, two-way repeated measures ANOVA; Figure 3.9d and Figure 3.10). Taken together, these data indicate that CRF acts in the nucleus accumbens to produce a positive affective state.

To test whether this positive affective state is dependent upon CRF's ability to increase dopamine release, we used the catecholaminergic-neuron-selective neurotoxin, 6-OHDA. Unilateral manipulations were used to avoid a potential confound of general deficits in performance or associative processes from bilateral dopamine depletion. Therefore, we first confirmed that infusions of CRF (500 ng/200 nl) into the nucleus accumbens of one hemisphere produced a conditioned place preference in control animals (conditioning by drug, $F_{1,12} = 11.77$, $p < 0.001$ two-way repeated-measures ANOVA; Figure 3.9d and Figure 3.10b). We then compared unilateral CRF place conditioning in animals that had received infusions (500 nl) of vehicle (0.09% NaCl,0.1% ascorbate) or 6-OHDA (2 μg) into the nucleus accumbens of the same side seven days earlier. 6-OHDA treatment produced a significant dopamine depletion on the side of the injection ($p < 0.001$; Figure 3.11a), but did not alter locomotor activity ($p > 0.05$, unpaired t-test, Figure 3.11b), demonstrating that the unilateral lesions did not produce a general deficit in motor function. CRF (500 ng in 200 nl) produced place preference in sham animals (conditioning by drug, $F_{1,18} = 6.95$, $p < 0.05$ two-way repeated-measures ANOVA; Figure 3.11c), of similar magnitude to controls (treatment by drug, $F_{1,30} = 0.35$, $p > 0.05$, two-way ANOVA). However, the subjective effects of intra-accumbens CRF in 6-OHDA-treated animals was significantly altered ($p < 0.05$, unpaired t-test, Figure 3.9d) to the extent where place preference was absent (conditioning by drug, $F_{1,18} = 0.00$, $p > 0.05$, two-way repeated-measures ANOVA; Supplementary Figure 3.11d). These data demonstrate that the positive affective state produced by CRF in the nucleus accumbens is dependent upon its ability to increase dopamine release.

This capacity for exogenously applied CRF to produce robust behavioral effects through regulation of dopamine transmission has important implications for the way the brain normally processes the environment to engage arousing stimuli. Therefore, we tested the role of *endogenously released* CRF in mediating the response to an arousing stimulus by assaying novel object exploration, a behavior that requires intact dopamine transmission[229,230]. We bilaterally infused the CRF antagonist, α-helical CRF (500 ng in 200 nl per side), into the nucleus accumbens, placed animals into an arena and then 15 minutes later, introduced a novel object into the center (Figure 3.12a). While α-helical CRF had no effect on baseline exploration of the center of the arena compared to vehicle (lactated ringer's with 1% acetic acid), it significantly attenuated the appetitive effects (i.e., eliciting of approach and exploration) of the novel object (treatment by stimulus, $F_{1,18} = 4.62$, $p < 0.05$, two-way repeated-measures ANOVA; Figure 3.12b). These data demonstrate that endogenous CRF in the nucleus accumbens is utilized under physiological conditions to mediate appetitive responses to arousing environmental stimuli.

Exposure to severe or chronic stress can produce profound alterations in normal stress signaling that can be detrimental to physical and mental health, predisposing individuals to depression[23,53]. To model this phenomenon, we employed a modified Porsolt paradigm in which mice are exposed to two days of repeated swim stress (Figure 3.13a). Animals were placed in a vessel of water (29.0 - 31.0 °C) for 15 minutes followed by four additional 6-minute swim sessions (separated by 6-minute recovery periods) 24 hours later. This protocol has been shown to produce escalating immobility across sessions indicating a depression-like phenotype[10]. We prepared coronal slices

of the nucleus accumbens from these animals thirty minutes after the final stress exposure and found that the ability for CRF to potentiate dopamine release was completely abolished (stress exposure by drug, $F_{4,116} = 12.61$, $p < 0.001$ two-way ANOVA, Figure 3.13b). This loss of CRF's action was also observed with the canonical Porsolt[33] paradigm (single 15-min swim; Figure 3.14). Notably, we established that this change in the ability of CRF to regulate dopamine release was not a generalized change in stress-related peptide signaling as the effect of a kappa-opioid agonist to reduce dopamine release was unaffected by the two-day stress-exposure paradigm (Figure 3.15). Therefore, these data demonstrate that severe stress selectively abolishes CRF's ability to modulate dopamine release in the nucleus accumbens. Surprisingly, there was no recovery of the action of CRF on dopamine release in the nucleus accumbens 7, 30 or even 90 days after stress exposure in animals that underwent the two-day forced swim stress paradigm (stress exposure by drug, $F_{4,116} = 4.852$, $p < 0.01$, two-way ANOVA; Figure 3.13b). This time period is consistent with the protracted course of stress-induced depressive disorders[231], and indeed, a depression-like phenotype was maintained across this 90-day post-stress period as assessed by swim immobility (Figure 3.16). Importantly, the loss of the CRF response was neither due to a baseline change in evoked dopamine release (Figure 3.17) nor simply an age-related phenomenon (Figure 3.18). Therefore, we have demonstrated that severe stress produces a persistent dysregulation of CRF-dopamine interactions that normally produces a positive affective state.

Stress-induced depressive disorders are associated with altered levels of several neurochemicals that interact with the CRF system, including serotonin[232,233],

dynorphin[234-236] and glucocorticoids[41,53,56,148,149]. Therefore, we targeted these systems to gain mechanistic insight into the stress-induced loss of CRF's regulation of dopamine release. We pre-treated animals (10 ml/kg intraperitoneal) with vehicle, fluoxetine (selective serotonin-reuptake inhibitor; 10 mg/kg), norBNI (kappa-opioid-receptor antagonist; 10 mg/kg) or RU486 (glucocorticoid-receptor antagonist; 30 mg/kg) prior to stress exposure on each of the swim-stress days. The animals were allowed to recover for seven days, then slices were prepared and the CRF response was tested (Figure 3.13c). The abolition of CRF modulation of dopamine release by stress was not significantly affected by fluoxetine or norBNI ($p > 0.05$; Figure 3.19) but was prevented by RU486 ($p < 0.001$; Figure 3.13d and Figure 3.19). Even at a lower dose (10 mg/kg), RU486 significantly reduced the effects of stress exposure on dopamine regulation by CRF ($p < 0.01$; Figure 3.19). These data demonstrate that glucocorticoid signaling is a critical component of the profound stress-induced dysregulation of CRF-dopamine interactions in the nucleus accumbens.

This robust loss of the neurochemical response to CRF in the nucleus accumbens following severe stress suggests a long-lasting alteration in its subjective qualities. To test this notion, we utilized the place conditioning paradigm in animals that had been exposed to the two-day swim-stress regimen. Mice that underwent repeated swim stress seven days prior to conditioning spent significantly less time in the CRF-paired chamber compared to the vehicle-paired chamber following conditioning, establishing that CRF in the nucleus accumbens is now aversive to these animals (conditioning by drug, $F_{1, 10} = 5.824$, $p < 0.01$, two-way ANOVA , Figure 3.20a-b). Therefore, severe stress produces a diametric shift in the subjective qualities of

CRF in the nucleus accumbens from positive to negative (Fig. 6c). Consistent with the enduring loss of CRF regulation of dopamine observed *in vitro*, the absence of CRF conditioned place preference persisted for at least 90 days following repeated stress exposure ($F_{2,20} = 6.870$, $p < 0.05$, one way ANOVA with Dunnett's post-hoc; Figure 3.20c and Figure 3.21). Likewise, endogenously released CRF no longer stimulated exploration of a novel object when tested seven days after stress exposure (stimulus by drug, $F_{1,16} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA; Figure 3.22) demonstrating that severe stress abolished the function of CRF in the nucleus accumbens to stimulate appetitive responses to arousing stimuli (unpaired t-test, $p < 0.05$, Figure 3.20d). Therefore, these findings demonstrate the long-term loss of a regulatory mechanism of motivated behavior following severe stress.

Major Depressive Disorder has a lifetime prevalence of 17 %, making it one the largest public health concerns in the US[1]; yet, its molecular foundation has been elusive. Patients suffering from this disorder present with constellations of symptoms including loss of affect, cognitive impairment and homeostatic imbalance[237], symptoms that are presumably precipitated by dysregulation of multiple brain regions[41]. It is established that glucocorticoid-dependent hippocampal atrophy is a critical mediator of cognitive impairment in depression such as memory loss[41,48]. More recently, disruption of nucleus accumbens function has been implicated in the affective symptoms of depression[41,91]. In the current work, we studied the actions of CRF on neurotransmission within this brain region in an attempt to connect pathological-stress-related neuroadaptation with the shift in affect observed in depressed patients.

CRF receptors are distributed widely throughout the brain[125] and mediate disparate effects. For instance, CRF increases motor activity when administered locally into ventral tegmental area[130] or nucleus accumbens shell[223], but not the prefrontal cortex[227], nucleus accumbens core[223] or bed nucleus of the stria terminalis[225], and can even elicit freezing behavior when injected into the periaqueductal gray[224], basolateral or central nucleus of the amygdala[226]. Likewise CRF produces conditioned place aversion when infused into the bed nucleus of the stria terminalis[225] or following ICV administration[131,205], yet we demonstrate that direct application to the nucleus accumbens produces conditioned place preference in naïve animals. This local effect of CRF in the nucleus accumbens is not surprising given its regulatory role on dopamine that we characterized, as dopamine agonist administration alone is sufficient to produce conditioned place preference[238-240]. Indeed, it was abolished by local dopamine depletion confirming the requirement for CRF to regulate dopamine in mediating this behavior. Furthermore, we demonstrate that endogenous CRF is present in the nucleus accumbens and promotes appetitive behavior towards arousing stimuli. Therefore, our data highlight the specificity of the local action of both exogenously applied and endogenously released CRF in the nucleus accumbens in producing a positive, rather than negative, subjective state.

Importantly, we demonstrate that severe stress disables this capacity of CRF to positively regulate dopamine, removing CRF's appetitive qualities, leaving a negative perceptual bias. This dysregulation is mediated by glucocorticoid, but not kappa, receptors. Glucocorticoid signaling has been shown to have genomic repressive effects of the CRF system[56], in particular the downregulation of CRF R1[148]. Indeed, genetic

deletion of the CRF R1 gene selectively from dopamine neurons increases anxiety-like behavior[241], further demonstrating that disruption of CRF-dopamine interactions alone is sufficient to produce a negative affective state similar to that following severe stress[242]. While acute regimens of the selective-serotonin reuptake inhibitor, fluoxetine, do not alleviate pre-existing depression-related symptoms in patients or animal models, they have been shown to prevent the induction of some depression-like responses to stress[243]. Nonetheless, acute administration of fluoxetine prior to stress exposure did not prevent the perturbation.

Collectively, we demonstrate a specific defect in the regulation of dopamine transmission in the nucleus accumbens as a consequence of exposure to stress that induces depression-like behavior. Indeed, depressive disorders produce a profound change in the perception and behavioral response to acute stressors and other arousing environmental stimuli that elicit CRF signaling. Taken together, our findings provide a neurobiological mechanism for the affective shift from engagement of the environment to withdrawal following severe stress, central to the manifestation of Major Depressive Disorder.

Methods

Subjects: Male C57BL/6 mice age > 50 days were maintained under a 12-h light–dark cycle (7a.m. to 7p.m. light) with access to standard food and water *ad libitum.* All procedures on animal subjects were approved by the University of Washington or Thomas Jefferson University IACUC committee. Mice housed together (2 - 4 per cage) were subjected to the same behavioral treatments.

***Immunohistochemistry*:** We used perfusion, cryosectioning and immunohistochemistry procedures as previously described[202]. Sections (30 μm) were then incubated with a mixture of mouse anti-tyrosine hydroxylase 1:1000 (Sigma, St. Louis, MO) and either rabbit anti-CRF (peptide) 1:150 (Sigma, St. Louis, MO) and chicken anti-ChAT antibody 1:150 (Invitrogen, Carlsbad, CA) or rabbit anti-CRF R1 or CRF R2 (Novus Biologicals, Littleton, CO) in blocking buffer for 24-36 hours at room temperature. Sections were then washed with PBS, and detection was performed using the fluorescent secondary antibody Alexa Fluor 488 goat anti-mouse IgG 1:500, Alexa Fluor 555 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-chicken IgG (Invitrogen, Carlsbad, CA) in blocking buffer for 2 hours at room temperature. Sections were washed in PBS 3 x 10 minutes and PB 2 x 10 minutes and mounted on Superfrost plus slides. Sections were imaged with epifluorescence (Nikon) and confocal microscopes (Leica).

Transmission electron microscopy: Mice were perfused and brains were sectioned as previously described. 100-nm sections were processed using standard transmission electron microscopy procedures[244]. Sections were incubated in mouse anti-TH (1:1,000; Immunostar Inc., Hudson, WI, USA) and rabbit anti-corticotropin-releasing factor receptor (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at RT. Immunoperoxidase detection of TH and silver-intensified immunogold localization of CRFr followed standard procedures[151]. Digital images were captured using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA). Only tissue sections with good preservation of ultrastructural morphology and with both TH and CRFr immunoreactivity clearly apparent in the tissue were used

for the analysis. For immunogold labeling, profiles with at least two immunogold-silver particles within a cellular compartment in a single thin section were considered immunolabeled[150,151]. The cellular elements were classified according to the description by Peters and colleagues[245,246].

Fast scan cyclic voltammetry: Mice were rapidly decapitated and the head placed in preoxygenated ice-cold artificial cerebrospinal fluid (aCSF) in which sucrose (248 mM) was substituted for NaCl. The brain was rapidly removed and blocked to isolate the anterior forebrain. 250-µm coronal slices containing the nucleus accumbens were prepared using methods previously described [167], placed in a recording chamber, and continuously perfused (1.5-2.0 ml/min) with oxygenated aCSF (in mM), NaCl 124, KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 2.0, $CaCl_2$ 2.0, Dextrose 10 and $NaHCO_3$ 26) maintained at 31-33 ^oC. Carbon fiber electrodes were fabricated using a Sutter P-97 puller. Carbon fiber electrodes (working electrodes) were hand cut to approximately 100-150 µm past the capillary tip. The potential at a carbon-fiber electrode was held at -0.4 V versus Ag/AgCl, ramped to +1.3 V and back to -0.4 V (400V/s) every 100 ms. A single bi-phasic electrical pulse (2 ms/phase, 100-500 µA) was applied to the slice to evoke dopamine release[247].

Swim stress: Mice were subjected to either a single 15 minute swim with a 24 hour recovery period, or a two-day swim stress in which they were exposed to a 15-minute swim session on day one, then 24 hours later on day two, were exposed to four 6-minute swim sessions separated by 6 minutes conducted under bright light (690-700 lux) conditions. Water temperature was maintained at 29.0 – 31.0 °C. Animals were

removed from the water if they became completely submerged for >1 sec at any time during the paradigm. Some animals were sacrificed at 30 minutes, 7, 30 or 90 days following the final swim session of the two-day protocol and nucleus accumbens slices were prepared.

***Cannulations*:** Animals were anesthetized with isoflurane and cannulation surgeries were performed using a stereotaxic alignment system similarly to methods previously described[202]. Double guide cannulas (26 gauge, 3.5 mm from pedestal, 2 mm separation; Plastics One, Roanoke, VA) were placed in the nucleus accumbens core at 1.0 mm/-1.0 mm lateral, 1.0 mm posterior from bregma, and 3.5 mm below the skull. Guide cannulas were anchored with screws (CMA/Microdialysis, Stockholm, Sweden) using dental cement, and dummy internal cannulas were placed inside until injection. In some cases, screws were replaced by a C & B Metabond priming solution (Parkel). Mice were injected intracerebroventricularly by placing a 33 gauge internal cannula (Plastics One, Roanoke, VA) into the guide cannula.

Conditioned place preference: Animals were allowed to recover from surgery for at least four days. All animals were handled for four days prior to the pre-test day. Animals assigned to the stress-exposed group were subjected to the two-day swim stress paradigm following recovery; animals were not included if they did not show normal swimming responses. Stress-exposed animals began CRF conditioning 7 or 90 days following the final swim session. A three-compartment place-conditioning apparatus was used to measure preference as previously described[202]. On days 2 and 3, mice received two injections per day: one injection of aCSF and one injection of CRF

(500ng/200nl) paired with different chambers at 125 nl/min. On day 4, mice were once again allowed free access to the entire apparatus for 30 minutes. Following the conclusion of behavioral testing, cannulae placements were assessed. Mice with cannula placements outside the accumbens were excluded from the study.

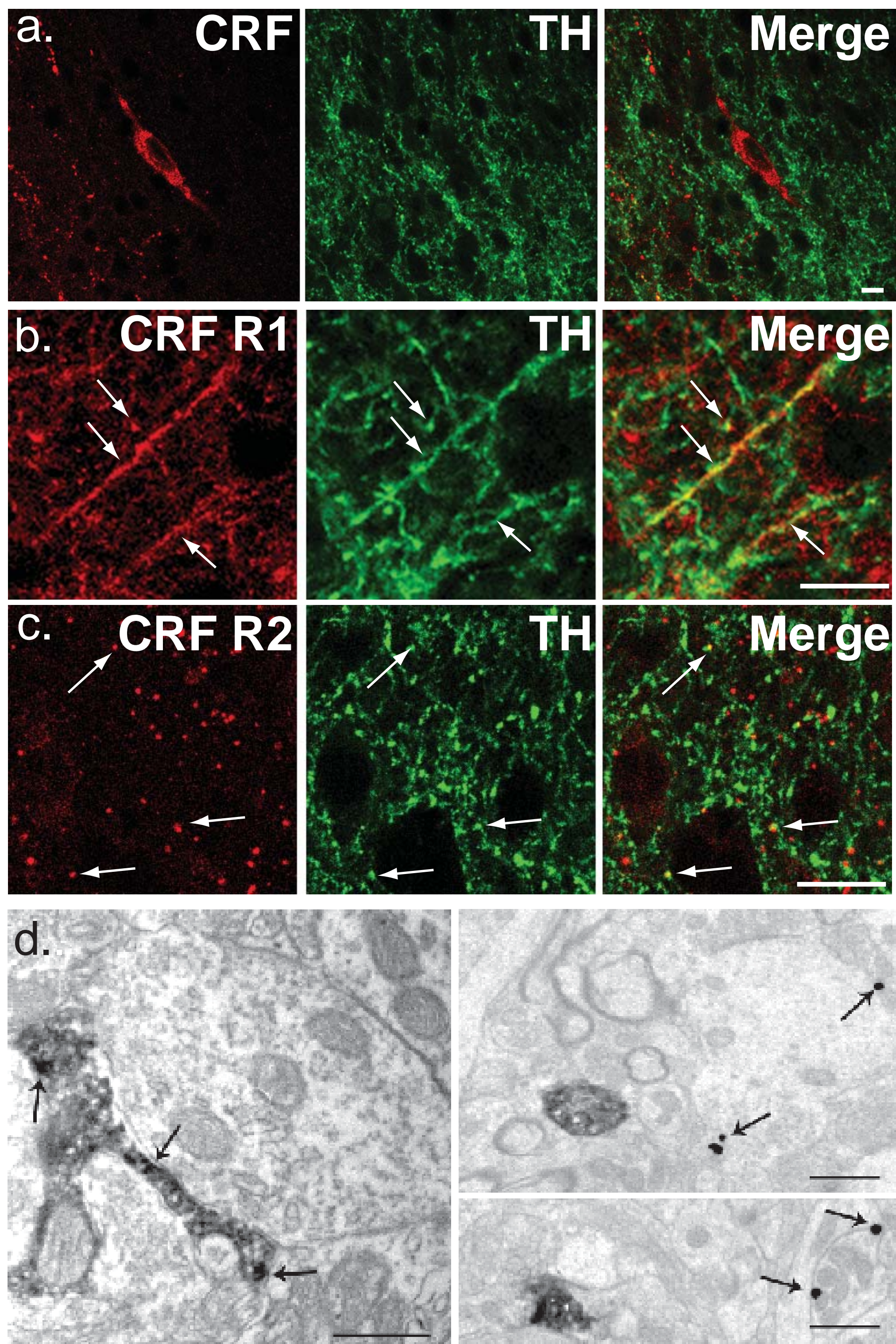
6-OHDA lesion and HPLC: Mice were injected with either 6-OHDA (Sigma) (2 µg/500 nl) or vehicle (0.9% NaCl, 0.1% ascorbate. Following the conclusion of behavioral testing, a tissue core (approximately 2 x 2 x 1mm) of the ipsilateral and contralateral accumbens of each animal was microdissected out, rapidly frozen in liquid nitrogen and stored in microcentrifuge tube at -80°C until processed for tissue dopamine content. High-performance liquid chromatography (HPLC) was used to measure monoamine content by the Neurochemistry Core Lab at Vanderbilt University's Center for Molecular Neuroscience Research[219].

Novel object exploration: Mice were cannulated, allowed to recover from surgery and handled for four days prior to being subjected to a novel object exploration assay similar to previously described[241]. Briefly, on test day 1, mice were given bilateral intra-accumbens microinfusions of either vehicle (lactated ringer's with 1% acetic acid) and were allowed to habituate in an open field for 15 minutes. Subsequently, a novel object was introduced and exploratory behavior of the novel object was measured for an additional 15 minutes. On test day 2, the animals received the alternative pharmacological treatment to what they received on day 1, were allowed to habituate again the open field and then exposed to a second novel object. Both pharmacological treatment and novel were counter-balanced across test days. Identically to the place

conditioning experiments, one group of mice were exposed to swim stress 7 days prior to test day 1.

Figure 3.1.Cellular localization of CRF peptide, CRF R1 and CRF R2 in the nucleus accumbens.

a. CRF peptide immunoreactivity (red) was present in large cell bodies located sparsely throughout the nucleus accumbens, as well as in small puncta apposed to TH positive fibers (green) (top panel). CRF R1 immunoreactivity had a diffuse, punctate pattern of staining. Notably, CRF R1 immunoreactivity (red) was localized to some TH positive fibers (arrows, yellow fluorescence) evidenced by both fluorescent co-labeling and congruent morphology (white arrows) (middle panel). CRF R2 immunoreactivity was localized primarily in the cytoplasm of medium sized cell bodies throughout the nucleus accumbens in addition to diffuse punctate staining, some of which was localized to TH positive puncta (white arrows, bottom panel). Scale bar = 10 μm. b. Transmission electron microscopy photomicrographs demonstrating the co-localization of CRFr (immunogold particles) with immunoperoxidase labeled TH positive profiles. CRFr (black arrows) were present on both TH positive and TH negative profiles. Left scale bar = 0.5 μm; right scale bars = 1 μm.


a.
CRF
TH
Merge
b.
CRF R1
TH
Merge
c.
CRF R2
TH
Merge
d.

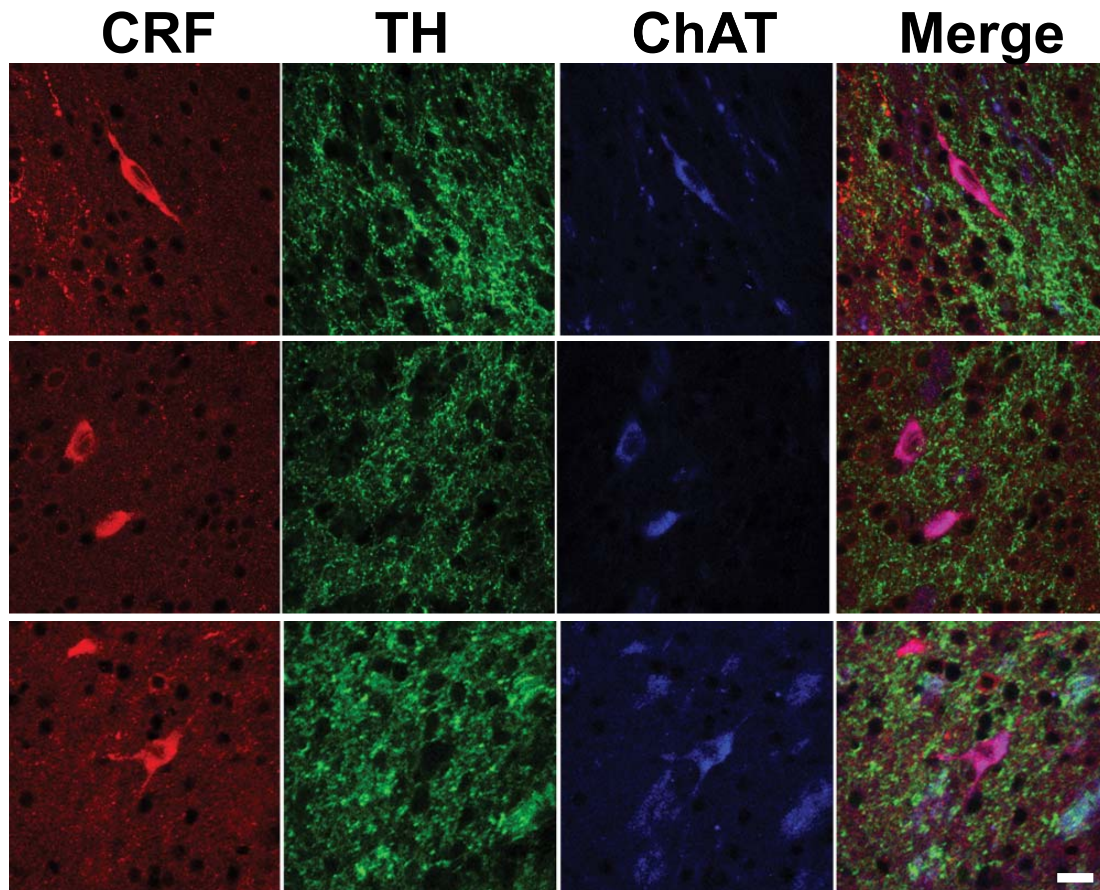


Figure 3.2. CRF peptide is localized cholinergic interneurons throughout the nucleus accumbens.

Immunofluorescent images demonstrating localization of CRF peptide, tyrosine hydroxylase (dopamine terminal marker) and choline acetyltransferase (ChaT) (acetylcholine marker) in the nucleus accumbens. CRF immunoreactivity was present in large cell bodies sparsely located throughout the nucleus accumbens. Three examples of confocal photomicrographs are shown, depicting CRF immunoreactivity (red) and ChAT immunoreactivity (blue) co-localization from three fields within the nucleus accumbens of three different mice. Scale bar = 20 μm.

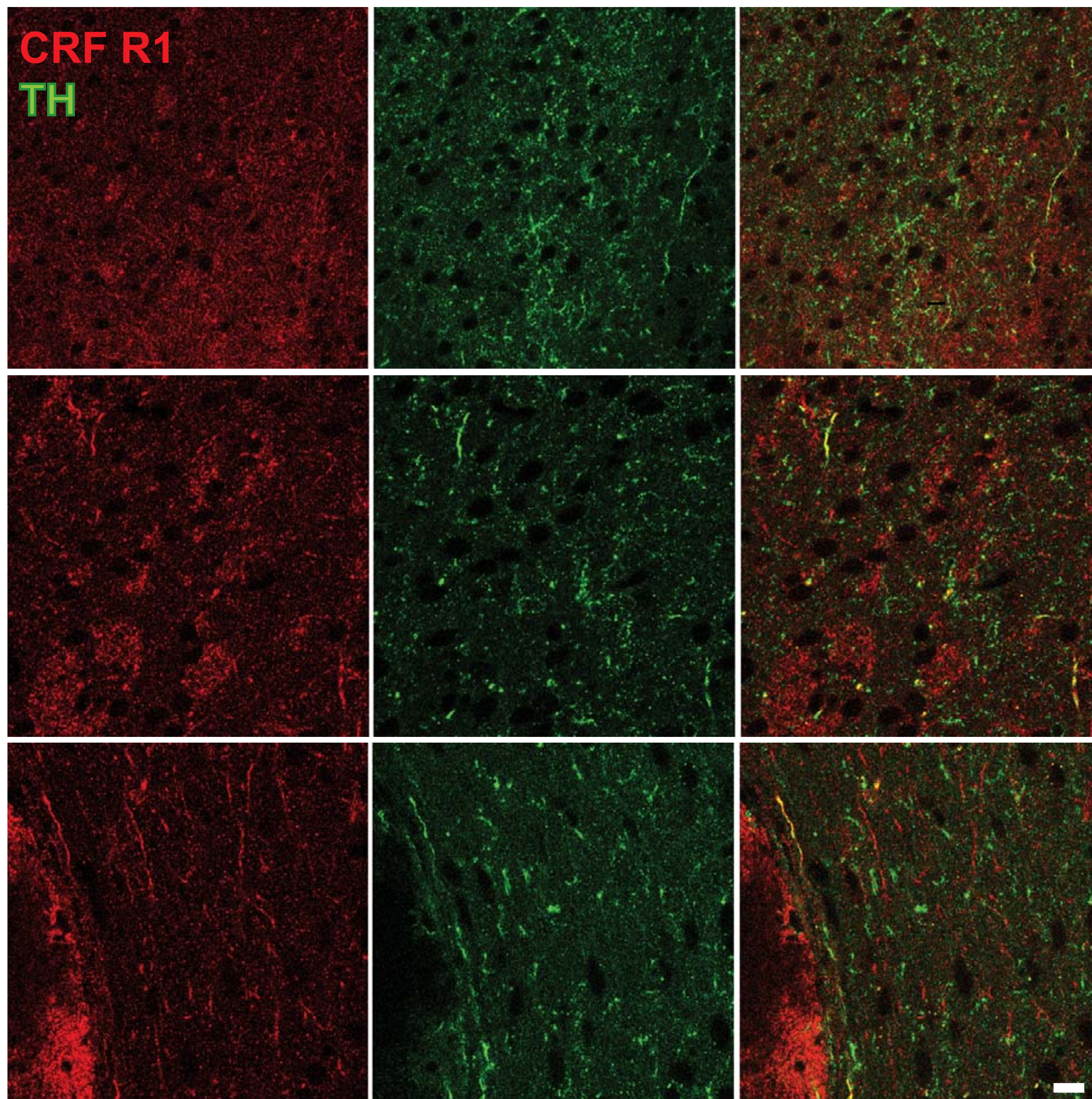


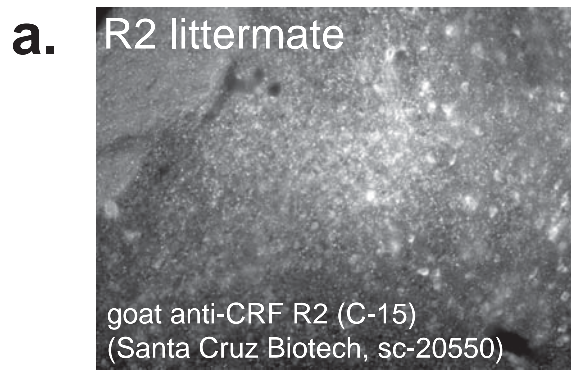
Figure 3.3. CRF R1 co-localization to TH positive fibers.

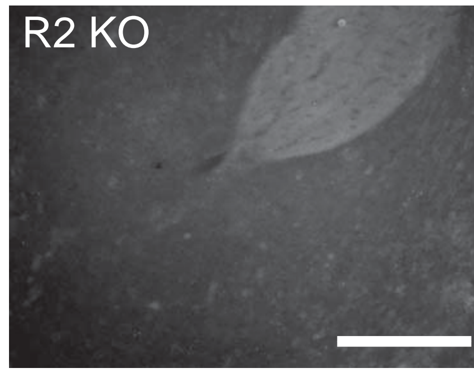
Additional 60x images from two additional animals demonstrating co-localization of CRF R1-IR to TH-IR in the nucleus accumbens core. Scale bar = 10 µm.

Figure 3.4. CRF R2 antibody validation and localization in the nucleus accumbens of WT littermate and R2 KO mice.

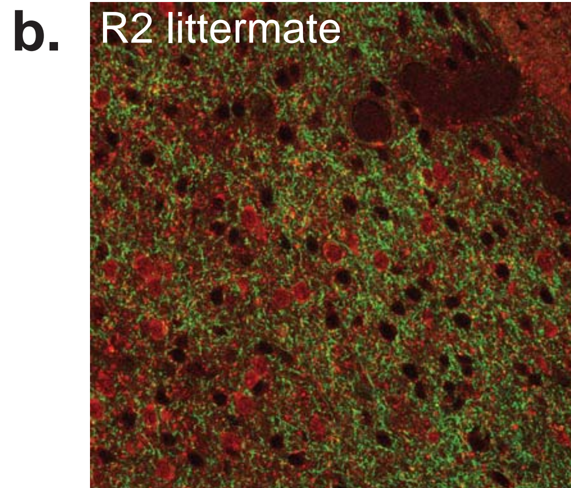
The CRF R2 KO animals used in the study were derived from the original R2 KO line generated by Bale & Vale (2000) in which exons 10-12 have been deleted creating a functional CRF R2 KO mouse. Non-specific antibody staining in R2 KO mice may be a result of antibody recognition of an epitope on a non-functional truncated protein that has been translated or cross-reactivity of the antibody to another protein. Thus, we used antibodies that recognized an epitope of the c-terminal tail of the R2 protein (Santa Cruz sc-20550) that should be not translated and a Novus Biologicals antibody (NBP1-00768). Sections were prepared, processed and imaged in parallel (i.e. on the same days). a. Low power (20x) epifluorescent images of the nucleus accumbens of WT (left) and R2 KO (right) using the c-terminal R2 antibody (Santa Cruz sc-20550). Scale bar = 100 μm. b. High power (60x) confocal merged images demonstrating CRF R2 (red) and TH (green) localization in the nucleus accumbens of WT (left) and KO (right) animals. Using confocal imaging, there is virtually no detectable CRF R2 red immunofluorescence in the nucleus accumbens of KO mice. Scale bar = 10 μm. c. Exclusively for this set of images, both images were thresholded so that only the top half of red fluorescent pixels are visible. Under this stringent thresholding, red fluorescent puncta remain in the WT image and are not apparent in the R2 KO image. Scale bar = 10 μm d. Low power (20x) epifluorescent images of the nucleus accumbens of WT (left) and R2 KO (right) using the Novus Biologicals R2 antibody (Novus Biologicals NBP1-00768). Scale bar = 100 μm. e. High power image (100x) demonstrating demonstrating co-localization of CRF R2 (Novus Biologicals NBP1-00768) with TH staining. The same pattern of staining seen in the c-terminal Santa Cruz antibody is also seen with the Novus Biologicals R2 antibody. Scale bar = 10 μm.

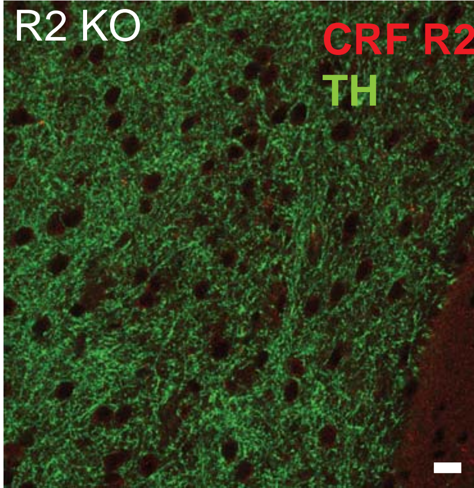
a.



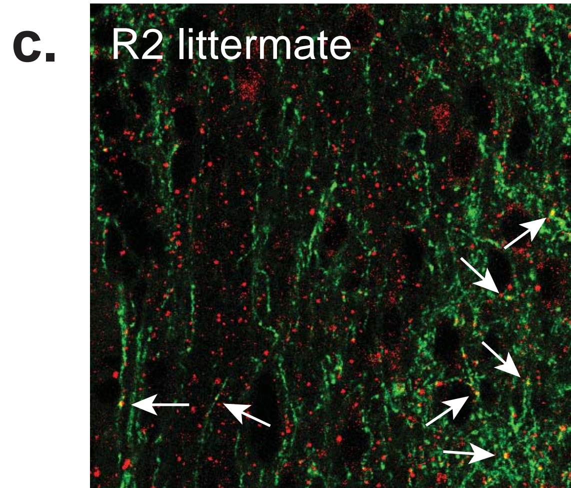


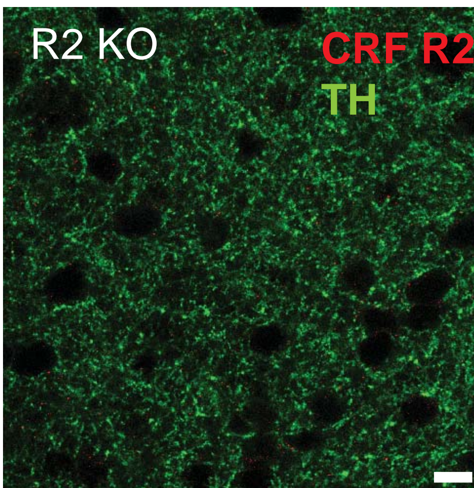
b.



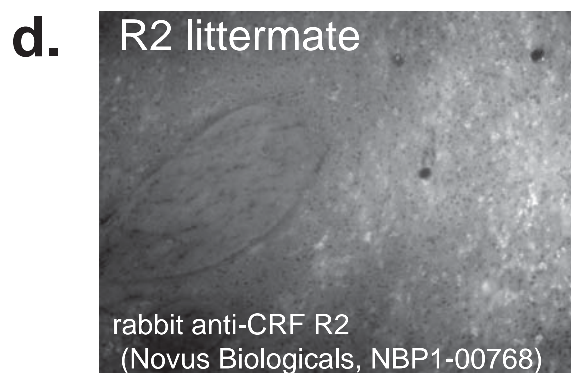


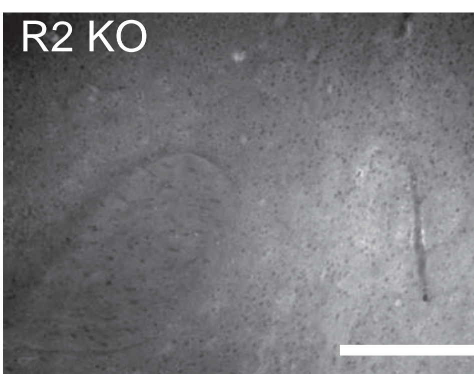
c.



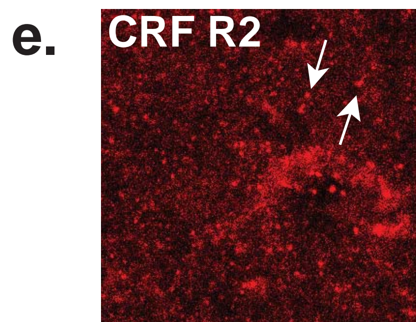


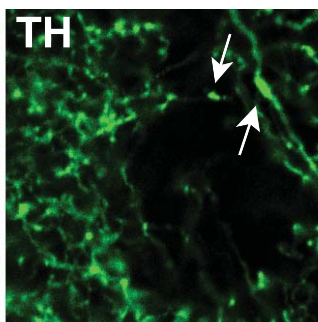
d.





e.





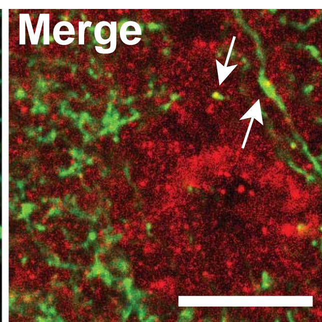
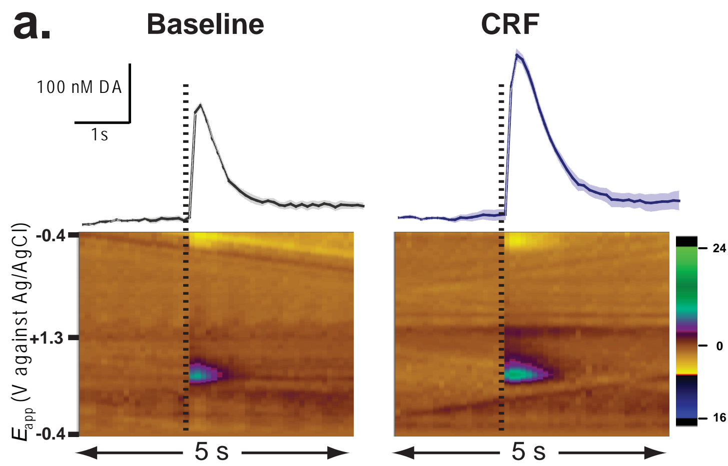
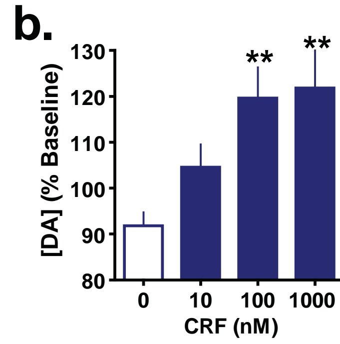
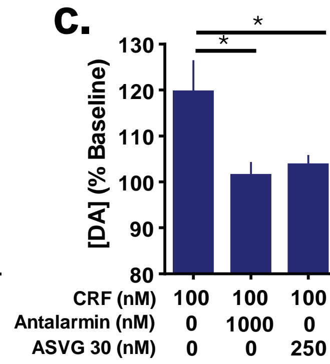


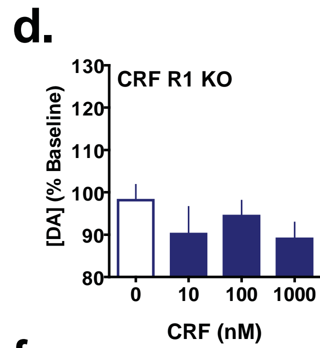
Figure 3.5. CRF increases dopamine release in the nucleus accumbens through co-activation of CRF R1 and R2.

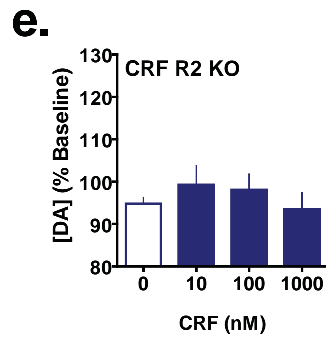
a. Representative evoked dopamine release before (grey line) and after (blue line) bath application of 100-nM CRF (top). Corresponding two-dimensional plots depicting changes in peak dopamine oxidation current (pseudocolor) with time as the abscissa and voltage potential as the ordinate (bottom). Dashed lines indicate the time at which stimulation was delivered. b. The mean + s.e.m. response to CRF (10, 100 or 1000 nM) or vehicle (0.07% acetic acid) in the last 10 min of 15-min application, normalized to pre-drug baseline. CRF (100, 1000 nM) increased dopamine release (Dunnet's post-hoc t-test, $p < 0.001$, n = 11-18). c. Pre-treatment with either the selective CRF R1 antagonist (antalarmin, 1000 nM) or the selective CRF R2 antagonist (anti-sauvagine 30, 250 nM) blocked the CRF mediated increase in dopamine release (Dunnett's post-hoc t-test, $p < 0.05$, n = 18-20). D & e. CRF (10,100, 1000 nM) did not did not increase evoked dopamine release in mice lacking the CRF R1 or CRF R2 receptor gene (gene by drug, $p > 0.05$, two-way ANOVA, n = 7-13). f. The selective CRF R1 agonist, stressin 1 (100 or 300 nM) did not increase evoked dopamine release ($F_{2,32} = 1.833$, $p < 0.05$ vs acetic acid vehicle, one-way ANOVA, n = 9-15). g. The selective CRF R2 agonist, urocortin 3 (100 or 300 nM) did not increase evoked dopamine release ($F_{2,18} = 1.036$, $p < 0.05$ vs DMSO vehicle, one-way ANOVA, n = 5-8). h. Co-application of stressin 1 and urocortin 3 (100 nM or 300 nM) significantly increased evoked dopamine release but not in the presence of antalarmin (1000 nM) and anti-sauvagine 30 (250 nM) together (Dunnett's post-hoc test, $p < 0.05$ vs acetic acid/DMSO vehicle, n = 8 - 15). * $p< 0.05$, ** $p < 0.01$, ***$p< 0.001$.

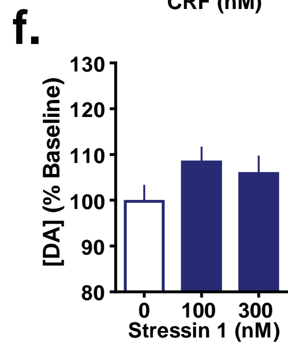

a.
Baseline
CRF
100 nM DA
1s
-0.4
+1.3
-0.4
E_{app} (V against Ag/AgCl)
5 s
5 s
24
0
16

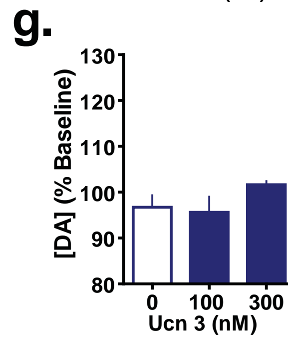

b.
[DA] (% Baseline)
**
**
0
10
100
1000
CRF (nM)

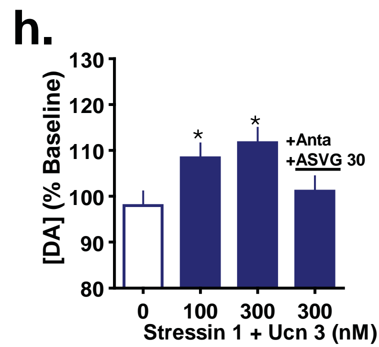

c.
[DA] (% Baseline)
*
*
CRF (nM) 100 100 100
Antalarmin (nM) 0 1000 0
ASVG 30 (nM) 0 0 250


d.
CRF R1 KO
[DA] (% Baseline)
0
10
100
1000
CRF (nM)


e.
CRF R2 KO
[DA] (% Baseline)
0
10
100
1000
CRF (nM)


f.
[DA] (% Baseline)
0
100
300
Stressin 1 (nM)


g.
[DA] (% Baseline)
0
100
300
Ucn 3 (nM)


h.
[DA] (% Baseline)
*
*
+Anta
+ASVG 30
0
100
300
300
Stressin 1 + Ucn 3 (nM)

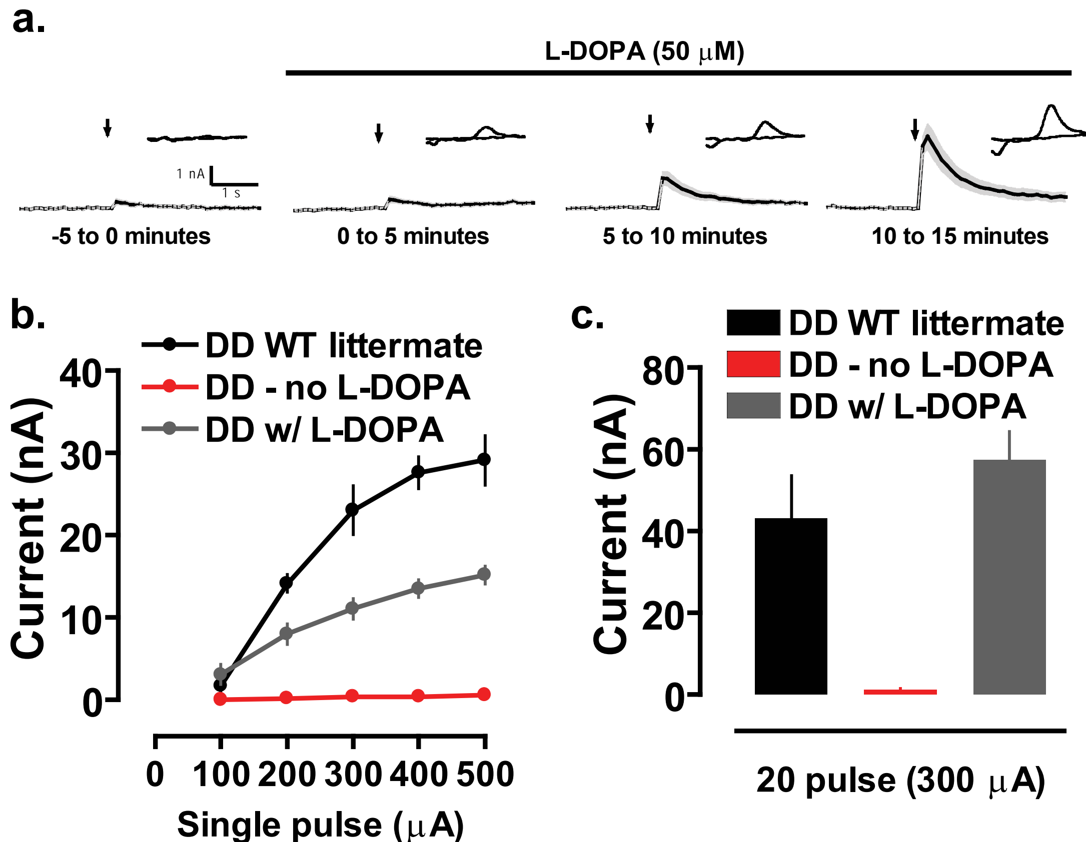


Figure 3.6. Evoked electrical currents detected by carbon fiber electrodes placed in the nucleus accumbens core are solely attributable to dopamine release.

Nucleus accumbens slices were prepared from "dopamine deficient" ($Th^{fs/fs}$; $Dbh^{Th/+}$) mice or littermate control mice in parallel. Following a baseline input-output curve, a dopamine recovery experiment was carried out. Following this recovery experiment, another input-output curve was obtained. Following five baseline stimulations, stimulations once every minute continued while L-DOPA (50 μM) was applied to slice. a. Averaged evoked responses before and after bath application of L-DOPA (50 μM). The insets are average cyclic voltammegrams (CV) corresponding to the averaged evoked response. As L-DOPA washes over the slice, the CV of the evoked response increasingly correlates with the stereotyped electrochemical fingerprint of dopamine. b. Input (electrical stimulation amplitude) – Output (subsequent current measured at the carbon fiber) for a single pulse stimulation in littermate control slices compared to slices from dopamine deficient mice before and after L-DOPA bath application. c. Evoked current elicited by a 20-p stimulation at 300 μA in slices prepared from littermate controls or slices from dopamine deficient mice either before or after L-DOPA bath application.

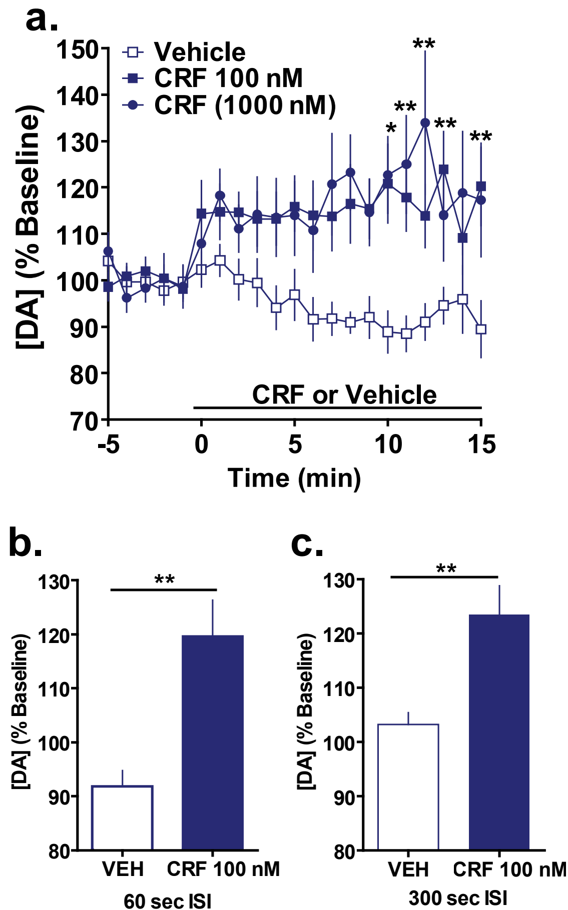


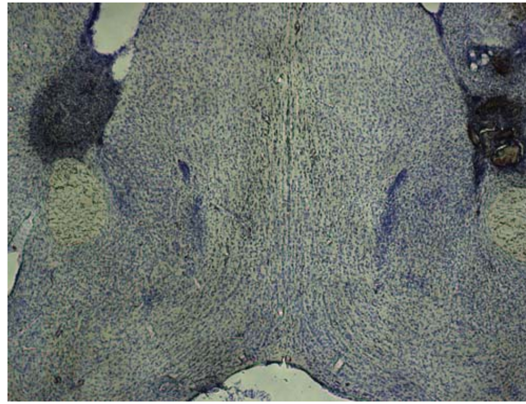
Figure 3.7. Time course of CRF or vehicle effect on evoked dopamine release.

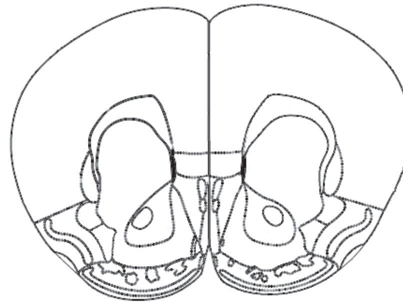
a. & b. A single bi-phasic stimulating pulse (100-500 µA) was applied to the slice once every minute and dopamine release was measured at the carbon fiber electrode placed in the nucleus accumbens core. Following a five stable baseline currents, CRF (100 nM, 1000 nM) or vehicle (0.07% acetic acid) was bath applied to the slice. There was a small (5-10%) depression in dopamine release over time apparent in the vehicle group ($p < 0.01$ vs. 100%). In contrast, CRF increased evoked dopamine release by 20% above baseline ($p < 0.001$ vs. 100%) and 27-30% above vehicle, n = 13 and 18 for vehicle and CRF experiments respectively (drug by time, $F_{20,560} = 2.994$, $p < 0.001$, two-way ANOVA). c. When a single pulse stimulation to the slice every 300 seconds which eliminated the run down demonstrated in the original vehicle group done with a 60 second inter-stimulus interval (ISI). Compared to the time-matched vehicle group, CRF (100 nM) significantly increased evoked dopamine release by 20.3% in the nucleus accumbens ($p < 0.01$, t-test, n = 8-10).

Figure 3.8. Cannula placements for naïve used in the conditioned place preference assay.

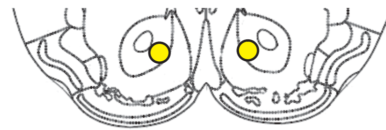
a. Representative cresyl violet image from naïve cohort and cannula placements for each individual animal in the naïve group. b. Representative cresyl violet image from stress-exposed cohort and cannula placements for each individual animal in the stress-exposed group.

Naive

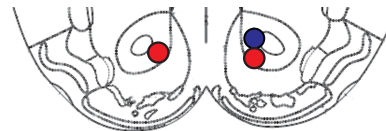




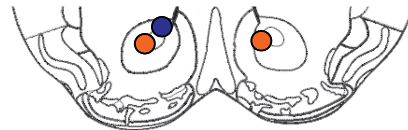
+1.58



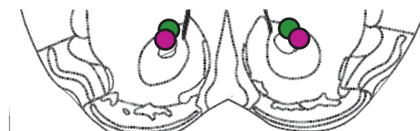
+1.46



+1.34

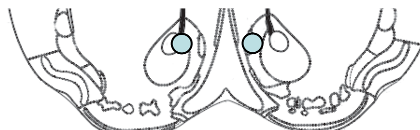


+1.22



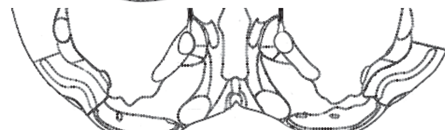
+1.10

+0.98



+0.86

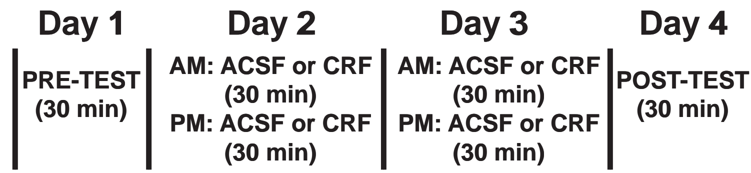
+0.74

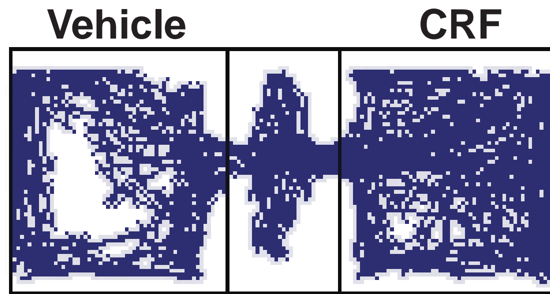


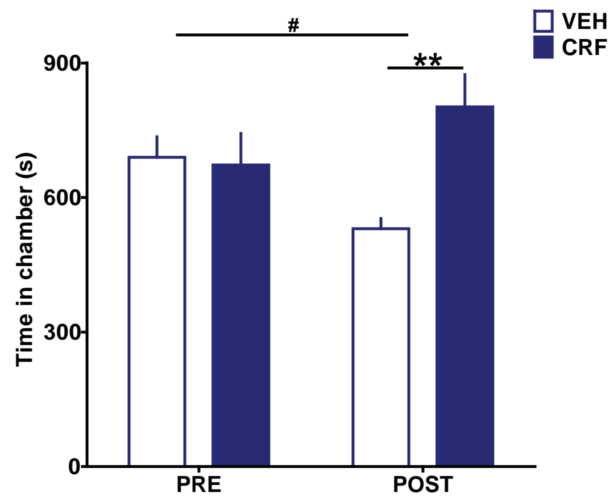
+0.62

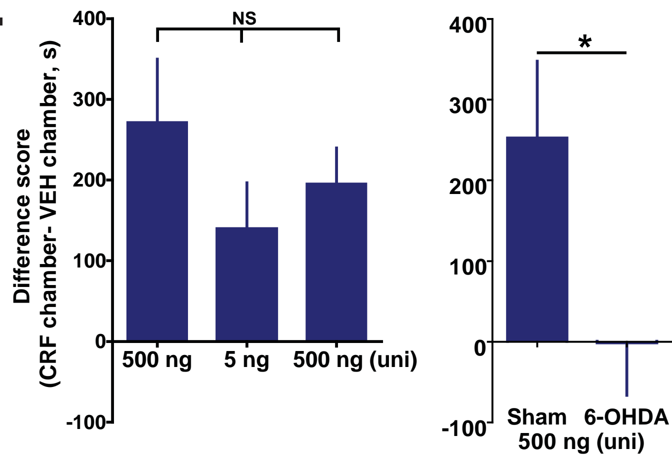
Figure 3.9. Intra-nucleus accumbens infusion of CRF produces a conditioned place preference.

a. Schematic of the CPP paradigm. b. Representative trace of time allocation on the CRF and VEH paired sides during the post-conditioning test. c. Mice spent significantly more time in the CRF-paired chamber post-conditioning than the VEH-paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,12} = 6.435$, $p < 0.001$ two-way repeated-measures ANOVA, n = 7 animals). d. Difference score from the post-conditioning test (CRF-paired chamber – VEH paired chamber) for intra-nucleus accumbens injections of CRF (500 ng/200 nl) bilateral, unilateral and CRF (5 ng/200 nl) bilateral. All three conditions yielded a significant preference ($p < 0.05$ for all conditions, one sample t-test) yet were not significantly different from each other ($F_{2,19} = 1.198$, $p = 0.3235$, one way ANOVA, n = 7-8) . Sham vehicle injected animals a significant preference for CRF similar to control animals, an effect that was absent in animals given a 6-OHDA unilateral lesion ($p < 0.05$, unpaired t-test, n = 10 for both groups).


a.
Day 1
PRE-TEST
(30 min)
Day 2
AM: ACSF or CRF
(30 min)
PM: ACSF or CRF
(30 min)
Day 3
AM: ACSF or CRF
(30 min)
PM: ACSF or CRF
(30 min)
Day 4
POST-TEST
(30 min)


b.
Vehicle
CRF


c.
VEH
CRF
#
**
Time in chamber (s)
900
600
300
0
PRE
POST


d.
Difference score
(CRF chamber- VEH chamber, s)
400
300
200
100
0
-100
NS
500 ng
5 ng
500 ng (uni)
*
Sham
6-OHDA
500 ng (uni)

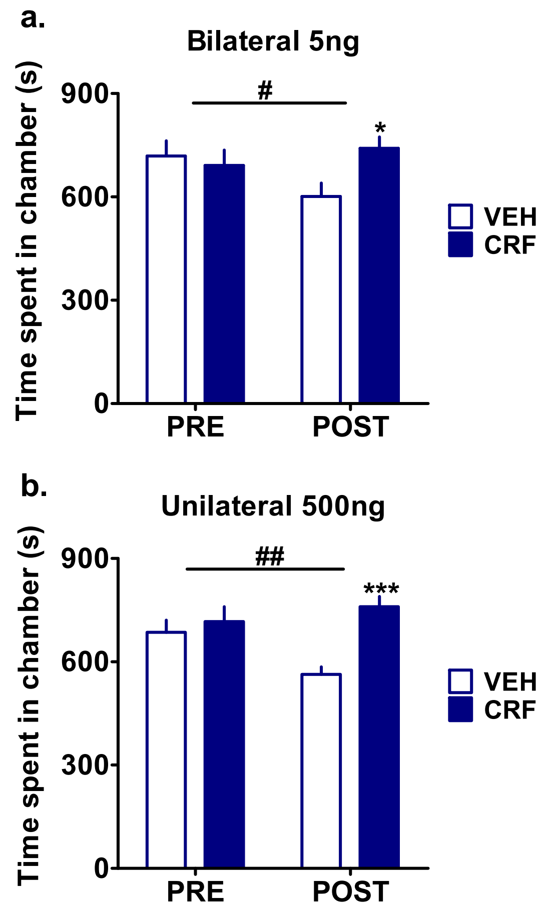


Figure 3.10. Pre- and post-test times for CRF (5 ng) bilateral injections and CRF (500 ng) unilateral injections.

a. CRF (5 ng) or VEH were administered bilaterally into the nucleus accumbens during conditioning days. Mice spent significantly more time in the CRF-paired chamber post-conditioning than the VEH paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,14} = 5.415$, $p < 0.05$ two-way repeated measures ANOVA, N= 8 mice). b. CRF (500 ng) or VEH were administered unilaterally into the nucleus accumbens during conditioning days. Mice spent significantly more time in the CRF-paired chamber post-conditioning than the VEH paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,12} = 11.77$, $p < 0.001$ two-way repeated measures ANOVA, N= 7 mice).

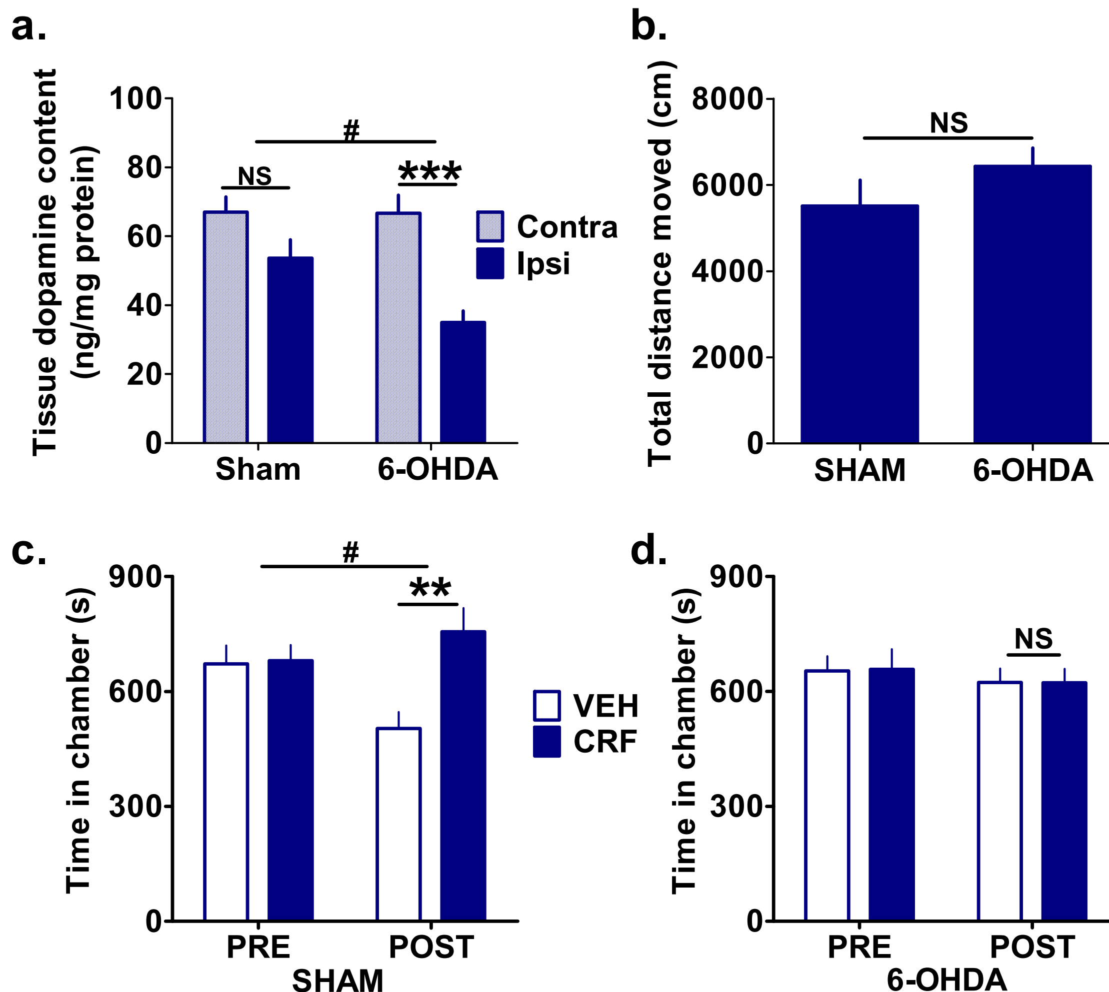


Figure 3.11. Intra-accumbens dopamine depletion with 6-OHDA blocks conditioned place preference for Intra-accumbens CRF microinfusion.

a. Unilateral injection of 6-OHDA (2 µg/500 nl) into the nucleus accumbens significantly decreased tissue dopamine content compared to both the uninjected contralateral side assessed with HPLC on fresh frozen tissue in contrast to Sham vehicle (0.9% NaCl, 0.1% ascorbate) animals that did not show significant dopamine depletion (Drug by side, $F_{1,18}$= 4.475, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc tests). b. Unilateral 6-OHDA lesion did not effect locomotor activity compared to Sham injected animals ($p > 0.05$, unpaired t-test). c. Sham vehicle injected animals significantly preferred the CRF paired chamber following conditioning (conditioning by drug, $F_{1,18}$ = 6.954, $p < 0.05$, two-way repeated measures ANOVA with Bonferroni post-hoct tests). d. 6-OHDA injected animals did not demonstrate a preference for the CRF paired chamber following conditioning (conditioning by drug, $F_{1,18}$ = 0.004, $p > 0.05$, two-way repeated measures ANOVA). # = $p < 0.05$ for the interaction, * $p < 0.05$, ** $p < 0.01$ post-hoc tests. n = 10 for both Sham and 6-OHDA groups.

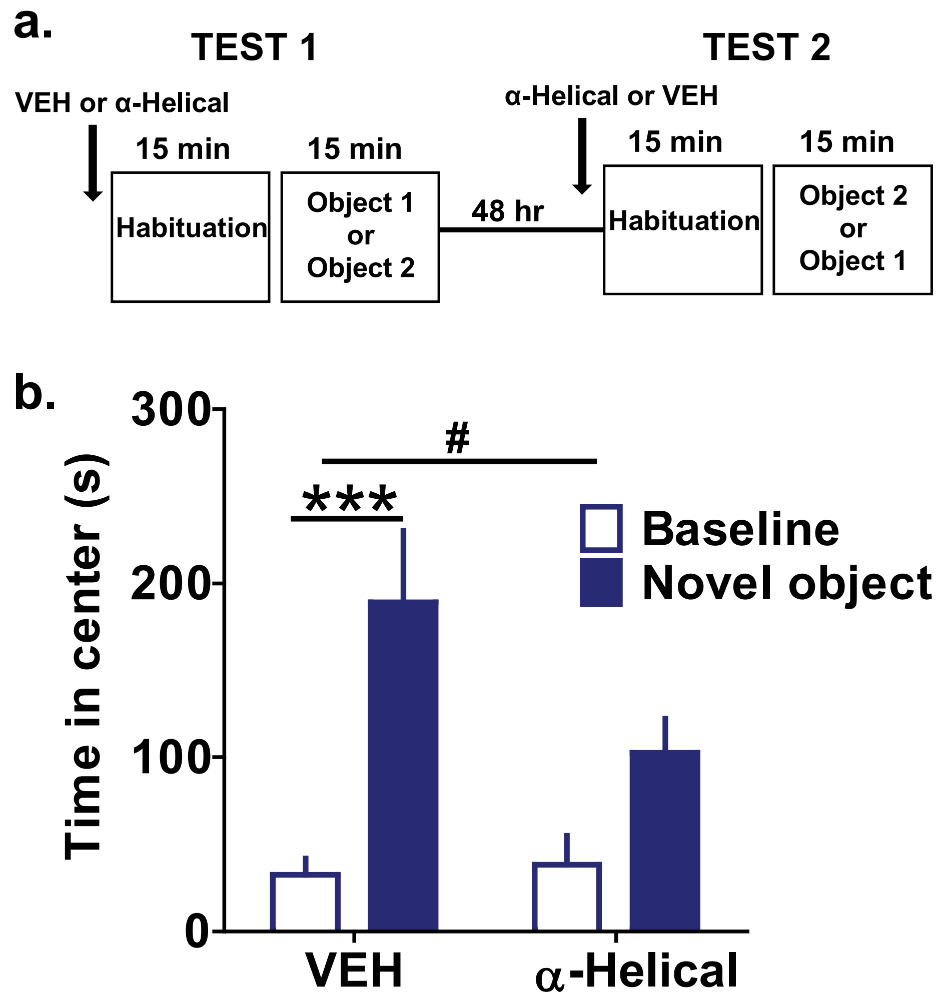
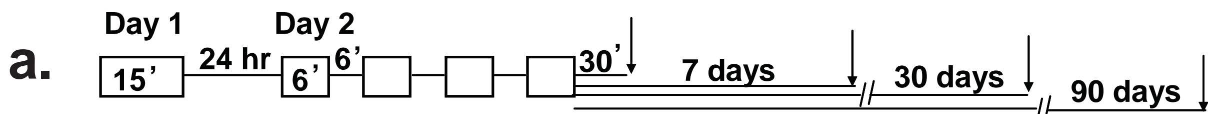


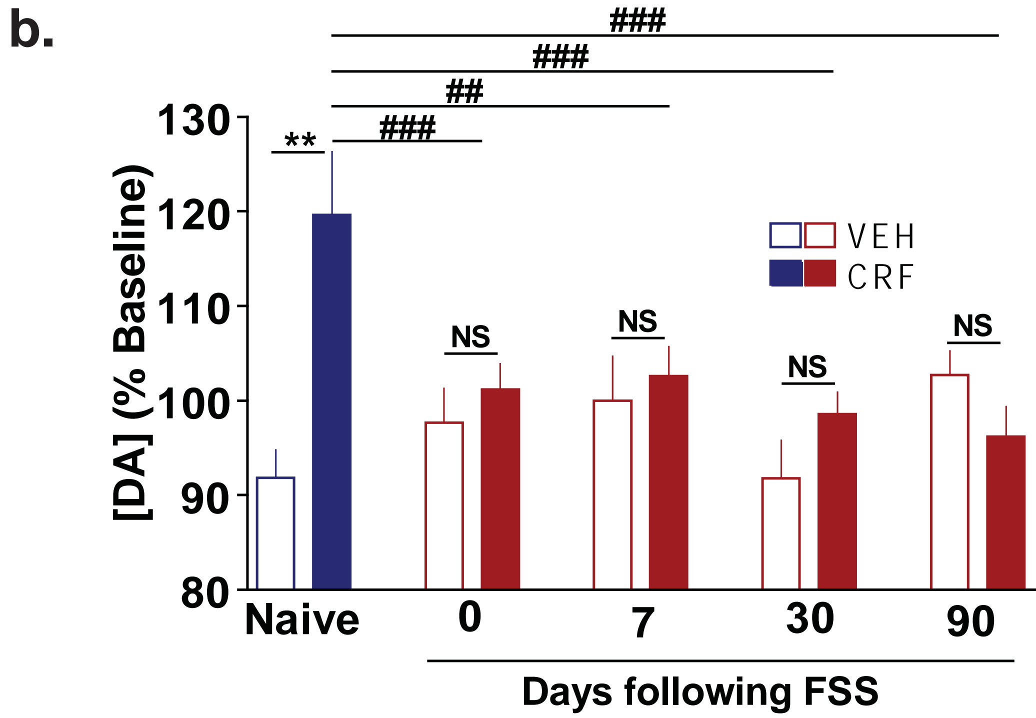
Figure 3.12. CRF is endogenously released in the nucleus accumbens to mediate exploration of a novel object.

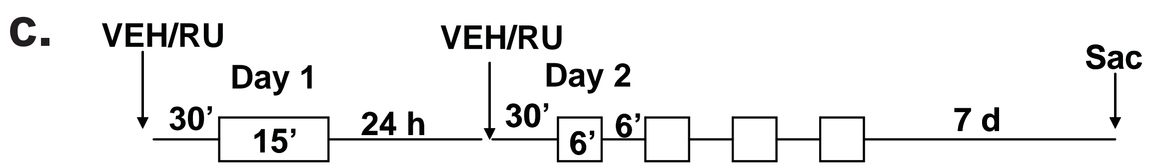
a. Schematic of novel object exploration assay. b. Animals spent significantly more time investigating a novel object placed in the center of an open field when treated with intra-accumbens bilateral microinfusions of vehicle (lactated ringer's with 1% acetic acid) compared to the non-selective CRF receptor antagonist, α-helical CRF (500 ng) (treatment by stimulus, $F_{1,18} = 4.62$, $p < 0.05$, two-way repeated-measures ANOVA, $n = 10$).

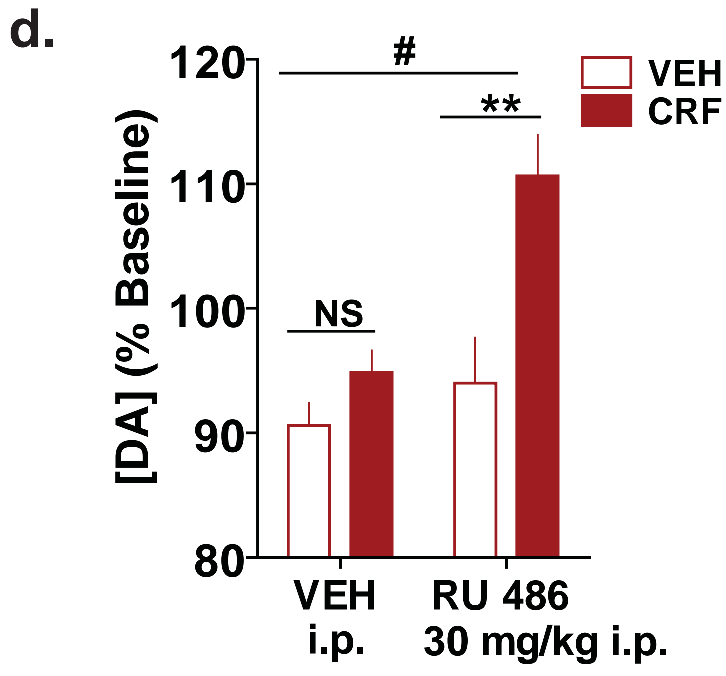
Figure 3.13. Stress exposure abolishes the CRF mediated increase in evoked dopamine release without recovery for at least 90 days.

a. Schematic of the swim stress paradigm and recovery periods. Nucleus accumbens slices were prepared 30 minutes, 7 days, 30 days or 90 days after the final swim session (black arrows). b. In contrast to naïve mice (blue bars), CRF (100 nM; closed bars) did not increase evoked dopamine release compared to vehicle (open bars) in nucleus accumbens slices from mice exposed to swim stress (red bars). The CRF response did not recover at any of the time points tested compared to naïve mice (stress exposure by drug, $F_{4,116} = 4.852$, $p < 0.01$, two-way ANOVA, n = 8 - 18). c. Time-line schematic of rescue experiments. Mice were pre-treated with either VEH (5% DMSO, 20% chremafor in saline) or RU 486 (30 mg/kg) 30 minutes prior to each swim stress day (arrows). Mice were allowed to recover for seven days and then slices were prepared. d. CRF significantly increased dopamine release compared to VEH in nucleus accumbens slices prepared from animals that had been pre-treated with RU486 (30 mg/kg) compared to VEH (i.p.) prior to each stress session (pre-treatment by drug, $F_{1,27} = 4.841$, $p < 0.01$, two way ANOVA with Bonferonni post-hoc-tests, n = 6-10).


a.
Day 1
15'
24 hr
Day 2
6'
6'
30'
7 days
30 days
90 days


b.
###
###
##
###
**
130
120
110
100
90
80
[DA] (% Baseline)
VEH
CRF
NS
NS
NS
NS
Naive
0
7
30
90
Days following FSS


c.
VEH/RU
Day 1
30'
15'
24 h
VEH/RU
Day 2
30'
6'
6'
7 d
Sac


d.
120
110
100
90
80
[DA] (% Baseline)
#
**
NS
VEH
CRF
VEH
i.p.
RU 486
30 mg/kg i.p.

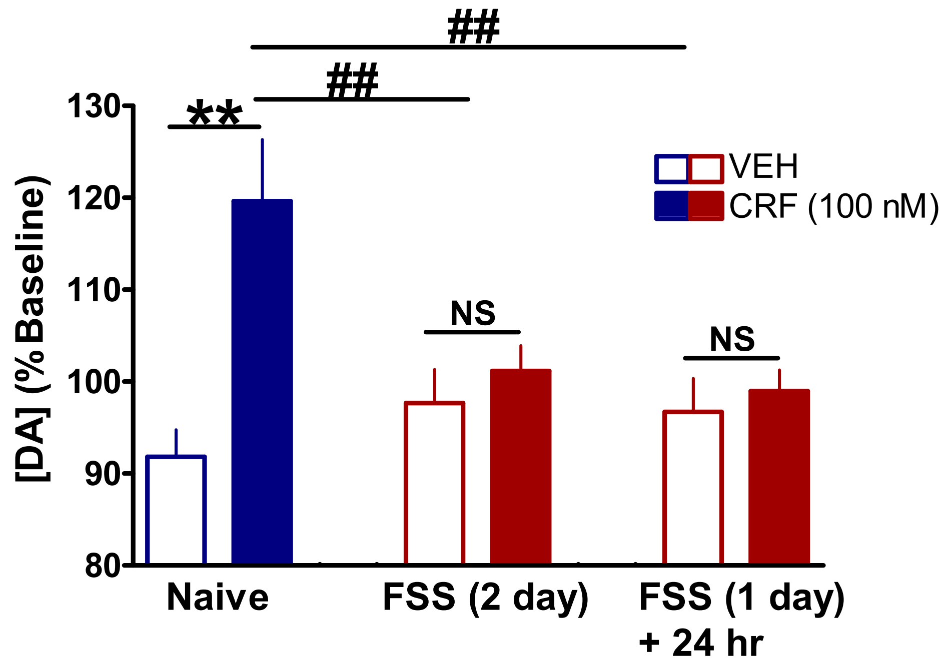


Figure 3.14. CRF is abolished following one- or two-day swim stress exposure.

In comparison to slices from naïve mice, exposure to either the modified two-day Porsolt paradigm used for all studies as well as the canonical one-day Porsolt paradigm significantly abolished CRF-mediated increase in evoked dopamine release. Animals exposed to one 15-minute swim stress were sacrificed 24 hours to time-match this set of animals with the two-day swim stress paradigm (stress exposure by drug, $F_{2,67}$ = 4.588, $p < 0.05$, two way ANOVA, n = 5-18).

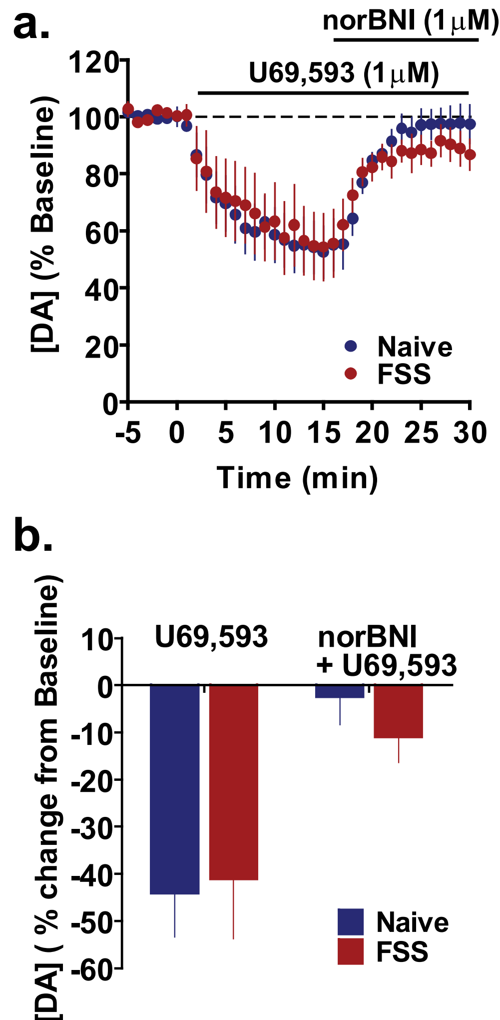


Figure 3.15. Kappa opioid regulation of dopamine release in the nucleus accumbens is unaffected in mice exposed to swim stress.

a. Time course demonstrating the effect of the kappa opioid receptor agonist U69,593 (1μM) and subsequent reversal by the kappa opioid receptor antagonist norBNI (1 μM) on stimulated dopamine release in the nucleus accumbens core of naïve and stress-exposed mice. There were no significant differences in kappa opioid receptor mediated inhibition of dopamine release in slices from naïve versus stress-exposed animals (time by stress exposure, $F_{1,14} = 0.3508$, $p > 0.05$, two-way repeated measures ANOVA). b. Mean data showing the percent change in dopamine release from baseline in the last ten minutes following U69,593 application and the last ten minutes of norBNI reversal. U69,593 produced on average a 44.08-% inhibition in nucleus accumbens animals in naïve animals and a 41.12-% in the nucleus accumbens of stress-exposed animals and in both cases was fully reversed by norBNI. There was no significant difference in mean responses between naïve and stressed groups ($p > 0.05$, Bonferroni post-hoc t-test, n = 6-10).

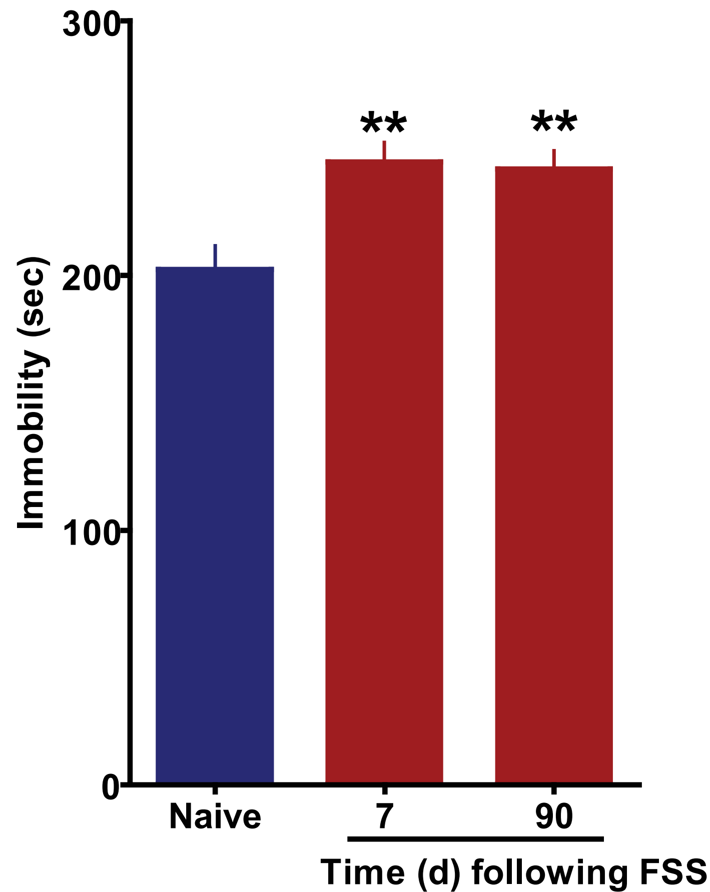


Figure 3.16. Animals displayed enhanced depression-like behavior compared to naïve animals even up to 90 days following initial stressor exposure.

Mice were given a five-minute forced swim test either in the absence of prior stress history or 7 or 90 days following exposure to an initial 2-day swim stress. Compared to stress-naïve animals, animals exposed to swim stress both 7 and 90 days prior showed significantly higher immobility during the FST indicating persistent depression-like behavior ($F_{2,25}$ = 8.287, $p < 0.01$, one way ANOVA with Neuman-Keuls.post-hoc t-test, n = 8-11).

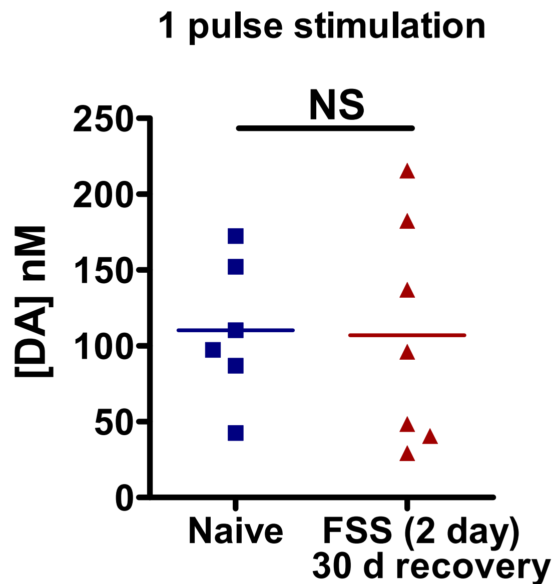


Figure 3.17. Basal evoked dopamine release was not affected by stress exposure.

Data were collected from nucleus accumbens slices prepared from naïve mice and stress-exposed mice that were allowed to recover for 30 days. A single-pulse electrical stimulation was delivered to three distinct sites in a nucleus accumbens slice and dopamine current was measured at each site. The evoked dopamine current for three sites was averaged. Following the conclusion of data collection, carbon fiber electrodes were calibrated using a flow cell system to 1 μM dopamine. Averaged current responses were converted to dopamine concentration. There were no differences in evoked dopamine release between naïve and stress-exposed animals (unpaired t-test, $p < 0.05$, $n = 6-7$).

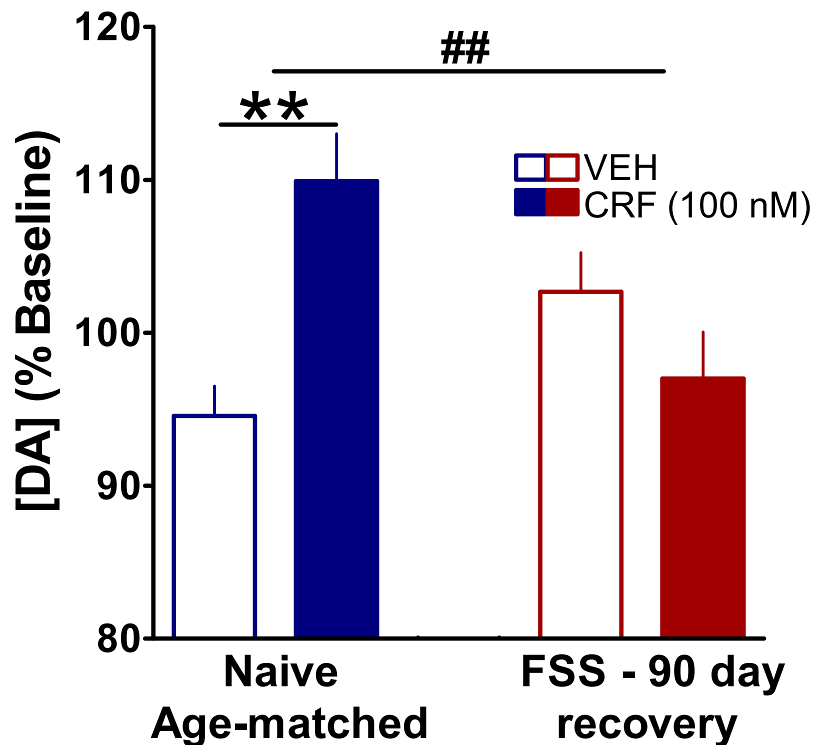


Figure 3.18. Loss of CRF response following stress exposure is not age related.

For all other experiments, animals were 60 -150 days old. However, stress-exposed animals allowed to recover for 90 days were >180 days old. To control for possible age-related effects on CRF responsivity, naïve-aged matched animals were interleaved (sacrificed and CRF response tested every other day) with stress-exposed animals allowed to recover for 90 days. Mice assigned to either the naïve or stress-exposed group were shipped on the same date and acclimated to the vivarium for the same amount of time. CRF significantly increased evoked dopamine release in nucleus accumbens slices from naïve age-matched mice compared to vehicle application, but had no effect on evoked dopamine release in the nucleus accumbens from stress-exposed animals that had recovered for 90 days compared to vehicle application (stress exposure by drug, $F_{1,42} = 10.97$, $p < 0.01$, two way ANOVA, n = 7-15).

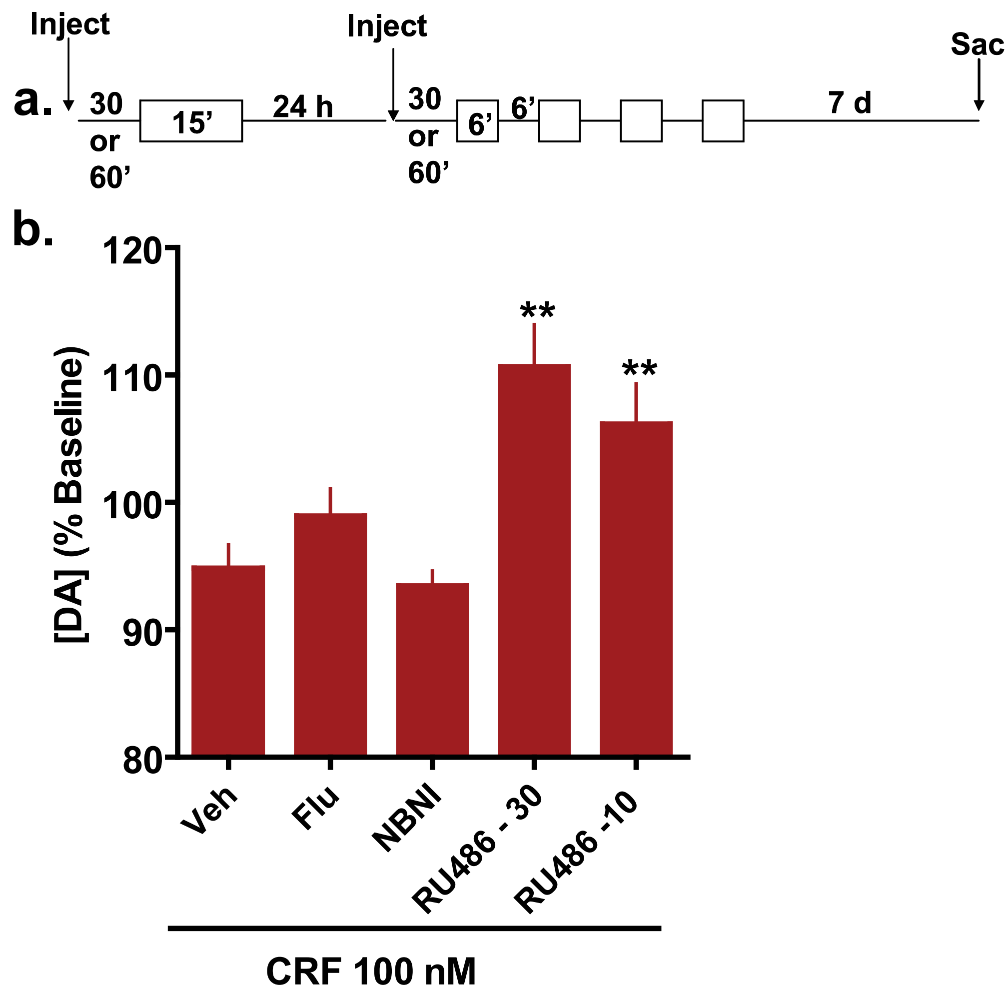
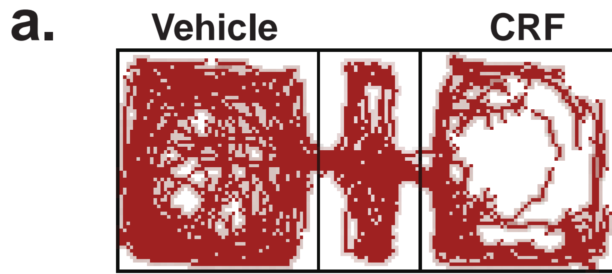


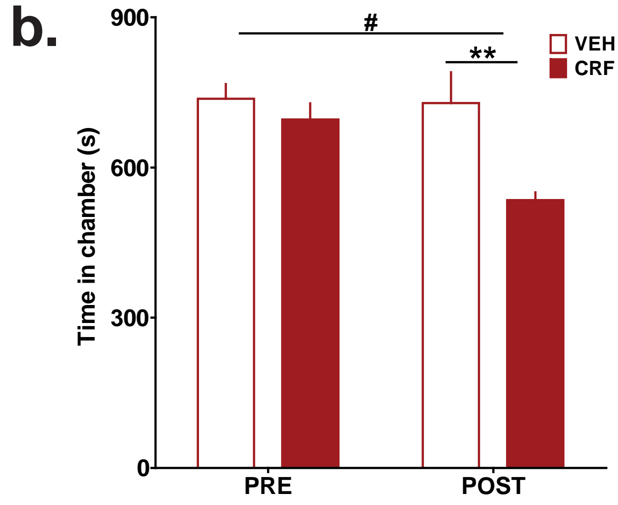
Figure 3.19. Pre-treatment with glucocorticoid receptor antagonist mifepristone prior to swim stress session protects CRF response.

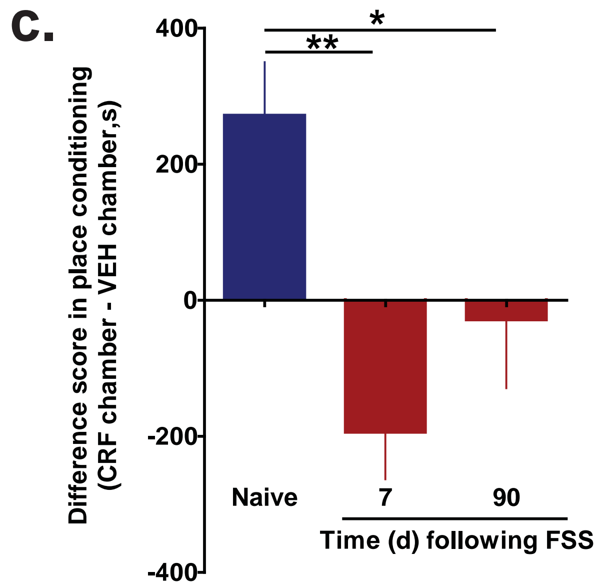
a. Mice were injected intraperitoneally (i.p.) with either vehicle (5% DMSO, 20% chremofor dissolved in saline), norBNI (kappa opioid receptor antagonist), fluoxetine (serotonin-selective reuptake inhibitor) or mifepristone/RU 486 (glucocorticoid receptor (GR) antagonist) at 10 mg/kg, 30 or 60 minutes prior to each swim session. Mice were allowed to recover seven days following the last stressor exposure. b. CRF (100 nM) significantly increased dopamine release in slices prepared from mice pretreated with RU-486 (10 or 30 mg/kg) compared to mice pretreated with vehicle. ($F_{4,48} = 6.858$, $p < 0.001$, one way ANOVA with Dunnett's post-hoc t-test compared to vehicle) but not mice pre-treated with fluoxetine or norBNI, ($F_{4,48} = 6.858$, $p > 0.05$, one way ANOVA with Dunnett's post-hoc t-test compared to vehicle n = 10-11).

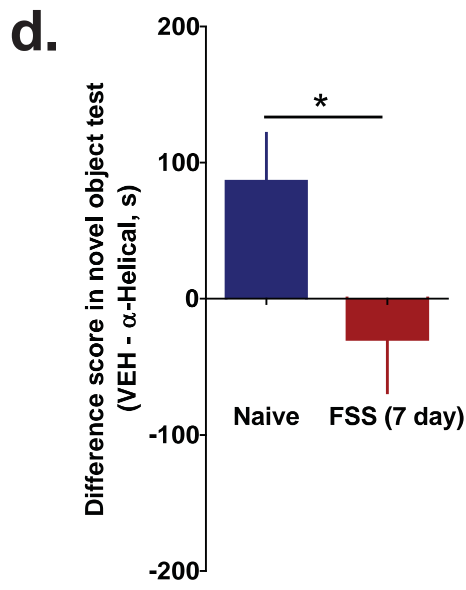
Figure 3.20. Stress exposure switches CRF action in the nucleus accumbens from appetitive to aversive in a conditioned place preference paradigm.

Seven days prior to conditioning, one group of animals was exposed to the same two-day stress paradigm used in Figure 4. Both stress-naïve and stress-exposed animals were handled daily for four days prior to conditioning. a. Representative trace of time allocation on the CRF and VEH paired sides during the post-conditioning test for stress-exposed animals. Stress-exposed mice spent significantly less time in the CRF-paired chamber post-conditioning than the VEH-paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,10} = 5.824$, $p < 0.01$ two-way repeated-measures ANOVA with post-hoc Bonferonni t-tests, n = 6 animals). d. Difference in time spent in CRF – vehicle paired chambers during the post-test for naïve (blue) or stress-exposed animals allowed to recover for 7 or 90 days (red). Difference scores were significantly different between naïve and stress-exposed animals ($F_{2,18} = 6.870$, $p < 0.01$, one-way ANOVA, n = 6-8). e. Stress exposure significantly abolished the stimulation of investigatory behavior of a novel object by CRF ($p < 0.05$, unpaired t-test, n = 9-10).


a.
Vehicle
CRF


b.
900
600
300
0
Time in chamber (s)
#
**
VEH
CRF
PRE
POST


c.
400
200
0
-200
-400
Difference score in place conditioning
(CRF chamber - VEH chamber,s)
*
**
Naive
7
90
Time (d) following FSS


d.
200
100
0
-100
-200
Difference score in novel object test
(VEH - α-Helical, s)
*
Naive
FSS (7 day)

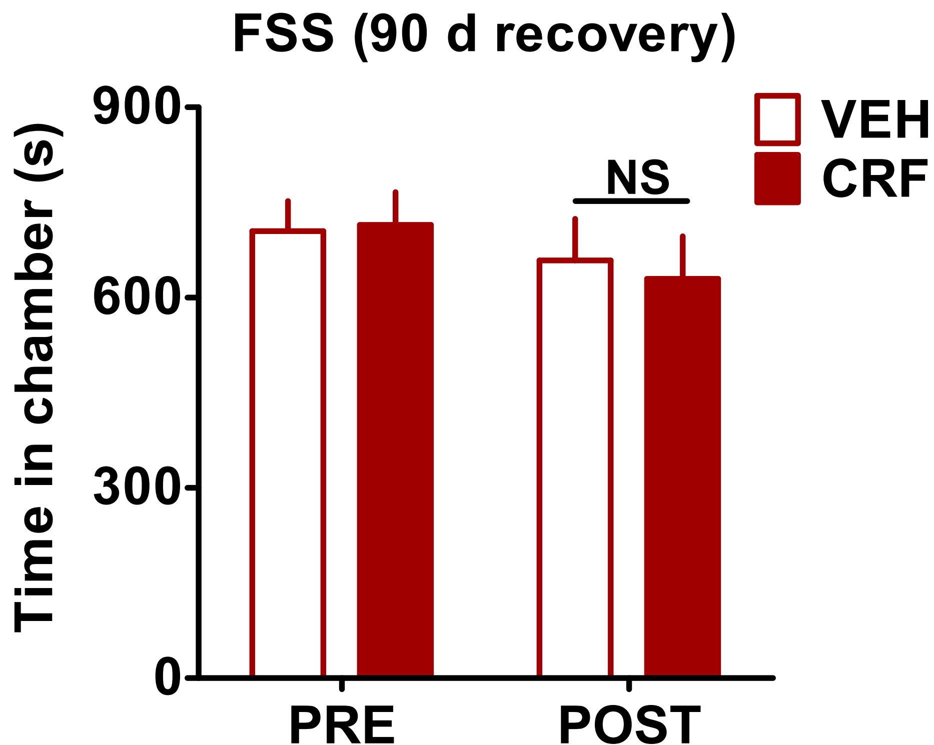


Figure 3.21. Pre- and post-test times for CRF conditioned place preference in mice exposed to two-day FSS and allowed to recover for 90 days.

Mice that had been exposed to 2-day swim stress 90 days prior to conditioning did not show a conditioned place preference to the CRF-paired context compared to naïve animals (conditioning by drug, $F_{1,14} = 0.1035$, $p > 0.05$, two-way repeated measures ANOVA, n = 8).

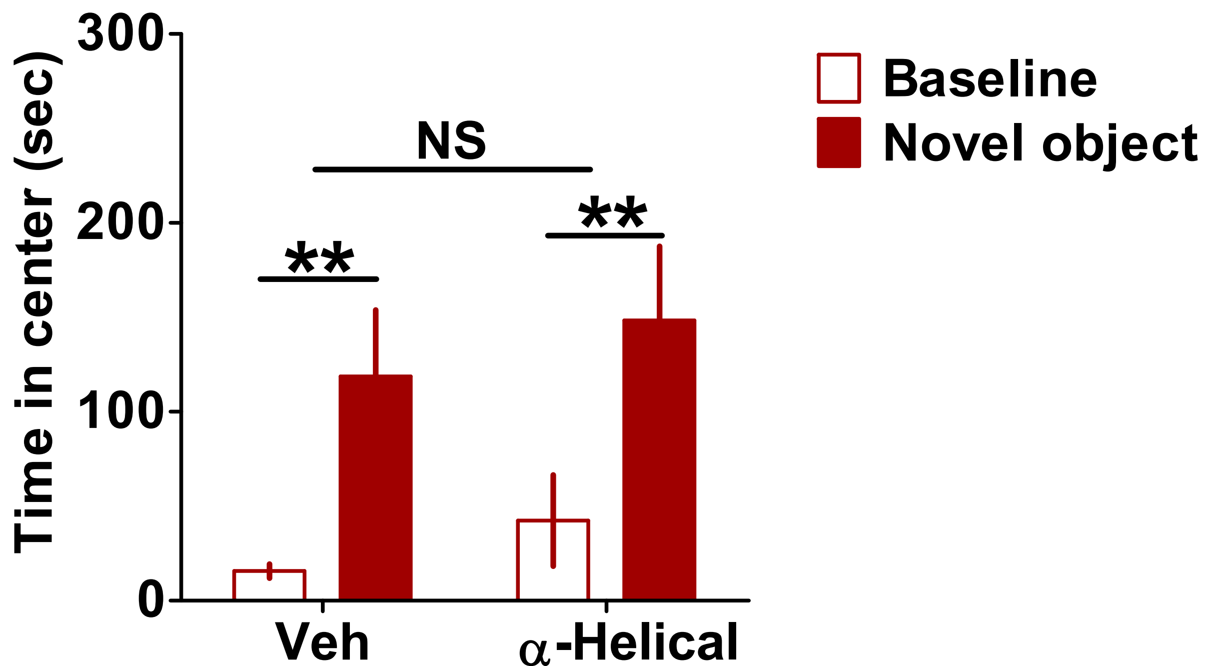


Figure 3.22. Stress exposure abolishes CRF-dependent component of novel object exploration.

Animals were exposed to two-day repeated swim stress 7 days prior to the first test day of the novel object exploration task. Identically to naïve animals, stress-exposed mice were given infusions of vehicle or α-helical CRF (500 ng) in a counter-balanced fashion across test days, prior to placement in an open field. While introduction of a novel object significantly increased center time in both drug conditions, there was not a significant drug interaction, indicating that stress exposure abolished the CRF-dependent component of novel object exploration (stimulus by drug, $F_{1,16} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA, n = 9). ** $p < 0.01$ for Bonferroni post-hoc t-tests.

Table 3.1. Quantification of EM labeling in 100-nm sections through the rostro-caudal axis of the nucleus accumbens to assess co-localization of CRF receptors and TH immunoreactivity.

	TH-axon terminals	CRFr in TH-at	Percentage
Mouse 1	299	41	13.71%
Mouse 2	367	38	10.35%
Mouse 3	390	75	19.23%
Total	1056	154	14.58%

To estimate the proportion of dopamine terminals that express at least one CRF receptor, we used the equation $P = 1 - (1 - p)^n$ where p is the probability of observing a CRF-receptor immunogold particle per section of a TH-positive terminal and n is the number of sections per terminal. CRF receptor immunoreactivity was observed on 14.58 % of section of TH-positive terminals (i.e., p = 0.1458). Given that dopamine terminals are approximately 1 μm in diameter, each total three-dimensional terminal profile occupies approximately ten 100-nm sections (i.e., n = 10). Therefore, we estimate that 80 % of dopamine terminals in the nucleus accumbens express CRF receptors.

Chapter 4: Repeated stress dysregulates kappa opioid receptor signaling in the dorsal raphe through a p38 MAPK dependent mechanism

The following chapter has been adapted from:

Lemos JC, Roth CA, Messinger DI, Gill HK, Phillips PEM, Chavkin C. Repeated stress dysregulates kappa opioid receptor signaling in the dorsal raphe through a p38 MAPK dependent mechanism**.** *Under review at Journal of Neuroscience*

Specific contributions:

J.C.L. carried out the electrophysiology experiments. J.C.L. and C.A.R conducted immunohistochemistry experiments. C.A.R. conducted quantitative analysis of immunohistochemistry experiments and conducted analgesia experiments. D.I.M. bred all transgenic mice lines. H.K.G. analyzed behavioral data. J.C.L., P.E.M.P. and C.C. developed the conceptual and experimental framework.

Introduction

Adaptive responses to stress exposure are critical to an organism's survival and rely upon a precisely tuned constellation of neurotransmitters and neurohormones acting both centrally and peripherally [30]. Moreover, the etiology of stress-induced mood disorders is thought to involve a combination of pathological neuroadaptations that include alterations in both stress-related neurocircuitry and signaling [23]. Dynorphins, the endogenous ligands for the kappa opioid receptor (KOR) [161], are released and up-regulated during stress exposure [202,236,248], and these endogenous opioid neuropeptides have emerged as critical mediators of several stress-related behaviors[196,249]. For example, genetic deletion of either prodynorphin or KOR or pharmacological blockade

by KOR antagonists reduce stress-induced anxiety-like, depression-like, and proaddictive behaviors (see[250]). However, the cellular and molecular mechanisms by which the dynorphin-KOR system regulates neuronal excitability in limbic regions and ultimately produces its behavioral effects remain unclear.

Activation of KOR either pharmacologically or following release of endogenous dynorphins results in both membrane-delimited Gβγ regulation of ion channel conductance and mitogen activated protein kinase (MAPK) signaling (Bruchas et al., 2010). Prolonged or repeated activation of KOR also stimulates the G-protein kinase 3 / β-arrestin signaling cascade that subsequently recruits and activates p38 MAPK [202,251,252], and p38 MAPK activation has been shown to be required for the aversive qualities of stress as well as promoting passive coping strategies [202,253]. Recently, we demonstrated that KOR-dependent activation of p38α MAPK in 5-HT neurons in the DRN is both necessary and sufficient to induce a negative affective state [253,254].

The importance of the DRN for stress-related behaviors is well established [13]. The DRN is a principal serotonergic nucleus in the brain that sends afferents to many forebrain limbic regions [255-258] and is specifically activated during exposure to uncontrollable stress [13,86,254,259,260]. Therefore, elucidating the regulation of DRN activity by stress-related neuropeptides in naïve and stress-exposed individuals is important to understand how the pathological effects of stress impact brain functioning.

In the present study, we measured the effects of KOR activation in DRN using whole-cell voltage clamp electrophysiological techniques to isolate glutamatergic and GABAergic fast synaptic activity as well as isolating post-synaptic G-protein gated

inwardly rectifying potassium (GIRKs, Kir3) currents. We then investigated how these processes were modified by repeated exposure to stress and specifically sought to identify p38 MAPK-dependent defects in this regulation caused by repeated stress. We hypothesized that repeated stress would result in sustained dynorphin release that would in turn disrupt KOR regulation of 5-HT neuronal excitability in the DRN via a p38α MAPK-dependent process.

Methods

Subjects: Male C57BL/6 mice, age >50 days, were maintained under a 12-h light–dark cycle (7a.m. to 7p.m. light) with access to standard food and water *ad libitum.* All procedures on animal subjects were approved by the University of Washington IACUC committee. Mice that were housed together (2 - 4 per cage) were subjected to the same behavioral treatment. Prodynorphin (Dyn -/-) knock-out mice and p38αCKO^{SERT} mice were derived as described in [208] and [253] respectively and have been backcrossed to C57BL/6 background >10 generations.

Swim stress: Mice were subjected to a two-day swim stress procedure in which they were exposed to a 15-min swim session on day one, then were exposed to four six-min swim sessions in 29.0 – 31.0 °C water, separated by six min and conducted under bright light (690-700 lux) conditions on day 2 as previously described [170].

***Tail flick*:** Stress-induced analgesia was assessed using the warm water (52.5°C) tail withdrawal assay as described [261,262].

Immunohistochemistry

Tryptophan hydroxylase (TPH) Immunohistochemistry staining and intracellular labeling

During electrophysiological recordings, cells were filled with biocytin (0.1%) (Sigma-Aldrich) present in the recording electrode. After recording, slices were fixed by submersion in 4% paraformaldehyde prepared in 0.1M phosphate buffer (PB; pH 7.4). Sections were incubated with mouse anti-TPH antibody (1:500, Sigma-Aldrich) for 12-16 hr at room temperature (RT). Subsequently, immunohistochemical labeling was visualized using Alexa Fluor 488 (1:500; Invitrogen) conjugated goat anti-mouse secondary antibody for 90-120 min at RT. Biocytin was visualized using streptavidin conjugated Alexa Fluor 647 (1:500, Invitrogen) contained in the same secondary antibody mixture. Between incubations, slices were rinsed with PBS (3 x 10 min) and all incubations were done with mild agitation on an orbital shaker.

Phospho-antibody Immunohistochemistry

Methods for immunohistochemistry experiments were similar to those reported previously (Bruchas et al. 2007). Mice were intracardially perfused with 4% paraformaldehyde in 0.1M PB. It has been our experience that phospho-protein immunoreactivity (ir) is of higher quality if PBS perfusion prior to 4% PFA is omitted. Brains were dissected and cryoprotected with 30% sucrose in 0.1M PB at 4°C overnight (or until the brains submerged), frozen and cut into 30 μm sections using a Leica SM200R microtome, and placed in 0.1M PB with 0.1% sodium azide until processing. We had previously generated a rabbit polyclonal antibody against the phosphorylated Ser369 residue of KOR [206] that is now available commercially at Abcam. We also generated a rabbit polyclonal antibody against the phosphorylated-Tyr 12 residue of K_{IR} 3.1 (GIRKp) as described in Ippolito et al. (2005). Standard immunohistochemical

procedures were performed to obtain phospho-KOR (KORp) (Abcam, ab63511), phospho-p38 MAPK (mouse monoclonal sc-7973) and p38α (rabbit polyclonal sc-535) (Santa Cruz) and pY12-K_{IR} 3.1staining. Briefly, floating sections were washed 3 x 10 min in PBS and then blocked for 60 min in 5% normal goat serum, 0.3% Triton-X in PBS. Importantly, we have found that for these phospho-antibodies use of floating sections produces higher quality staining than thaw mounted sections. Sections were incubated in primary antibody at different concentrations, temperatures and incubations for each antibody: KORp (1:200, 72 hr at 4°C); mouse anti-phospho-p38 MAPK/ rabbit anti-p38α (Santa Cruz) (1:50, 36 hr RT or 72 hr at 4°C); affinity purified GIRK-p (1:100 for 72 hr at 4°C; the concentration is based on a 750 μg protein/ml yield as described (Ippolito et al., 2005)). Often slices were co-labeled with mouse anti-TPH (Sigma) at 1:1000; guinea pig anti-Dynorphin B (Peninsula Laboratories) was used at 1:200. Slices are then washed in PBS 10-15 min x 3 and incubated in goat Alexa Fluor antibody (mouse or rabbit, respectively) at 1:500 to 1:750 for 2 hr at RT. Following secondary antibody incubation, slices were washed 3 x 10 min in PBS, then 2 x 10 min in 0.1M PB. Sections were then mounted on Superfrost plus slides and converslipped with Vectashield (Vector Laboratories).

Standard epifluorescent and confocal microscopy techniques (Nikon Eclipse E600 and Leica SL, respectively) were used to image sections for both TPH confirmation and phospho-antibody staining. For comparisons of phospho-ir from different behavioral treatment groups, three animals for each group were perfused, sectioned and processed in parallel. Imaging of sections from animals exposed to different treatments were done at the same time with the same camera settings. As it is

typical for different rounds of staining to yield different immunoreactive intensities, images from each treatment group displayed in this study came from the same round of staining. The pattern of KORp-ir and GIRKp-ir across treatment groups seen in the dorsal raphe was similar to that previously reported both in vitro and in vitro [181,202,251,263,264].

Phospho-p38-ir quantification

The selectivities of both the p38α and phospho-p38 MAPK antibodies were previously established using both viral vector expression and conditional gene knock-out approaches [253]. We quantified phospho-p38 MAPK-ir using Metamorph software as a ratio of phospho-p38 MAPK positive cells to p38α positive cells (i.e. co-localized). Similar quantification of GIRKp-ir and KORp-ir could not be performed since the antibodies against the unphosphorylated forms of GIRK and KOR were also raised in from rabbits. We have previously confirmed that p38α is the isoform that is phosphorylated following KOR activation [253]. Cells are considered positive for the protein if the intensity of the immunoreactivity is one-standard deviation above the average background pixel intensity.

Electrophysiology

Procedures were similar to those previously reported [88,265]. Mice were decapitated, head placed in ice cold sucrose buffer during the dissection, and the brain rapidly removed and blocked. The blocked tissue was mounted on the stage of a Leica VT1000 S vibratome and surrounded in oxygenated sucrose buffer. Sections (200 μm thick) were cut through the raphe nuclei in sucrose and then placed in oxygenated

95%O_2/5%CO_2 ACSF incubated in a 35-37˚C bath for 1 hr. The slices were then removed from the bath and kept in oxygenated ACSF at RT. ACSF was composed of (mM): NaCl 124, KCl 2.5, NaH_2PO_4 2, $CaCl_2$ 2.5, Dextrose 10 and $NaHCO_3$ 26. The sucrose buffer was ACSF in which NaCl was replaced by 248 mM sucrose.

Slices were placed in a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with oxygenated ACSF at approximately 1.5 ml/min, maintained at 30-32˚C by an inline solution heater (TC-324, Warner Instruments). For recording EPSCs or GIRK currents, the resistance of the electrodes was 5-10 MΩ when filled with an internal solution of (mM) K-gluconate 130, NaCl 5, Na phosphocreatine 10, $MgCl_2$ 1, EGTA 0.02, HEPES 10, MgATP 2, Na_2GTP 0.5, 0.1% Biocytin, pH 7.3. For measuring IPSCs, the resistance of the electrodes was 4-8 MΩ when filled with an intracellular solution of (mM) K-gluconate 70, KCl 70, NaCl 2, Na phosphocreatine 10, EGTA 4, HEPES 10, MgATP 2, Na_2GTP 0.3, 0.1% Biocytin, pH 7.3. Whole cell recordings in voltage clamp were performed on cells in the ventromedial and dorsomedial aspect of the DRN. The cell was voltage clamped at -70 mV using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA). Signals were digitized by a Digidata 1440 A/D converter (Molecular Devices) and stored using pClamp 10.2 software (Molecular Devices). EPSCs were isolated using picrotoxin (10 µM) (Sigma-Aldrich) and CGP 55845 (1 µM) (Tocris Biosciences). IPSCs were isolated using DNQX (20 µM) and APV (50 µM) (Tocris Biosciences). For evoked recordings, the stimulating electrode was placed 150-200 µm dorsolateral of the recording site. Paired pulse stimulation (1 msec pulse width, 50 msec inter-stimulus interval, 100-1000 µA) was delivered every 10 sec. A mid-range output response was used as baseline for each

cell (e.g. approximately the middle of an input-output curve) to allow for observation of either positive or negative regulation by U69,593 (National Institute of Drug Abuse Drug Supply Program). For mPSCs, TTX (1 μM) (Calbiochem) was added to the bath. For GIRK current experiments, once a recording had been established, the slice was bathed in 5.5 mM K^+ ACSF (from 2.5 mM K^+) to enhance the currents in the cells at hyperpolarized potentials. As expected, when the slice was incubated in high [K^+], the cells became more depolarized and inward rectification was more apparent. This increased current was blocked by 100 μM $BaCl_2$ [266]. The cells were subjected to a voltage ramp protocol in which they were brought from -120 mV to -50 mV over 10 sec. After three stable ramp measurements (the average of three sweeps) were obtained, U69,593 (1000 nM) was bath applied and the slice was allowed to equilibrate for 3 min. Subsequently three ramp measurements were taken every 3 min for 12 additional min and the last three ramp measurements (after 15 min of U69,593 application) were averaged. Following U69,593 application and stable current responses, $BaCl_2$ (100 μM) was added to distinguish Ba^{2+}-sensitive GIRK currents.

Data analysis and statistics

Electrophysiological data were analyzed using Clampfit 10.2 (Molecular Devices) and MiniAnalysis 6.0.7 software (Synaptosoft, Inc.). One-way repeated measure ANOVAs or paired t-tests were used for within-cell experiments (pre-drug baseline vs. post-drug effect). We used unpaired t-tests as well as one-way and two-way ANOVAs as specified in the Results, for comparisons made across behavioral treatment groups and genotypes. The responses of individual cells were considered separate N, as is standard for electrophysiology experiments, however, when comparing differences

between different behavioral treatment groups (i.e. naïve vs. stress), a minimum of 3 animals from at least two different cages were used per group.

Results

The dorsal raphe is a heterogeneous nucleus containing approximately 70% 5-HT and 30% non 5-HT containing neurons [267]. Recordings were confined to the dorso- and ventro-medial aspect of the dorsal raphe (see representative low power image; Figure 1a, left panel), which has the highest density of the 5-HT-containing neurons. Additionally, during electrophysiological recordings, cells were filled with biocytin and subsequently slices were post-fixed and processed using standard immunohistochemical procedures to distinguish serotonergic (TPH-ir positive) and non-serotonergic cells (Figure 4.1a). Only 12% of cells were not positively identified as serotonergic neurons, and these were not included in the subsequent analysis.

Kappa opioid activation in serotonergic dorsal raphe neurons has a net inhibitory effect

Following 5-10 min of stable baseline evoked EPSC amplitude responses, the kappa opioid receptor agonist, U69,593 (500 nM, then 1000 nM) was bath applied to the slice; both eEPSC amplitude and paired paired-pulse ratio were measured. Representative traces are provided demonstrating that KOR activation by U69,593 decreased eEPSC amplitude of the first evoked EPSC (red line) compared to baseline responses (black line) (Figure 4.1b, inset). U69,593 at 500 nM and 1000 nM significantly inhibited eEPSC amplitudes by 31 ± 6% and 37 ± 8% respectively ($F_{2,29}$ =13.53, $p < 0.0001$, one-way repeated measures ANOVA with Dunnett's post-hoc t-test, Figure 4.1b and c). We verified that cumulative dosing did not result in KOR desensitization by showing that 5

μM U69,593 added to a previously untreated slice did not produce a greater inhibition than 500 or 1000 nM added cumulatively (data not shown). Inhibition of eEPSC amplitude significantly correlated with the increase in paired pulse ratio ($r^2 = 0.39$, $p < 0.05$) (Figure 4.1d), indicating that KOR activation reduced probability of glutamate release via a presynaptic mechanism.

To further assess the site of KOR action, we also measured the effect of U69,593 (1000 nM) on miniature EPSC (mEPSC) frequency and amplitude, and representative traces are shown (Figure 4.2a). Cumulative histograms of both the inter-event interval (Figure 4.2b) and amplitude (Figure 2c) corresponding to these traces are also shown. U69,593 significantly decreased mEPSC frequency from baseline (from 7.1 ± 1.8 Hz to 4.1± 1.1 Hz, $p < 0.05$), which was blocked by pre-treatment with the KOR antagonist norBNI (1 μM) (from 7.6 ± 1.3 Hz to 6.1± 1 Hz, $p > 0.05$) (norBNI treatment by time, two-way repeated measures ANOVA, Bonferroni post-hoc, * $p < 0.05$, Figure 4.2d). U69,593 did not significantly affect mEPSC amplitude in either the presence or absence of norBNI (norBNI treatment by time, two-way repeated measures ANOVA, Bonferroni post-hoc, $p > 0.05$, Figure 4.2e). These data confirm that acute activation of KOR presynaptically depresses excitatory synaptic transmission.

Because KORs regulate both glutamatergic and GABAergic transmission with similar efficacies in other brain regions [111,113,174,268,269], we next assessed the effects of U69,593 on both evoked inhibitory post-synaptic current (eIPSC) amplitude and miniature IPSC frequency and amplitude. As in the eEPSC experiments, a paired pulse stimulation was delivered to the slice and baseline recordings of pharmacologically isolated eIPSCs (e.g. in the presence of DNQX and APV) were obtained. KOR

activation by U69,593 had no significant effect on eIPSC amplitude (representative traces, Figure 4.3a; time course, Figure 4.3b; $p > 0.05$) nor did it significantly affect paired pulse ratio ($p > 0.05$). Similarly, U69,593 had no effect on mIPSC (representative traces, Figure4. 3c), frequency (paired t-test, $p > 0.05$, Figure 4.3d), or amplitude (paired t-test, $p > 0.05$, Figure 4.3e). These data demonstrate that KORs presynaptically inhibit excitatory synaptic input without affecting GABAergic transmission in DRN 5-HT neurons, producing a net reduction in excitability.

KOR activation has also been shown to have direct, post-synaptic effects in the cells of the spinal trigeminal nucleus [270]. However, this has not previously been examined in DRN. Both the conductance (slope) and peak inward currents were determined for pre-drug and post-drug voltage ramp measurements (see representative traces, Figure 4.4a). U69,593 significantly increased both the conductance (3.7± 0.4 nS to 4.5 ± 0.4 nS, paired t-test, $p < 0.01$, Figure 4.4b) and the peak Ba^{2+}-sensitive inward current (87.8 ± 24.1 pA to 145 ± 24.0 pA, paired t-test, $p < 0.01$, Figure 4.4c). U69,593 increased the GIRK current 48.7 pA above baseline, and this effect was blocked by 1 μM norBNI pretreatment (unpaired t-test, $p < 0.001$, Figure 4.4d). These data suggest that in stress-naïve animals, KOR activation has an inhibitory effect on serotonergic neuronal excitability through both pre- and post-synaptic sites of action.

Repeated stress exposure reduces KOR inhibitory regulation on 5-HT neuronal excitability post-synaptically

We found evidence for dynorphin B-ir within the DRN, but in contrast to a previous study by Fu et al. (2010), this staining was present in both TPH-ir positive and

TPH-ir negative cells (Figure 4.5a, yellow and white arrows, respectively), suggesting that dynorphin may be locally released from a heterogeneous population of cells that includes serotonergic neurons. To evoke endogenous dynorphin release in DRN, we used the previously validated two-day repeated force swim stress (FSS) paradigm [170,271] (Figure 4.5b). This modified Porsolt procedure produced escalating immobility within the first 15-min session as well as enhanced immobility over the second day of swim sessions ($F_{6,54} = 40.59$, $p < 0.0001$, one-way repeated measures ANOVA, Figure 4.5a), indicative of increased passive coping behavior [34]. In agreement with our previous findings [170], this two-day swim paradigm produced significant stress-induced analgesia (as measured with the warm water tail immersion assay) that could be prevented by norBNI pre-treatment (time by behavioral treatment, $F_{2,36} = 9.969$, $p < 0.001$, two-way repeated measure ANOVA, Figure 4.5d), indicating that this stress exposure evoked dynorphin release. We previously showed that dynorphin release can be detected by an increase in KOR phosphorylation (KORp-ir) (McLaughlin et al., 2003b); agonist activated KOR is a substrate for G-protein Receptor Kinase (GRK3) which phosphorylates ser369 in the carboxy-terminal tail of KOR. Endogenous dynorphin released by neuropathic pain stress or administration of the stress-related peptide corticotropin releasing factor intracerebroventricularly increased KORp-ir in wild type mice but not in mice lacking the gene for preprodynorphin (Dyn -/-) [205,207]. Using increases in KORp-ir to detect sites of endogenous dynorphin action in the present study, we found that KORp-ir was significantly increased in the dorsal raphe nucleus evident 30 min following the cessation of the last swim (Figure 4.5e). This elevation in KORp-ir returned to basal levels in animals that were allowed to recover for 24 hr

following the last swim session (Figure 4.5e). At higher magnification (60 and 100 x), we observed that KORp-ir puncta were robustly localized to the somata of DRN cells and to a lesser extent outside of the somata, interdigitated with synaptophysin or vGlut1 postive puncta (data not shown). However, even at this resolution we could not distinguish KORp-ir in presynaptic from post-synaptic subcellular profiles. These results confirmed that the two-day swim stress paradigm produced escalating depression-like behaviors, robust and transient activation of KORs through release of endogenous dynorphins, and dynorphin-KOR-dependent stress induced analgesia.

Repeated swim stress exposure significantly reduced subsequent in vitro postsynaptic responses to U69,593 in slices prepared 30 min after the final swim session (representative traces, Figure 4.6a). There was no significant difference in basal GIRK current between naïve and swim-stressed groups (Naïve: 76.4 ± 23 pA; FSS (2d): 66 ± 19.5 pA, unpaired t-test, $p > 0.05$). The increase in GIRK current caused by U69,593 (1 μM) was 51% smaller in serotonergic neurons recorded in slices from FSS-treated compared with those from naïve mice (Figure 4.6b). The reduction in KOR stimulated GIRK currents was not evident in slices from mice allowed to recover for 24 hr after the final swim session ($F_{2,19} = 4.432$, $p < 0.05$, one way ANOVA, Figure 4.6b). Mice exposed to additional five-minute daily swim sessions for 5 more days (FSS 7d) did not further decrease subsequent in vitro KOR responses (Naïve: 48.7 ± 8.6 pA; FSS (2d): 23.7 ± 6.4 pA; FSS (7d): 25.7 ± 3.6 pA) ($F_{2,18} = 4.217$, $p < 0.05$, one-way ANOVA). The dysregulation of the KOR response caused by forced swim was not evident in Dyn-/- mice ($p > 0.05$, unpaired t-test, Figure 4.6b). Thus, although multiple neurotransmitters were likely released during swim stress exposure [34], the most parsimonious explanation

for stress-induced dysregulation of KOR responsivity is that dynorphins were being repeatedly released to activate KORs. While this does not preclude the involvement of other stress-related neurotransmitters, these data demonstrate that the reduction in KOR regulation of inward current required stress-induced endogenous dynorphin release.

Interestingly, repeated stress exposure had no significant effect on KOR regulation of excitatory presynaptic transmission recorded in serotonergic neurons (Figure 4.7). U69,593 had a similar inhibitory effects on evoked EPSC amplitude in slices from naïve and FSS exposed mice (Naïve: 38% inhibition for 1000 nM; FSS: 43% inhibition for 1000 nM; $p > 0.05$, two-way repeated measure ANOVA, Figure 4.7a). Cells recorded from stress-exposed mice had a significantly larger mEPSC amplitudes compared to naïve animals (two-way repeated measure ANOVA, main effect of behavioral treatment, $F_{1,10} = 16.08$, $p < 0.01$) suggesting that these cells were more excitable following FSS. The inhibition of both mEPSC frequency (behavioral treatment by time, $F_{1,10} = 0.3210$, $p > 0.05$, two-way repeated measures ANOVA) and amplitude (behavioral by time, $F_{1,10} = 3.024$, $p > 0.05$, two-way repeated measures ANOVA) by U69,593 was not significantly different in 5-HT neurons from naïve compared with stress-exposed animals (Figure 4.7b and c). The trend for U69,593 to slightly decrease mEPSC amplitude observed in naïve animals was significant in stress-exposed animals (post-hoc t-test, $p < 0.01$). The lack of a sustained effect of dynorphin release on the excitatory inputs suggests that either the presynaptic KORs were not desensitized or that their desensitization quickly recovered. Taken together, these data demonstrate that repeated stress exposure selectively disrupts the inhibitory actions of KOR on

excitability post-synaptically while keeping the pre-synaptic inhibition of glutamatergic synaptic drive intact (represented schematically in Figure 4.7d).

Stress-induced reduction in KOR signaling in 5-HT neurons of the DRN is p38α MAPK dependent

Animals perfused 30 min following the final repeated stress exposure had significantly increased phospho-p38-ir compared to naïve animals in DRN (Figure 4.8a and b). The increased phospo-p38-ir recovered to baseline levels 24 hr following the swim stress, and the increase was blocked by norBNI administered prior to the swim stress (representative images, Figure 4.8a; $F_{3,12} = 4.630$, $p < 0.05$, one way ANOVA, Figure 4.8b). Stress exposure did not change the number of p38α positive cells within the DRN ($p > 0.05$, Figure 4.8c).

Using a conditional knock-out animal (p38αCKO^{SERT}) in which p38α is specifically deleted from SERT-containing neurons[253] , we assessed whether p38α MAPK activation following swim stress mediated the stress-induced reduction in KOR activation of GIRK current. Compared to wild type (WT) littermates that have p38α expressed in both TPH-ir positive and negative neurons (Figure 4.8d, top panel), in p38αCKO^{SERT} animals, p38α-ir was only detected in TPH-ir negative neurons. These mice also ubiquitously express a ROSA-eYFP stop-floxed reporter, where Cre recombinase expression induces a YFP signal; we also used this signal as an indirect proxy of the floxed p38α gene excision. Thus, to ensure that the analyzed cells from CKO animals were both TPH-ir positive and had p38α MAPK excised, we only included biocytin filled cells that were both TPH-ir positive and YFP-ir positive (Figure 4.8e).

Prior studies using *in vitro* transfected cell culture systems had shown that opioid receptor activation of p38 MAPK caused Src phosphorylation of tyrosine-12 (Y12) in the amino terminal domain of Kir3.1 that enhances GIRK channel deactivation[263,272]. GIRK channel phosphorylation by this mechanism might have been responsible for the reduction in response to U69,593 evident in slices from FSS-exposed mice. Consistent with this hypothesis, p38α MAPK excision from serotonergic neurons prevented the stress-induced reduction in KOR-activated GIRK current (stress exposure by genotype, $F_{1,34} = 4.344$, $p < 0.05$, two-way ANOVA, Figure 4.8f). To further test this hypothesis, we used a previously characterized antibody, GIRKp, able to detect phosphorylation of the Y12 residue of $K_{IR}3.1$ [263] after either kappa agonist treatment with U50,488 (20 mg/kg, i.p.) or repeated swim stress. Both of these manipulations produced robust increases in GIRKp-ir in TPH-ir positive neurons compared to basal levels (Figure 4.9a). The increase in GIRp-ir caused by FSS was blocked by pretreatment with 10 mg/kg norBNI (Figure 4.9a). Stress-induced phosphorylation of p38 MAPK and $K_{IR}3.1$ co-localized in individual cells within the DRN (Figure 4.9b).

To determine whether p38 MAPK activation also promoted phosphorylation at the KOR ser-369 site important for receptor desensitization we conducted IHC experiments in which WT or p38αCKO[SERT] animals were exposed to repeated swim stress and compared to naïve controls. Serial sections of the DRN from these different animals were stained for either KORp-ir or GIRKp-ir. As previously shown, repeated swim stress increased both KORp-ir and GIRKp-ir with the DRN compared to naïve WT animals (Figure 4.10a, left side). Interestingly, in p38αCKO[SERT] animals, the stress-induced increase in KORp-ir was still apparent compared to naïve CKO animals (Figure

4.10a, top panel). In contrast, stress-induced GIRKp-ir was markedly blunted in CKO animals when compared to both naïve CKO animals and stress-exposed WT animals (Figure 4.10a, bottom panel). These results are an *in vivo* demonstration that p38α MAPK activation is required for the phosphorylation of the Y12 residue of $K_{IR}3.1$, but p38α MAPK deletion does not affect the phosphorylation of the Ser369 residue of KOR [202,252]. A schematic summary of these results is shown (Figure 4.10b).

Discussion

In this study, we demonstrate that KOR activation produces a net inhibitory regulation of 5-HT neuronal excitability through pre-synaptic inhibition of glutamatergic synaptic activity onto serotonergic neurons coupled with an increase in post-synaptic GIRK current activation. We found that the post-synaptic, but not the pre-synaptic inhibitory effects of KOR activation were reduced following repeated stress-exposure, demonstrating a stress-induced change in the regulation of serotonergic neuron excitability. We interrogated the mechanism underlying this stress-induced dysregulation of KOR function through use of functional neuroanatomy (i.e. phospho-antibody immunohistochemistry) as well as p38αCKO^{SERT} transgenic animals and found that stress-induced activation of p38α MAPK was responsible for the reduction in acute KOR-activated GIRK current seen in 5-HT cells in the DRN of stress-exposed animals. Consistent with our previous work [181,252,263], we found that p38α MAPK-dependent inhibition of KOR function was likely a consequence of the tyrosine phosphorylation of the GIRK channel initiated by p38α MAPK, following p38α MAPK's activation by KOR.

Mechanism of agonist-induced reduction of KOR activated GIRK current ex vivo

There are several possible mechanisms that could underlie our observation that KOR activation of GIRK current was reduced in stress-exposed animals. First, stress may desensitize KOR signaling through a β-arrestin dependent mechanism. KOR desensitization and internalization caused by phosphorylation of Ser369 by GRK3 can result in sustained analgesic tolerance that can take weeks to recover[273]However, we found that within the DRN, stress-induced KORp-ir as well as reduction of KOR mediated GIRK current recovered to initial baseline levels in 24 hr, suggesting that phosphorylation of KOR itself was not responsible for the observed dysregulation of response.

Alternatively, there is evidence suggesting that tyrosine phosphorylation at the N-terminal cytoplasmic domain of the GIRK channel is a potential mechanism for the p38-dependent reduction of GIRK current. In previous *in vitro* work, we showed that phosphorylation of two obligatory/cooperative tyrosine residues (demonstrated through BDNF exposure and activation of TrkB receptors) on $K_{IR}3.1$ (Y12 and Y67) or $K_{IR}3.4$ (Y32 and Y53) inhibit U69,693 evoked GIRK current through accelerated deactivation [272,274]. Phosphorylation of these residues does not cause basal reduction in current, consistent with our observations that basal GIRK currents were not affected by stress [272,274]. Moreover, suppression of U69,593-induced GIRK current was dependent on p38 MAPK activation of Src kinase [263]. *In vivo*, neuropathic pain or swim stress caused an increase in phospho-GIRK-ir in the spinal cord that was absent in mice lacking GRK3 [181,263], which would in turn prevent p38 MAPK recruitment. Here we show p38α MAPK excision from serotonergic neurons was sufficient to block both stress-induced reduction of KOR-induced GIRK as well as stress-induced phosphorylation of K_{IR} 3.1, while

having no appreciable effect of phosphorylation of KOR. Since p38 is a ser/thr-selective kinase, tyrosine phosphorylation of the channel likely indirectly follows from the MAPK activation of a src-like kinase in serotonergic neurons, as previously demonstrated in transfected AtT20 cells[263] Thus, our findings provide a functional consequence of repeated stress exposure in vivo that is consistent with prior work in heterologous systems.

Disparate actions of stress on pre- versus post-synaptic KOR function

The disparate effect of stress exposure on pre-synaptic versus post-synaptic KOR actions points to a fundamental difference in the molecular machinery regulating these two subcellular compartments. Recently, agonist sensitivity and differences in regulatory mechanisms of opioid receptors based on subcellular localization has been examined in pro-opiomelanocortin (POMC) neurons. Pennock and Hentges (2011) found that agonist-induced desensitization occurs post-synaptically, but not pre-synaptically. Moreover, these investigators found that there was greater efficacy of opioid agonism at pre-synaptic receptors compared to post-synaptic receptors, indicating that there may be more spare receptors available pre-synaptically. While clearly there are temporal differences between these findings on acute desensitization and the current study, the results of Pennock and Hentges underscores differences in agonist-induced regulation of a receptor that are dependent on subcellular localization.

Furthermore, in the current work, it is likely that the differences in pre- versus post-synaptic regulation by stress are related to subcellular differences in expression of GRK3, p38α MAPK and Src kinase. For example, we only observe p38 MAPK-ir in the soma and dendrites of serotonin-containing neurons. Moreover, it is likely that KOR-

dependent GIRK current activation did not regulate glutamatergic release probability. It has been shown that KOR inhibition of excitatory transmission in the hippocampus is dependent on dendrotoxin-sensitive Shaker-type voltage gated potassium channels [275]. There has been one prior report of KOR regulation of EPSPs in the DRN in putative rat 5-HT neurons using current clamp techniques with the selective KOR agonist enadoline [276]. However, it was important to further characterize the pre- and post-synaptic regulation by KOR using voltage clamp techniques and a combination of evoked and miniature PSCs to advance our understanding of stress-induced alterations of KOR function. Here we found that behaviorally evoked endogenous ligand release can have different effects on pre- versus post-synaptic receptors within the same cell type of a brain region.

Serotonin, stress coping and mood disorder

While the role of serotonin in cognition and emotion is generally well appreciated, the mechanisms of serotonin action have been difficult to define because of its wide regional distribution and large number of cloned receptors [15,72,73]. There have been two prevailing theories of serotonin function that have been posited and tested. First, is the hypothesis that reduction in serotonin tone/release below basal levels enhances the perception of threat or punishment and promotes a negative affective bias or aversion [72,74]. This hypothesis has been supported experimentally through serotonin depletion experiments in rats, monkeys and humans and is also supported by clinical evidence that enhancement of serotonin through selective serotonin reuptake inhibitors (SSRIs) ameliorates depression [15,72]. The second theory, also supported by preclinical evidence, posits that there is a direct relationship between serotonin levels and

behavioral or response inhibition (i.e. increase in serotonin would cause an increase in behavioral inhibition) [73]. Furthermore, it is plausible that both decreases or increases in behavioral inhibition in stressful situations translates to active versus passive coping strategies [277]. It is likely that serotonin has a role in both these processes, however it also possible that they are supported by serotonin acting disparate regions. For example, it has been shown that an acute stress exposure causes elevation in serotonin the dorsolateral striatum (behavioral inhibition) and simultaneous decreases in serotonin in the lateral septum and the amygdala (aversive learning) [77]. Importantly, dysregulation of either or both of these behavioral processes can lead to stress vulnerability by producing behavioral hyper-responsivity/enhanced passive coping and enhanced perception of punishment or aversion. Understanding the regulation of dorsal raphe firing is critical in order to disentangle the role of serotonin in both adaptive behaviors and psychiatric disease.

The current work demonstrated that in naïve animals, KOR activation in the dorsal raphe caused in net reduction in 5-HT neuronal excitability, which may act to return firing back to homeostatic levels after heightened periods of activation. KOR regulation of excitatory synaptic input in DRN is consistent with similar inhibitory effects on glutamatergic transmission in the hippocampus [183,191]. Importantly, KOR activation in DRN of naïve animals may simultaneously encode the negative affective properties of a stressor through acute reduction in serotonin tone in limbic regions as a transient teaching signal. Stress-induced dynorphin release is likely to produce a net reduction in serotonergic transmission by both presynaptic inhibition of excitatory drive and post-synaptic hyperpolarization of TPH-ir neurons. Sustained dynorphin action caused a

p38α MAPK-dependent reduction in the postsynaptic inhibitory regulation by dynorphin/KOR; however presynaptic inhibition of excitatory drive was not attenuated. We also found that stress-exposed mice had an increased basal glutamatergic drive onto 5-HT neurons similarly to what has been reported in stress exposed rats [86]. While these observations suggest enhanced serotonergic neuronal excitability following stress, further study is necessary to define net effect on DRN circuit excitability, However, p38-mediated dysregulation of the serotonergic output has been previously shown to contribute to the dysphoric effects of stress[205,253] and is likely to involve local circuit adaptations in DRN.

We have shown that in addition to mediating the dysphoric properties of stress, p38 MAPK activation within the DRN contributes to the increased immobility or passive coping behaviors evident in the swim paradigm following prior stress exposure [253]. Our current data are consistent with the notion that prior stress experience promotes a shift to passive coping/behavioral inactivation to subsequent stress exposures caused by an enhanced stress-induced release of 5-HT into the forebrain. This shift resulting from pathogenic stress exposure may lead to an enhanced aversive state in the presence of mild stressors or even non-noxious stimuli. In conclusion, these data offer a mechanism for stress-induced dysregulation of the excitability of neurons in the DRN, a critical locus of stress neurocircuitry and identify a functional target of stress-induced p38MAPK activation that may mediate some of the negative aspects of pathological stress.

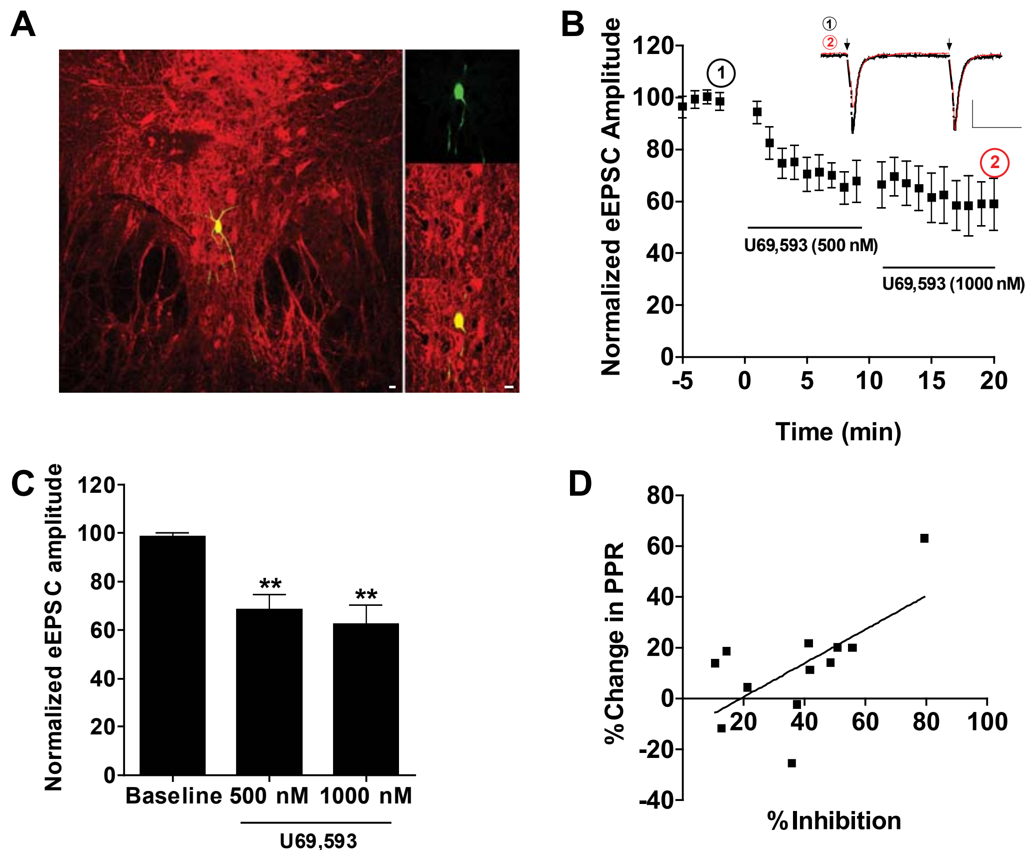


Figure 4.1. KOR activation by U69,593 depresses evoked glutamatergic EPSCs recorded in 5-HT neurons of the DRN.

A. 20x and 40x (inset) confocal fluorescent images of a representative recording site within the DRN. Cells were filled with biocytin and post-fixed following the conclusion of recording to assess for the presence of TPH. Cells were considered 5-HT positive if the biocytin-filled cell (green) co-localized with TPH (red). Recordings were confined to the dorsal and ventromedial aspect of the mid to caudal DRN. Scale bar = 20 µm. B. Representative traces (inset; scale bar = 25 msec, 100 pA) and time course of normalized evoked EPSC amplitude prior to and following U69,593 (500 nM, 1000 nM) application. Electrical stimulation (100-1000 µA) was delivered in a pairwise fashion to the slice with an inter-stimulus interval of 50 msec (arrows). C. Four responses from baseline, U69,593 500 nM and 1000 nM were averaged to get mean drug responses relative to baseline (**p < 0.01). Both concentrations of significantly decreased the normalized eEPSC amplitude compared to baseline. D. There was a significant inverse correlation between the % inhibition of eEPSC amplitude and the % increase in paired pulse ratio (r^2 = 0.3912, $p < 0.05$) N = 12 for all graphs.

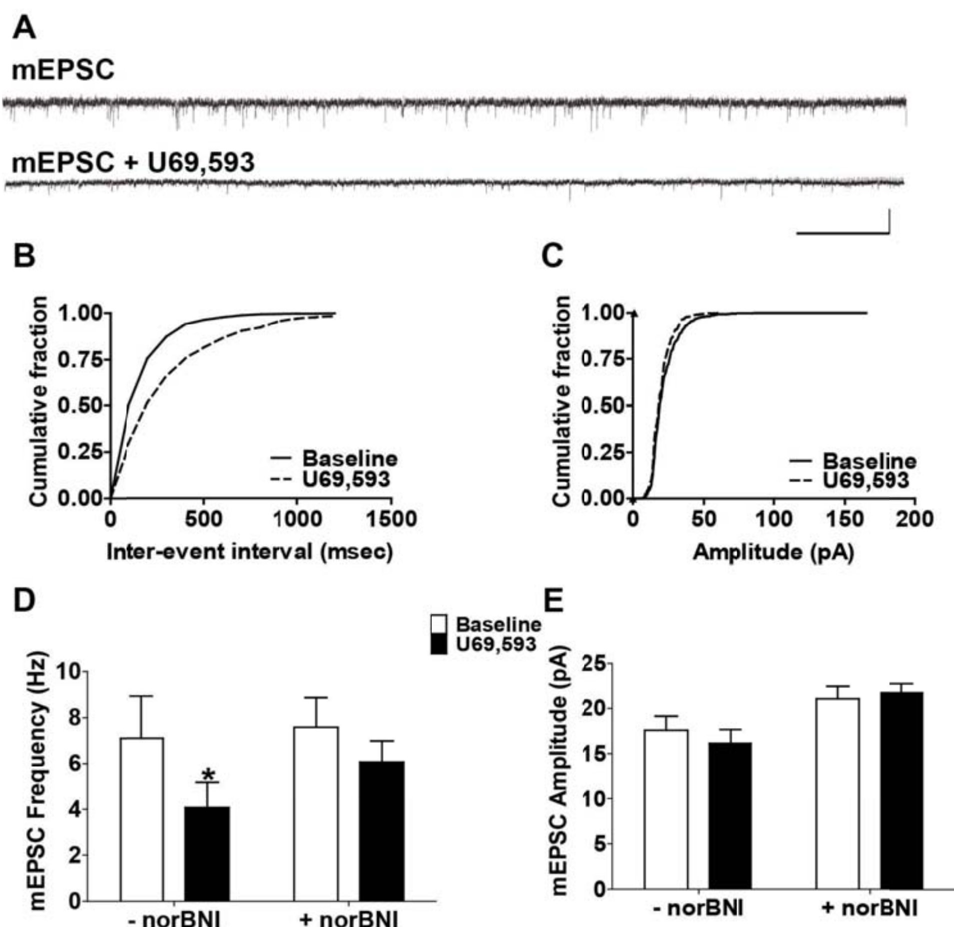


Figure 4.2. U69,593 produces a norBNI-sensitive decrease in mEPSC frequency and amplitude.

A. Representative traces of pharmacologically isolated mEPSCs recorded from 5-HT DRN neurons prior to and following U69,593 (1000 nM) application (Scale bar = 40 pA, 1 sec). B. Cumulative histogram of the inter-event interval of baseline mEPSC and that after U69,593 wash-in corresponding to representative traces in A. C. Cumulative histogram of mEPSC amplitudes prior to and following drug wash-in corresponding to the representative traces in A. D. U69,593 significantly decreases mEPSC frequency by 43% on average that was blunted in slices incubated with norBNI following a stable baseline Bonferroni post-hoc, * $p < 0.05$, N = 7 for both groups). E. U69,593 had no effect on mEPSC amplitude in slices with or without norBNI ($p < 0.05$, N =7 for both groups).

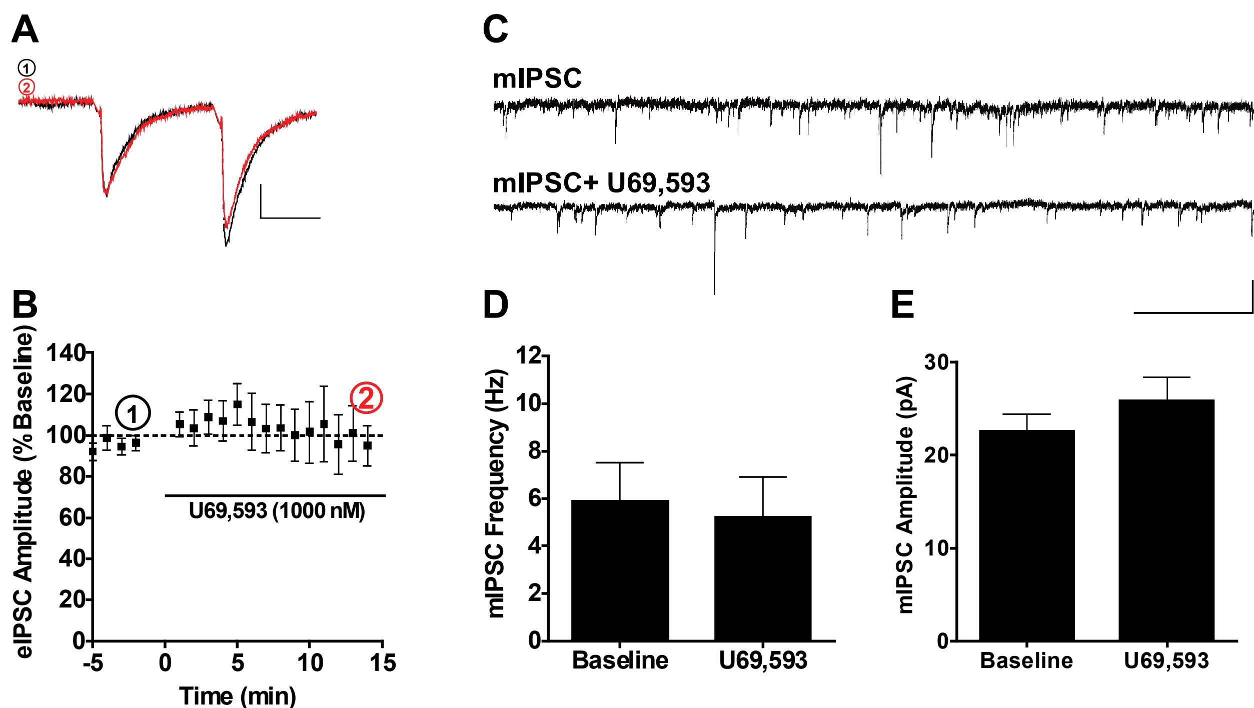


Figure 4.3. KOR activation by U69,593 has no effect of evoked GABAergic IPSCs or mIPSCs.

A. Representative traces of eIPSCs prior to and following U69,593 (1000 nM) application to the slice (Scale bar = 50 msec, 200 pA). B. Time course of normalized evoked IPSC amplitude responses to U69,593 (1000 nM) relative to baseline demonstrating no effect of KOR activation on GABAergic synaptic transmission (N = 9). C. Representative traces of mIPSCs before and after U69,593 (1000 nM) bath application.(Scale bar = 40 pA, 1 sec). D. & E. U69,593 had no effect of mIPSC frequency or amplitude (N = 6).

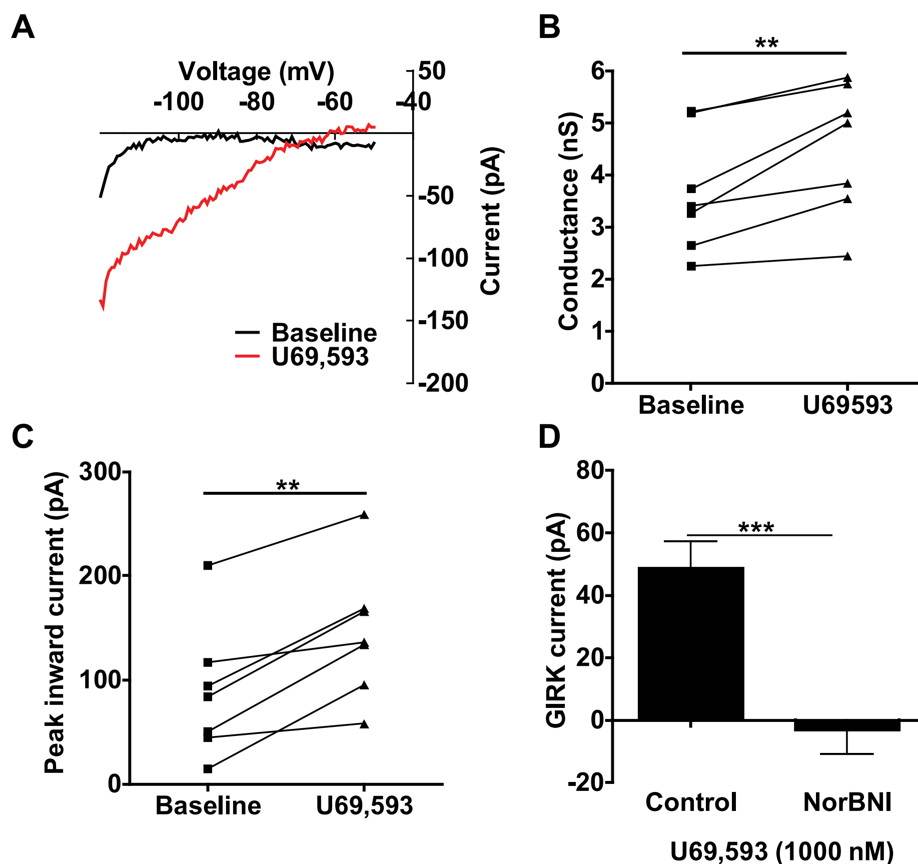
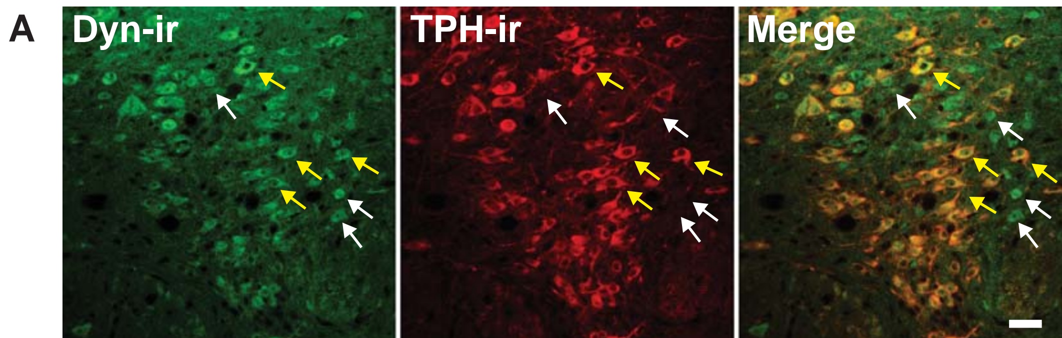


Figure 4.4. KOR activation increases GIRK currents post-synaptically.

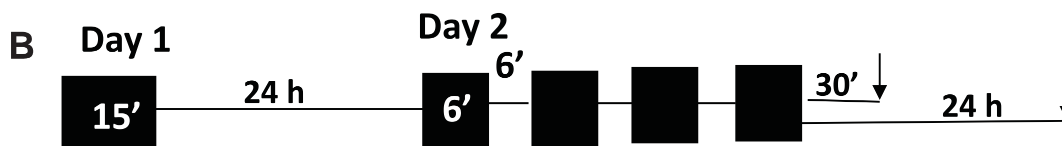
Representative traces of baseline inward rectifying currents and KOR-activated (U69,593-induced) currents in which the Ba^{2+}-insensitive current has been subtracted. B. and C. U69,593 increases significantly increases the conductance (slope) and the peak inward current (measured at -120 mV) compared to baseline measurements (** $p < 0.01$, N = 7). D. For both untreated slices and slices pre-incubated with norBNI (1000 nM), the baseline current was subtracted from the current following U69,593 application to get an absolute change in peak inward current (denoted as GIRK current). U69,593 produced a 48.7 pA GIRK current that was absent in cells that had been pre-incubated with norBNI (** $p < 0.01$, N =7 for both groups).

Figure 4.5. Repeated forced swim stress causes the release of dynorphin and KOR activation.

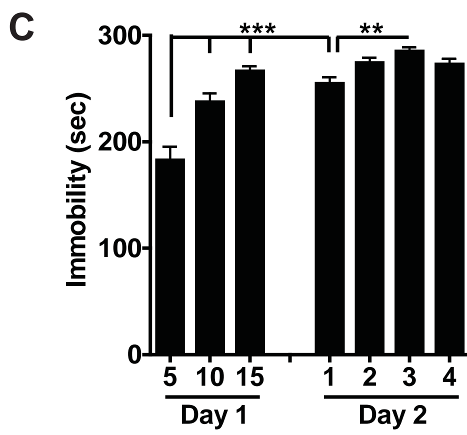
A. 20x fluorescent images of dynorphin and TPH co-localization with the DRN. There was evidence of dynorphin positive cells in both TPH negative (white arrows) and TPH positive cells (yellow arrows) within the DRN. Scale bar = 40 μm. B. Schematic of the two-day repeated forced swim paradigm used in the study. C. Mice displayed significantly escalating immobility within the first 15 min session on day 1, from day 1 to day 2 and also across sessions on day 2 (** $p < 0.01$, *** $p < 0.001$, N =10). D. Mice showed a significant stress-induced increase in tail-withdrawal latency following exposure to the two-day swim paradigm that could be blocked by norBNI (10 mg/kg) pretreatment (### $p < 0.001$ interaction; *** $p < 0.001$ post-hoc Bonferonni, N = 7-21). E. Two-day repeated swim stress produces an increase in phospho-KOR-ir compared to naïve animals that returns to basal levels 24 hr after the last swim session (scale bar = 100 μm).



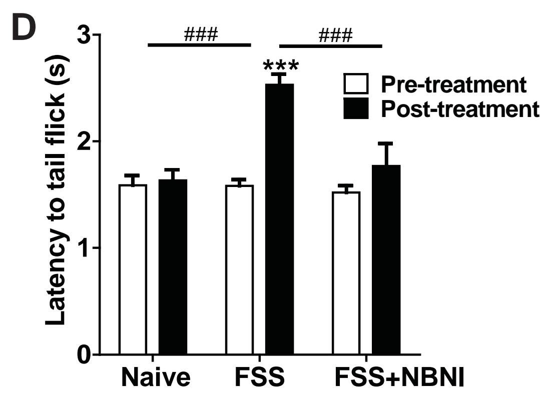
A
Dyn-ir
TPH-ir
Merge



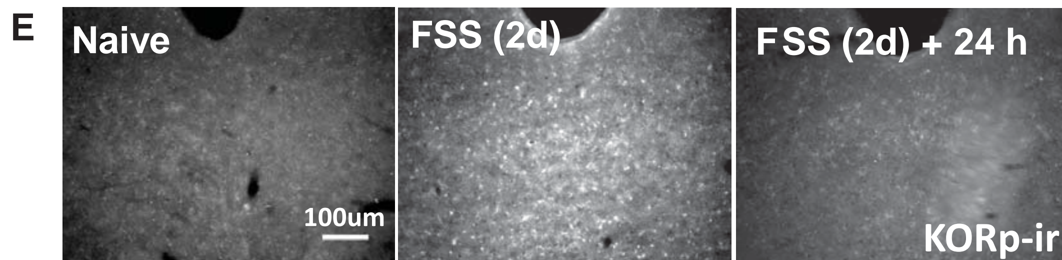
B
Day 1
15′
24 h
Day 2
6′
6′
30′
24 h



C
Immobility (sec)
300
200
100
0
***
**
5
10
15
1
2
3
4
Day 1
Day 2



D
Latency to tail flick (s)
3
2
1
0
###
###
***
Pre-treatment
Post-treatment
Naive
FSS
FSS+NBNI



E
Naive
100um
FSS (2d)
FSS (2d) + 24 h
KORp-ir

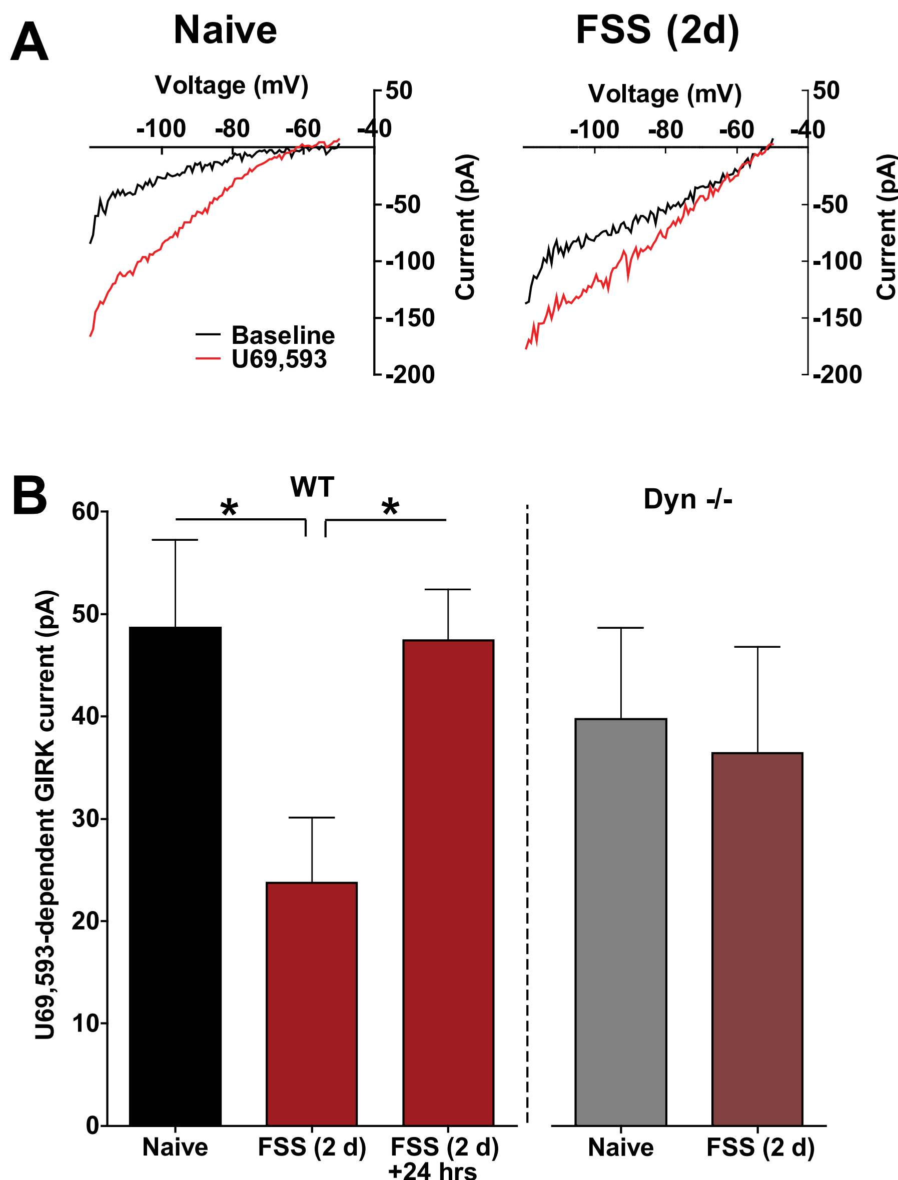
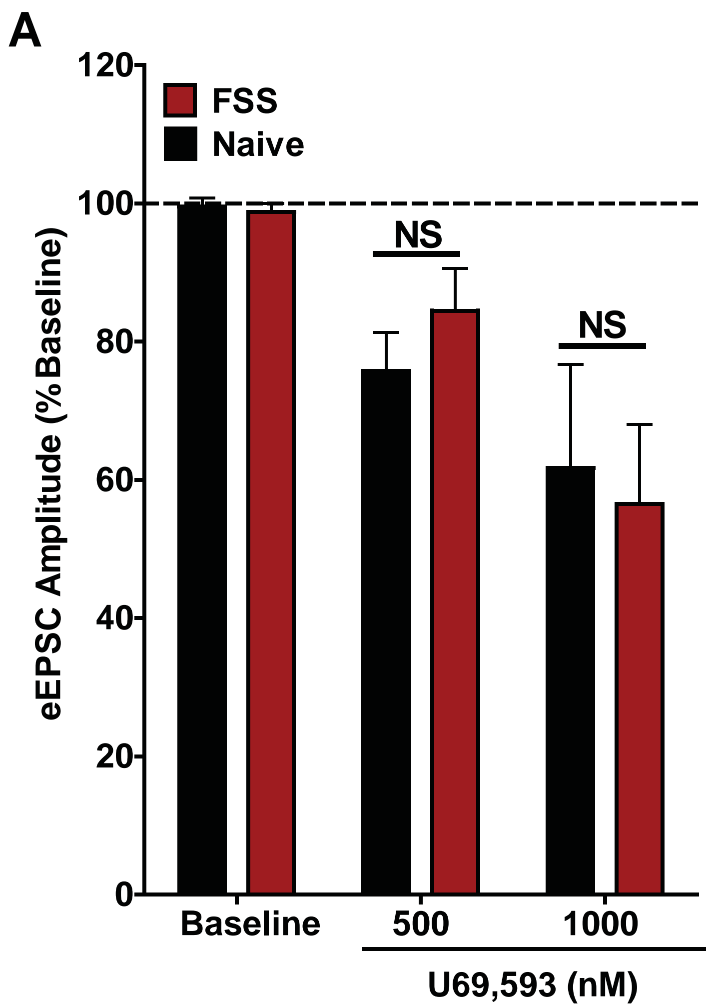


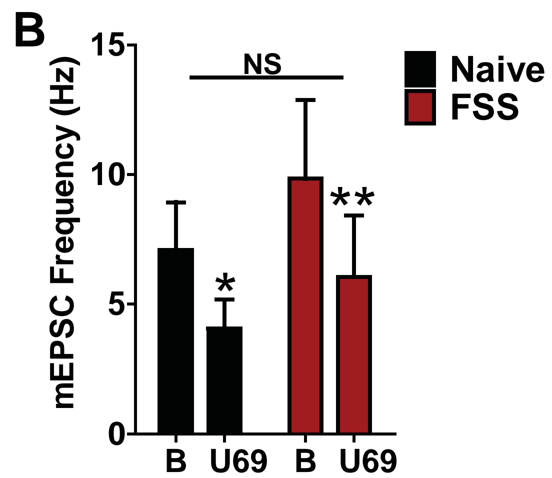
Figure 4.6. Repeated forced swim stress causes a reduction of KOR-activated GIRK current.

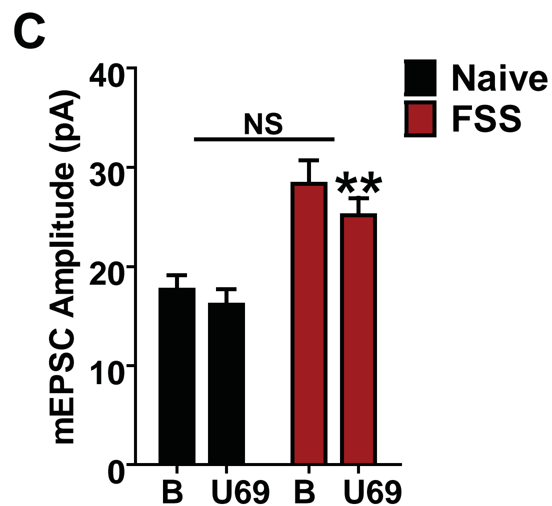
A. Representative traces of baseline and KOR-induced GIRK currents in 5-HTcells recorded from the DRN of a naïve or stress-exposed mouse. B. In WT animals, repeated swim stress causes a significant decrease in KOR-activated GIRK current that recovered 24 hr following the final swim session (* $p < 0.05$, N =7-8). In animals lacking the preprodynorphin gene (Dyn -/-), stress exposure did not significantly decrease KOR mediated GIRK current relative to naïve animals (N = 7-9).

Figure 4.7. Repeated stress exposure does not alter KOR mediated depression of glutamatergic synaptic transmission.

A. Repeated swim stress did not alter KOR inhibition of normalized eEPSC amplitude at either 500 or 1000 nM relative to measurements obtained in stress-naïve animals (N = 10-12). B. U69,593 (1000 nM) caused a similar inhibition of mEPSC frequency in 5-HT cells recorded from naïve versus stress-exposed mice (behavioral treatment by time, $F_{1,10} = 0.3210$, $p > 0.05$, two-way repeated measures ANOVA) (Naïve: 62 ± 7.0% of baseline; FSS: 58 ± 7.8 % of baseline, Bonferroni post-hoc tests, * $p < 0.05$, ** $p < 0.01$, N = 5-7. C. 5-HT cells recorded from stress-exposed animals had significantly larger mEPSC amplitudes (two-way repeated measure ANOVA, main effect of behavioral treatment, $F_{1,10} = 16.08$, $p < 0.01$). There was not a significant difference in U69,593-induced inhibition of mEPSC amplitude between naïve and stress-exposed mice (behavioral by time, $F_{1,10} = 3.024$, $p > 0.05$, two-way repeated measures ANOVA) (Naïve: 92 ± 3.3 % of baseline; FSS: 89 ± 2.4 % of baseline). There was a trend for a small (8%) decrease in mEPSC amplitude by U69,593 in naïve animals, but this was not significant (Bonferroni post-hoc test, $p > 0.05$). U69,593 did produce a small (10%), but significant decrease in mEPSC amplitude in 5-HT cells of stress-exposed animals (Bonferroni post-hoc t-test, ** $p < 0.01$) , N = 5-7. D. Summary of KOR regulation of DRN 5-HT neuronal excitability pre- and post-synaptically in stress-naïve and stress-exposed mice.


A
FSS
Naive
eEPSC Amplitude (% Baseline)
120
100
80
60
40
20
0
NS
NS
Baseline
500
1000
U69,593 (nM)


B
mEPSC Frequency (Hz)
15
10
5
0
NS
*
**
B
U69
B
U69
Naive
FSS


C
mEPSC Amplitude (pA)
40
30
20
10
0
NS
**
B
U69
B
U69
Naive
FSS

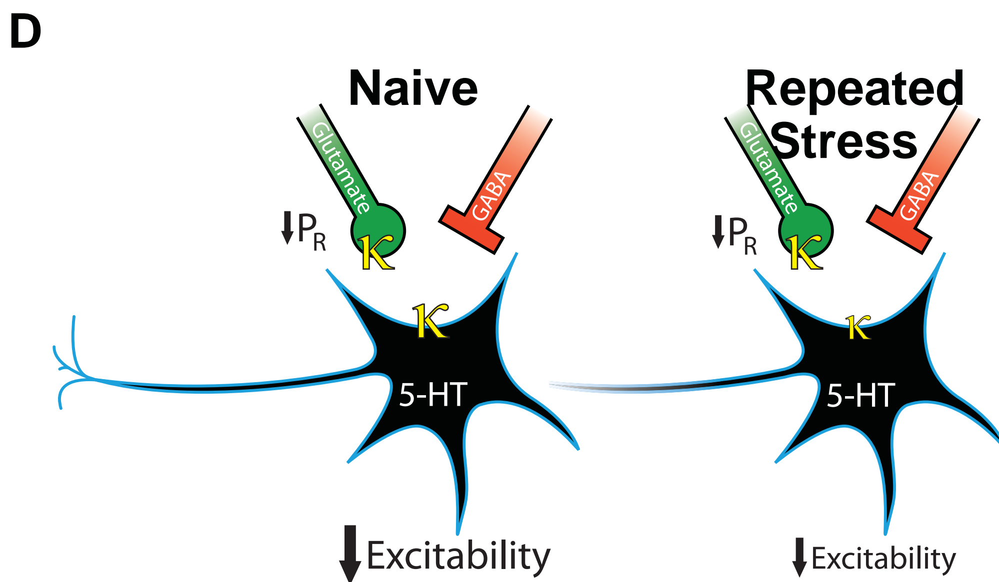
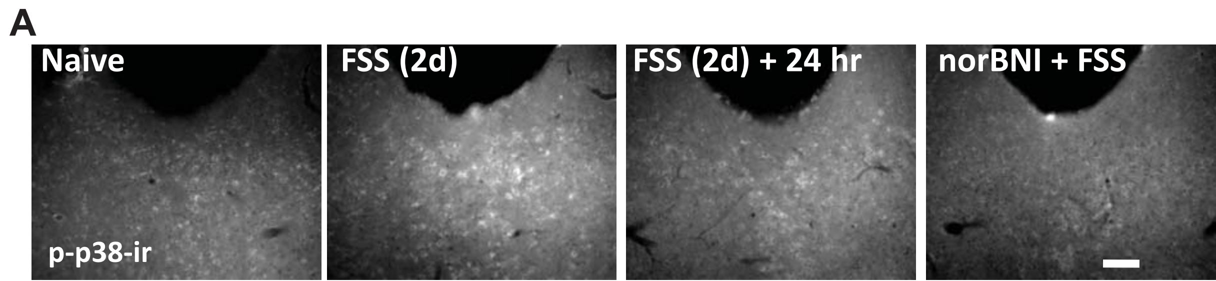
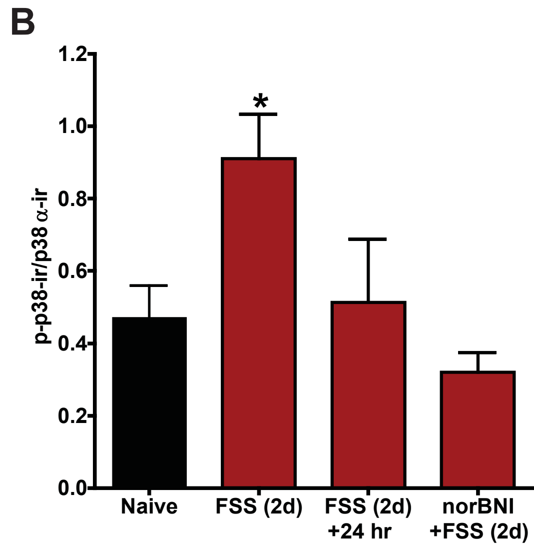
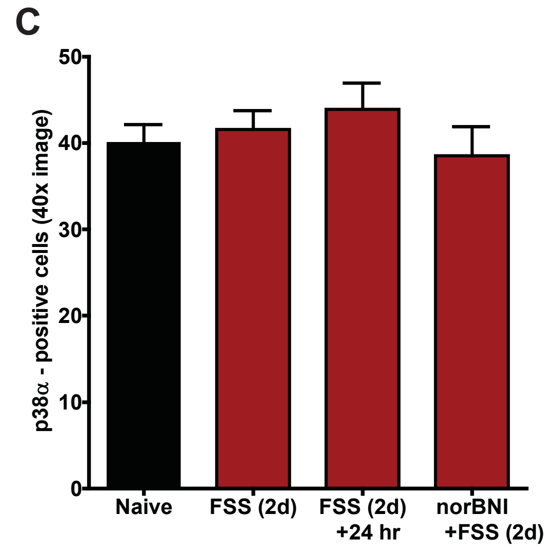

D
Naive
Glutamate
GABA
↓PR
κ
5-HT
↓Excitability
Repeated
Stress
Glutamate
GABA
↓PR
κ
5-HT
↓Excitability

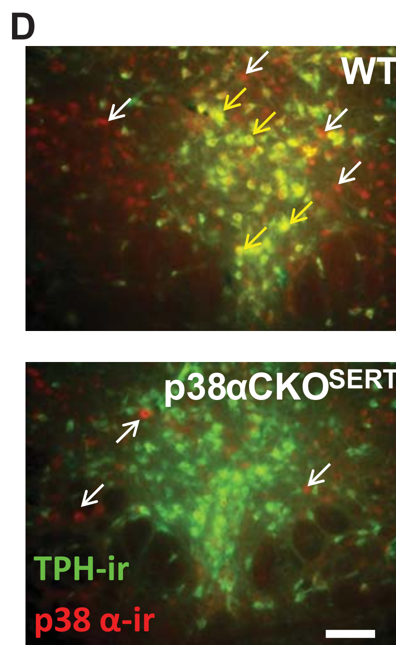
Figure 4.8. p38MAPk mediates the stress-induced reduction in KOR-activated GIRK current.

A. Representative greyscale fluorescent images of phospho-p38 MAPK-ir in the DRN in naive animals, stress-exposed animals, stress exposed animals allowed to recover for 24 hr and stress-exposed animals pre-treated with norBNI (scale bar = 200 μm). B. Two-day swim stress significantly increased the ratio of phospho-p38 MAPK-ir: p38α-ir cells compared to naïve animals sampled from a 40x image of the medial DRN. Following 24 hr of recovery, this ratio returned to baseline levels. norBNI pre-treatment significantly blocked stress-induced activation of phospho-p38MAPK-ir (*$p < 0.05$, N = 3-5 animals, triplicate sampling per N). C. There was no significant difference in total number of p38α positive cells across groups (N = 3-5 animals, triplicate sampling per N). D. Representative fluorescent images of p38α-ir (red fluorescence) and TPH-ir (green fluorescence) co-localization in the DRN of WT and p38αCKO^{SERT} animals. Co-localization of both proteins is indicated by the presence of yellow fluorescence above background. p38α-ir was present in both TPH positive (yellow fluorescence, yellow arrows) and TPH negative (red fluorescence only, white arrows) within the DRN of WT animals. In contrast, p38α-ir was only present above background staining in TPH negative cells in p38αCKO^{SERT} (red fluorescence only, white arrows) (scale bar = 100 μm). E. Slices obtained from p38αCKO^{SERT} animals used for electrophysiological recording were post-fixed following recordings and triple-labeled for biocytin (blue), TPH (red) and YFP (green) to confirm that the recorded cells were both TPH positive and YFP positive, indicating p38α had been excised (scale bar = 50 μm). F. Stress-induced reduction in KOR-activated GIRK current in 5-HT cells was absent in 5-HT neurons from stress-exposed p38αCKO^{SERT} animals compared to their naïve counterparts (# $p < 0.05$ interaction, N = 7-12).


A
Naive
FSS (2d)
FSS (2d) + 24 hr
norBNI + FSS
p-p38-ir


B
p-p38-ir/p38 α-ir
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Naive
FSS (2d)
FSS (2d) +24 hr
norBNI +FSS (2d)


C
p38α - positive cells (40x image)
50
40
30
20
10
0
Naive
FSS (2d)
FSS (2d) +24 hr
norBNI +FSS (2d)


D
WT
p38αCKO^SERT
TPH-ir
p38 α-ir

E
TPH
Biocytin
YFP
Merge

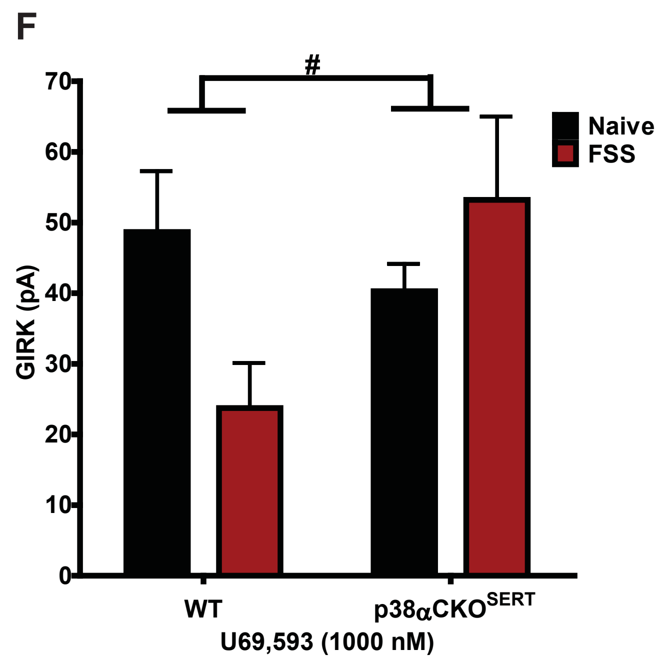
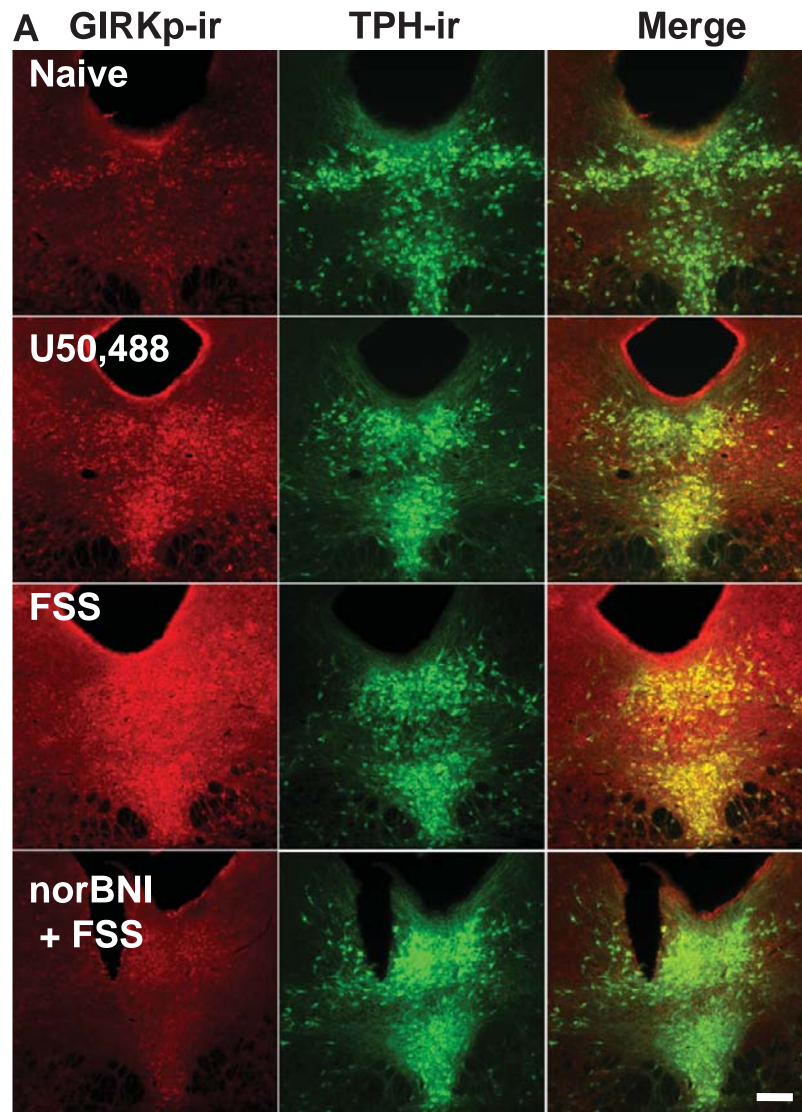

F
GIRK (pA)
70
60
50
40
30
20
10
0
#
Naive
FSS
WT
p38αCKO^SERT
U69,593 (1000 nM)

Figure 4.9. Repeated stressor exposure produces a KOR-dependent increase in phosphorylation of the tyrosine 12 residue of KIR 3.1 in the DRN.

A. Fluorescent images of phospho-GIRK-ir and TPH labeling in the DRN of a stress-naïve, U50,488 (20 mg/kg) injected, stress-exposed and norBNI + stress exposed animal. Relative to basal levels of phospho-GIRK-ir present in naïve animals, U50,488 or repeated swim stress causes robust increases in GIRKp-ir in TPH positive cells that was blocked by norBNI pre-treatment (scale bar = 200 μm). B. 40x fluorescent images demonstrating co-localization of phospho-p38MAPK-ir and phospho-GIRK-ir. Stress exposure increased phospho-p38MAPK-ir and phospho-GIRK-ir within the same cells in the DRN (scale bar = 50 μm).


A
GIRKp-ir
TPH-ir
Merge
Naive
U50,488
FSS
norBNI
+ FSS

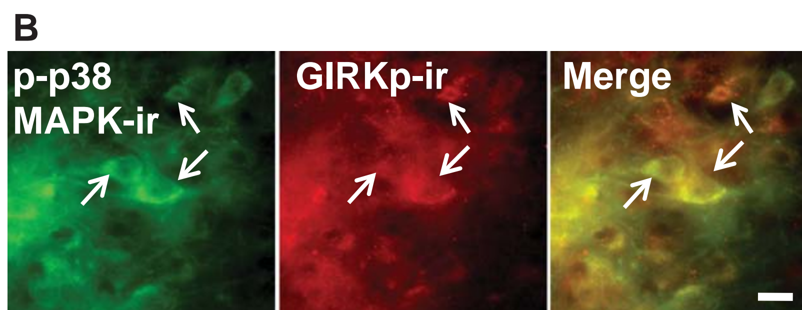
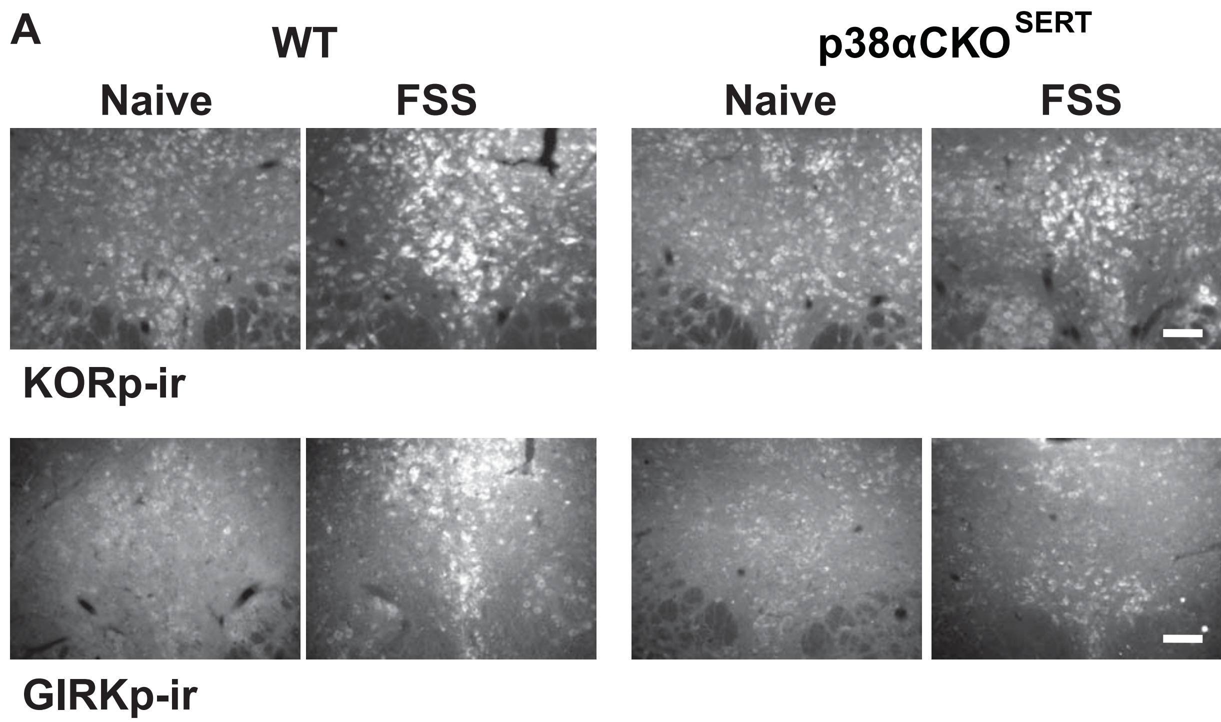
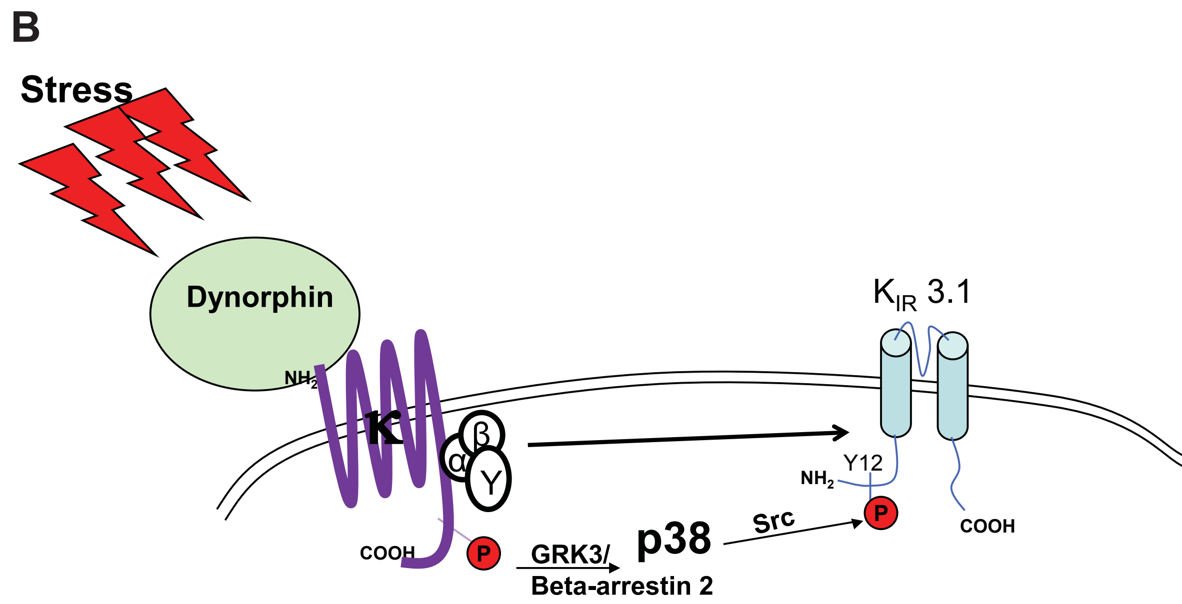

B
p-p38
MAPK-ir
GIRKp-ir
Merge

Figure 4.10. Excision of p38α from 5-HT neurons blocks stress-induced phosphorylation of GIRK, but not KOR.

A, top panel. Repeated swim stress increases phospho-KOR-ir in the DRN compared to naïve animals in both WT and p38αCKO^{SERT} animals. A, bottom panel. Excision of p38α reduced stress-induced phospho-GIRK-ir in the DRN compared to WT animals (scale bar = 100 μm). B. Schematic depicting indirect tyrosine phosphorylation of the GIRK channel by p38α MAPK following stress-induced dynorphin release and subsequent KOR activation.


A
WT
p38αCKO SERT
Naive
FSS
Naive
FSS
KORp-ir
GIRKp-ir


B
Stress
Dynorphin
NH2
K
β
α
γ
KIR 3.1
Y12
NH2
P
COOH
COOH
P
GRK3/
Beta-arrestin 2
p38
Src

Chapter 5: Bi-directional alteration of 5-HT1A autoreceptor function following different stress-exposure paradigms requires activation of the kappa opioid system.

These data are unpublished.

Specific contributions:

J.C.L. collected all of the data presented in this chapter.

Introduction

The 5-HT_{1A} autoreceptor is present on serotonin-containing neurons of the dorsal and median raphe nucleus (DRN and MRN respectively) and upon activation, dampens firing of these neurons and subsequent release of serotonin into the forebrain[278]. 5-HT_{1A} autoreceptors are not tonically active, but rather are engaged during heightened periods of neuronal excitability[278]. The 5-HT_{1A} receptor is a $G_{i/o}$ coupled receptor that activates G-protein activated inward rectifying potassium (GIRK) channels through $G_{\beta\gamma}$ gating mechanisms[266]. Alterations in 5-HT_{1A} receptor levels and function have been found in patients with PTSD, anxiety and depression[2,15,84,278,279]. Likewise, pre-clinical roden models of stress-induced anxiety and depression have also described alterations in 5-HT_{1A} receptor function. However, these studies have yielded conflicting results. For example, studies have shown that 5-HT_{1A} autoreceptor function in DRN neurons can either be sensitized or desensitized as a result of a stress challenge to the animal depending on the type of stress insult (e.g. footshock, swim stress, restraint stress, social defeat stress) and the chronicity and contrallability of the stress. In some of these studies, acute or sub-chronic stress has no effect on 5-HT_{1A} function, whereas chronic

stress causes desensitization of receptor function[280,281]. In contrast, other studies report 5-HT_{1A} receptor sensitization with both an acute and chronic stress challenge [85,86]. Part of the problem when trying to directly compare these studies is that across studies, investigators use different pharmacological agents at a broad range of concentrations to activate 5-HT_{1A} receptors, use different species (rat versus mouse) and use either *in vitro* or *in vivo* electrophysiological techniques to measure 5-HT_{1A} activation of GIRK and suppression of cell firing. Therefore, I sought to conduct a parametric set of experiments examining the effect of chronicity on 5-HT_{1A} autoreceptor function using the same type of stress exposure (i.e. forced swim stress or FSS) using the same 5-HT_{1A} agonist in the same species.

In addition to this ambiguity, even less is known about the mechanisms underlying stress-induced regulation of 5-HT_{1A} in the DRN. The dynorphin-kappa opioid receptor system is engaged during stress-exposure, dynorphin is released in the DRN and KOR activation regulates 5-HT neuronal excitability (see chapter 4). I hypothesized that stress induced activation of the dynorphin-KOR system may be, in part, responsible 5-HT_{1A} adaptations caused by stress.

Methods

Subjects: Male C57BL/6 mice, age >50 days, were maintained under a 12-h light–dark cycle (7a.m. to 7p.m. light) with access to standard food and water *ad libitum.* All procedures on animal subjects were approved by the University of Washington IACUC committee. Mice housed together (2 - 4 per cage) were subjected to the same behavioral treatment.

Swim stress: Mice were subjected to a two-day swim stress procedure in which they were exposed to a 15-min swim session on day one, then on day two, were exposed to four six-min swim sessions in 29.0 – 31.0 °C water, separated by six min and conducted under bright light (690-700 lux) conditions as previously described [170]. A subset of mice were given a six minute swim stress exposure every day, for five additional days. Another group of animals was treated with norBNI (10 mg/kg) i.p. 24 hours prior to the stress exposure. These groups had accompanying control animals that were injected with norBNI (10 mg/kg) and were sacrificed in a time matched fashion to the two stress conditions.

Electrophysiology

Procedures were similar to those previously reported and described in chapter 4 [88,265]. Sections that were 200 μm thick were cut through the raphe nuclei in sucrose and then placed in oxygenated 95%O_2/5%CO_2 ACSF incubated in a 35-37˚C bath for 1 hr. The slices were then removed from the bath and kept in oxygenated ACSF at RT. ACSF was composed of (in mM): NaCl 124, KCl 2.5, NaH_2PO_4 2, $CaCl_2$ 2.5, Dextrose 10 and $NaHCO_3$ 26. The sucrose buffer was ACSF in which NaCl was replaced by 248 mM sucrose.

Slices were placed in a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with oxygenated ACSF at approximately 1.5 ml/min, maintained at 30-32˚C by an inline solution heater (TC-324, Warner Instruments). The resistance of the electrodes was 5-10 MΩ when filled with an internal solution of (in mM) K-gluconate 130, NaCl 5, Na phosphocreatine 10, $MgCl_2$ 1, EGTA 0.02, HEPES 10,

MgATP 2, Na_2GTP 0.5, 0.1% Biocytin, pH 7.3. The cells were voltage clamped at -70 mV using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA). Signals were digitized by a Digidata 1440 A/D converter (Molecular Devices) and stored using pClamp 10.2 software (Molecular Devices). To measure GIRK currents, once a recording had been established, the slice was bathed in 5.5 mM K^+ ACSF (from 2.5 mM K^+) to enhance the currents in the cells at hyperpolarized potentials. As expected, when the slice incubated in high [K^+], the cells became more depolarized and inward rectification was more apparent. This increase in conductance was sensitive to 100 μM barium chloride [266]. The cells were subjected to a ramp protocol in which they were brought from -120 mV to -50 mV over 10 sec. After three stable ramp measurements (the average of three sweeps) were obtained, the 5-HT_{1A} agonist 5-carboxamidotryptamine (5-CT) (1 or 30 nM) was bath applied and the slice was allowed equilibrate for 3 min. Subsequently three ramp measurements were taken every 3 min. For cumulative concentration response experiments, each concentration was applied to the slice for 8-10 minutes or until the cell reached a stable new equilibrium (i.e. three stable GIRK current responses). Following 5-CT application and stable current responses, $BaCl_2$ (100 μM) was added to distinguish Ba^{2+}-sensitive currents.

TPH Immunohistochemistry staining and intracellular labeling

During electrophysiological recordings, cells were filled with biocytin (0.1%) (Sigma-Aldrich) present in the recording electrode. After recording, slices were fixed by submersion in 4% paraformaldehyde prepared in 0.1M phosphate buffer (PB; pH 7.4). Sections were incubated with mouse anti-TPH antibody (1:500, Sigma-Aldrich) for 12-16 hr at room temperature (RT). Subsequently, immunohistochemical labeling was

visualized using Alexa Fluor 488 (1:500; Invitrogen) conjugated goat anti-mouse secondary antibody for 90-120 min at RT. Biocytin was visualized using streptavidin conjugated Alexa Fluor 647 (1:500, Invitrogen) contained in the same secondary antibody mixture. Between incubations, slices were rinsed with PBS (3 x 10 min) and all incubations were done with mild agitation on an orbital shaker.

Results

Baseline and 5-CT induced inward currents (measured at -120 mV) were obtained and Ba^{2+}-insensitive currents were subtracted from the total current. 5-CT (30 nM) significantly increased GIRK current from baseline in TPH positive serotonin containing neurons (paired t-test, $p < 0.01$, Figure 5.1a) as previously described[266]. 5-CT activated GIRK currents at concentrations of 1-30 nM applied cumulatively to the slice. There was no significant difference between 5-CT stimulated GIRK responses at 30 nM applied cumulatively, 30 nM applied directly (non-cumulatively) and 100 nM applied directly (non-cumulatively) ($F_{2,24} = 0.1728$, $p > 0.05$, one-way ANOVA, Figure 5.1b) indicating that there was no acute desensitization apparent in the cumulative concentration response experiment and that 30 nM 5-CT produced maximal activation of GIRK currents.

DRN 5-HT cells were recorded from animals exposed to two-day swim stress (schematized in Figure 5.2a) and 5-CT (30 nM) activated GIRK current responses were measured. Compared to that obtained from naïve animals, FSS (2d) did not significantly alter 5-CT activated GIRK current responses when 30 nM was administered either non-cumulatively (unpaired t-test, $p > 0.05$, Figure 5.2a) or cumulatively

(Bonferroni post-hoc test, $p > 0.05$, Figure 5.2b). However, we did find that at 1 nM, a submaximal concentration, 5-CT responses were sensitized in 5-HT cells recorded from the DRN of FSS (2d) exposed animals (time by behavioral treatment, $F_{3,51} = 5.358$, $p < 0.01$, two-way repeated measures ANOVA, Figure 5.2b). In contrast, in animals that were exposed to the same two-day swim stress and then exposed to one six-minute swim session every day for five additional days had desensitized 5-CT responses at maximal concentrations (10 and 30 nM) compared to naïve animals (time by behavioral treatment, $F_{3,42} = 6.814$, $p < 0.001$, two-way repeated measures ANOVA, Figure 5.2b). We were curious if the sensitization observed in animals exposed to two-day swim stress returned to naïve levels 24 hrs after the cessation of the final swim stress exposure. Surprisingly, the sensitization observed after two-day stress exposure, was also observed following a 24-h recovery period ($F_{2,22} = 4.086$, $p <0.05$, one-way ANOVA, Figure 5.2c). Moreover, though not significantly different, the sensitization observed at 24 hrs following stress exposure, was qualitatively larger than that observed when the animals were sacrificed 30 minutes after the final swim.

The dynorphin-KOR system is activated during swim stress and regulates serotonin containing neurons of in the DRN. We assessed the effects of stress exposure both at two days and seven days on 5-CT activation of GIRK currents in animals that were pre-treated with the selective KOR antagonist norBNI (10 mg/kg) i.p. We have previously shown that norBNI is a long-lasting antagonist (up to 21 days)[261,282,283] and thus we only administered norBNI once, 24 hrs prior to the first stress exposure. Compared to time-matched animals treated with norBNI, but not exposed to stress, norBNI treated animals exposed to two-day swim stress did not have

sensitized 5-HT_{1A} responses at a submaximal (1 nM) concentration of 5-CT (time by behavioral treatment, $F_{3,36} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA, Figure 5.3a). Similarly, seven-day stress exposure did not cause significant desensitization of 5-HT_{1A} responses at maximal (10 and 30 nM) concentrations of 5-CT in norBNI treated animals compared to time-matched norBNI treated animals (time by behavioral treatment, $F_{3,33} = 0.2120$, $p > 0.05$, two-way repeated measures ANOVA, Figure 3b). Interestingly, norBNI pre-treatment seven days prior to slice preparation significantly elevated 5-HT_{1A} responses at all concentrations compared to naïve animals (main effect, $F_{2,63} = 4.325$, $p < 0.05$, Figure 3b) suggesting that the long-lasting impact of norBNI administration seven days prior to testing sensitizes 5-HT_{1A} receptors. Collectively this data suggests that the dynorphin-KOR system in 5-HT neurons of the DRN regulates 5-HT_{1A} responsivity.

Discussion

The principal conclusion from the current work is that stress exposure can bi-directionally alter 5-HT_{1A} autoreceptor responsivity in serotonin containing neurons of the DRN that is dependent on the chronicity of the stress exposure. Importantly, we used the same mode of stress exposure and only varied the number of days the animal was exposed to stress. Two-day swim stress exposure produces a sensitization of 5-HT_{1A} activation of GIRK currents at submaximal concentrations of the 5-HT_{1A} agonist 5-CT. In contrast, seven-day stress exposure produces desensitization of 5-HT_{1A} activation of GIRK currents at maximal concentrations of 5-CT. Interestingly, stress-induced sensitization of 5-HT_{1A} induced GIRK current did not recover in 24 hours after two-day stress exposure and in fact was more robust. A similar sensitization of 5-HT_{1A}

response has been observed in mice exposed to social defeat stress for 10 days[85]. The stress-induced sensitization of 5-HT_{1A} in the DRN may, in part, lead to increased anxiety-like and despair-like behavior observed in stress-exposed animals. Enhanced autoinhibition would lead to chronically lower serotonin tone in the forebrain. Indeed, transgenic animals that have higher 5-HT_{1A} autoreceptor levels demonstrate enhanced behavioral despair-like behavior [284]. The desensitization of 5-HT_{1A} activation of GIRK currents has also been reported in animals exposed to chronic variable stress[280,285] or animals exposed to uncontrollable foot-shock[281]. Interestingly, chronic SSRI treatment also causes 5-HT_{1A} autoreceptor desensitization[286,287] suggesting that stress-induced desensitization of 5-HT_{1A} responsivity may be a compensatory response to stress insult.

Very little is known about the mechanisms underlying stress-induced regulation of 5-HT_{1A} function. Recently, it has been demonstrated that that stress-induced activation of glucocorticoid receptors in the DRN and subsequent activation of HDAC6 mediates 5-HT_{1A} autoreceptor sensitization. In the current work, we show that stress-induced alterations in 5-HT_{1A} function are prevented by pre-treatment with norBNI to the whole animal prior to stress-exposure. These data indicate that the dynorphin-KOR system is also involved in stress-induced regulation 5-HT_{1A} autoreceptor sensitivity. Interestingly, norBNI administration in and of itself, when administered seven days prior to testing, robustly increased 5-HT_{1A} sensitivity at all concentrations tested. Previous work from the laboratory has shown that norBNI, like stress and KOR agonism with U50,488, activates c-Jun kinase (JNK)[261,282]. Since stress, KOR agonism and norBNI all lead to enhanced activation of JNK, it is possible that both stress-induced sensitization of 5-HT_{1A} and norBNI-sensitization of 5-HT_{1A} require JNK activation.

Future research must be done to test this hypothesis directly. This line of evidence provides further insight into the mechanisms underlying bi-directional regulation of 5-HT_{1A} autoreceptor function following exposure to sub-chronic and chronic stress.

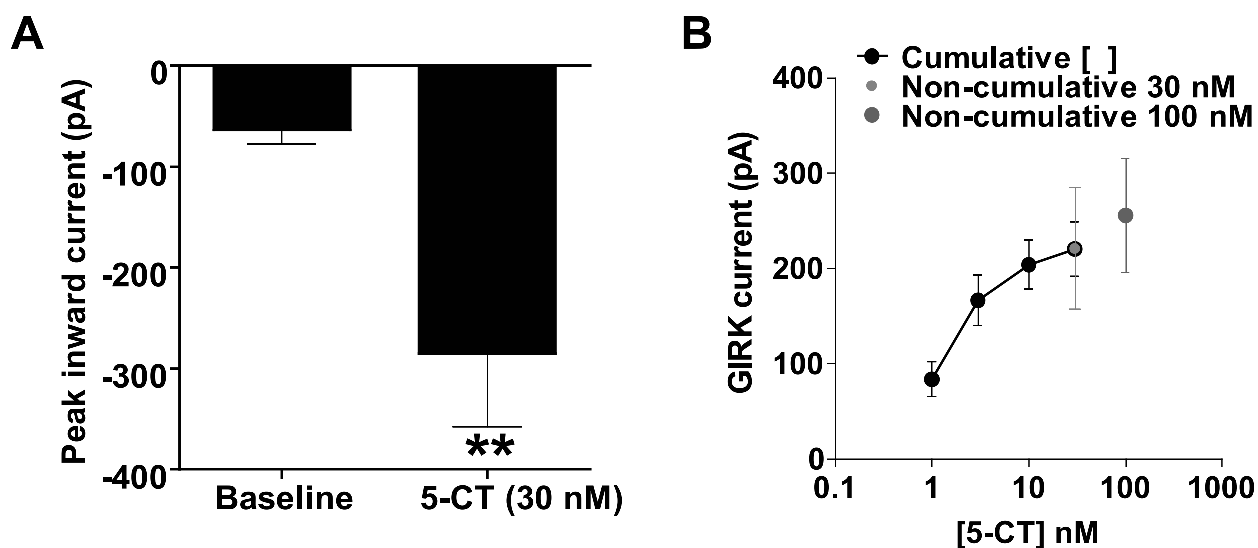


Figure 5.1. Activation of 5-HT_{1A} by 5-CT produces a concentration dependent increase in GIRK current in 5-HT neurons of the DRN.

A. Peak Ba^{2+}-sensitive inward current prior to and following bath application of 5-CT (30 nM) to the slice. 5-CT significantly increases GIRK current in 5-HT DRN cells (paired t-test, $p < 0.01$, N = 7). B. Cumulative concentration response curve of 5-CT activation of GIRK current (1,3,10, 30 nM). There was no difference between 5-CT responses elicited by 30 nM applied cumulatively or non-cumulatively or compared to 100 nM applied directly ($F_{2,24} = 0.1728$, $p > 0.05$, one-way ANOVA, N = 7-11).

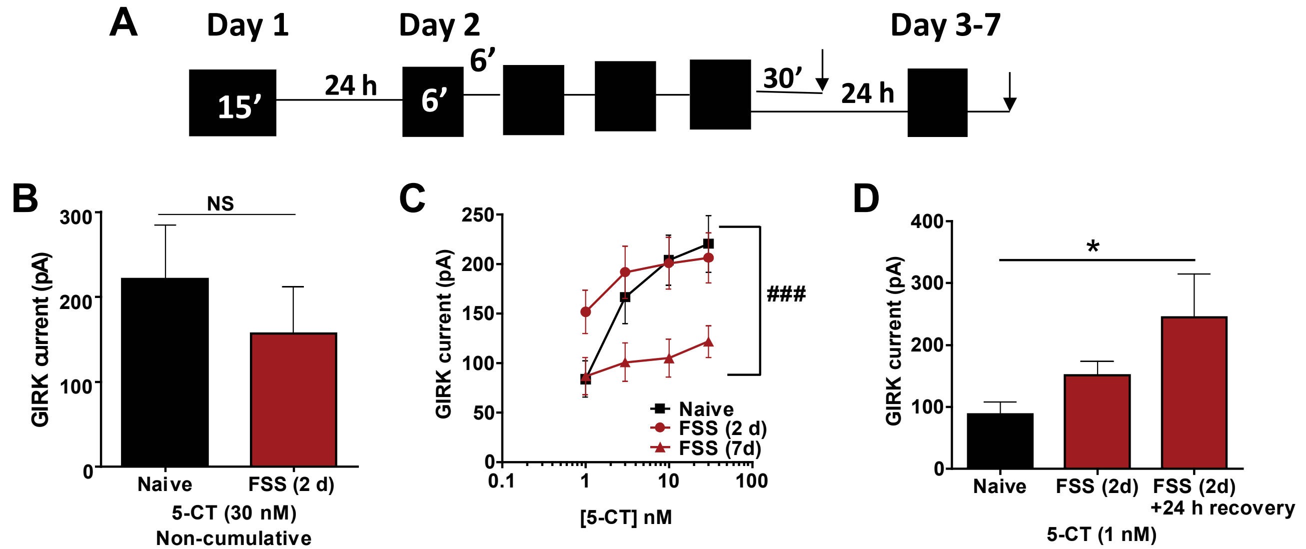


Figure 5.2. Two-day and seven-day stress exposure causes bi-directional adaptations to 5-HT_{1A} receptor function.

A. Schematic of stress paradigm used in these studies. B Compared to that obtained from naïve animals, FSS (2d) did not significantly alter 5-CT activated GIRK current responses when 30 nM 5-CT was administered non-cumulatively (unpaired t-test, $p > 0.05$, N = 6-7). C. Cumulative concentration response curves of 5-CT in animals exposed to two-day swim stress (time by behavioral treatment, $F_{3,51} = 5.358$, $p < 0.01$, two-way repeated measures ANOVA) and seven-day swim stress (time by behavioral treatment, $F_{3,42} = 6.814$, $p < 0.001$, two-way repeated measures ANOVA) were significantly different from that obtained from naïve animals (N = 5-11). D. The sensitization of a submaximal concentration of 5-CT (1 nM) that was observed after two-day stress exposure, was also observed following a 24-h recovery period ($F_{2,22} = 4.086$, $p <0.05$, one-way ANOVA, N = 7-10).

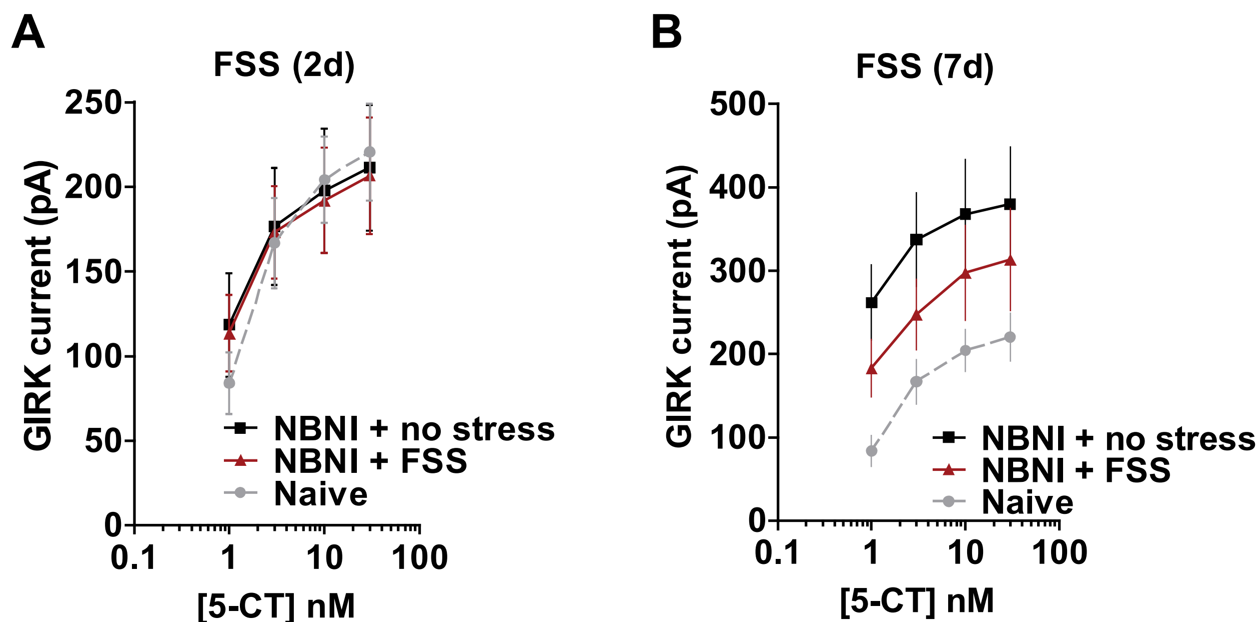


Figure 5.3. NorBNI pre-treatment prior to stress-exposure blocks stress-induced alterations in 5-HT1A function.

A. The cumulative concentration response curve of 5-CT in norBNI treated animals exposed to two-day swim stress was not significantly different from norBNI treated time matched control animals (time by behavioral treatment, $F_{3,36} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA, N = 6-8). B. Similarly, seven-day stress exposure did not cause significant desensitization of 5-HT_{1A} responses at maximal (10 and 30 nM) concentrations of 5-CT in norBNI treated animals compared to time-matched norBNI treated animals (time by behavioral treatment, $F_{3,33} = 0.2120$, $p > 0.05$, two-way repeated measures ANOVA, N = 5-11). norBNI pre-treatment seven days prior to slice preparation significantly elevated 5-HT_{1A} responses at all concentrations compared to naïve animals (main effect, $F_{2,63} = 4.325$, $p < 0.05$).

Chapter 6: Dissertation Conclusions

Over the last several decades, basic scientists have taken on the challenge of identifying biological substrates that underlie complex psychiatric diseases such as anxiety, depression and addiction. A basic strategy used to address this challenge was to dissect complex syndromes such as Major Depressive Disorder (MDD) into small discrete components that might be modeled in animals. In doing so, we have been able to link discrete neurochemicals with specific aspects of psychiatric disease such as negative affect, withdrawal, hyperarousal and anhedonia. We have now identified several neuropeptides, small molecule neurotransmitters and downstream signaling molecules as well as specific brain regions that are critically involved in arousal, affect and motivation in the normal state and become altered or engaged in the pathological states. This reductionist approach taken up until the early 2000s has provided a wealth of information regarding which molecules and which brain regions are involved in certain aspects of behavior, yet have been studied in a modular fashion. Another population of scientists has taken a ‘top-down’ approach by attempting to study the activity of whole brain regions both in human and non-human primates under different theoretical frameworks that include learning and economic theories. The next wave of research as well as a primary goal of this thesis work is the merger of these two different scientific approaches. We sought to understand how these molecules interact with each other within discrete limbic brain regions to produce behavioral responses; how they feed forward and feedback on one another and how they are released temporally in response to stress and arousing environmental stimuli. From this line of

work as well as many other studies we are beginning to more fully understand behavior and psychiatric diseases on the microcircuit level.

New Insights on neuropeptidergic regulation of monoamines

Hans Selye was the first to describe the concepts of eustress and distress[26], which has since been expounded on by McEwen and Sapolsky[29,47,48]. Here we have tried to tease these two concepts apart biologically by examining first, acute neuropeptidergic regulation of monoamines and then examining adaptations of those interactions following exposure to severe stress. Adaptive responses to acute stressors in the environment first require the engagement of arousal systems. In order to adaptively and actively cope with a stressor, there must be a motivational component that allows the individual to respond to the stimulus. This motivational component may be positive or negative meaning that it enables an individual to either actively approach the stimulus in spite of potential risk or actively avoid the stimulus. Finally, it is useful to the organism to learn that some stressors are dangerous or threatening. In order to do this, stressors must elicit some negative affective state, whether it be dysphoria, anxiety or fear, that allows the animal to associate certain stimuli with negative outcomes. It has been shown that acute stressors elicit the release of CRF extra-hypothalamically[210,288]. CRF administered i.c.v and therefore, made available to several brain regions, is aversive to the animal and causes the animal to avoid riskier environments such as the open arms of an elevated plus maze or an open field[131,167,205,250]. However, it is possible that these effects are mediated by CRF acting on a subset of brain regions. When CRF is administered directly into the BNST, a brain region that has been implicated in anxiety responses[70], it also produces an aversion[225]

indicating that this brain region is critical in encoding CRF-induced aversion. We have established that CRF causes the release of dynorphin and activation of KORs in several limbic brain regions; most notably the BLA and DRN[205]. It is this engagement of the dynorphin-KOR system by CRF that underlies both the aversive and anxiogenic qualities of CRF[167,205,250]. Importantly, when KOR activation is blocked in the BLA via site-specific injection of norBNI, that negative affective state assayed by place aversion and elevated plus maze are blocked[250] suggesting that CRF-mediated KOR activation in the BLA is a critical for informing the animal that stressful stimuli are threatening or should be avoided. We have also found that conditioned place aversion produced by direct KOR agonism with U50,488 is blocked by prior norBNI injection into the DRN[254] demonstrating that the aversive component of KOR activation also requires the DRN. The DRN is also of interest since it has one of the densest concentrations of CRF R2[125] receptors in the brain and the reader may recall that the CRF-induced aversion was mediated by CRF R2[205]. Interestingly, we also found that KOR activation in the DRN was also necessary to suppress nociceptive responding during stress events[254]. These two pieces of evidence suggested that KOR acting acutely in the DRN acts as a "negative affect" signal. I were particularly interested in the KOR regulation of the DRN because of the large body of evidence suggesting that serotonin is instrumental in regulating mood or emotional states, however, KOR regulation of serotonergic DRN cell excitability has never been fully characterized. As part of this thesis work, we surveyed the functional actions of KOR in serotonin-containing cells of the DRN using electrophysiological techniques. I measured the actions of KOR activation on fast excitatory and inhibitory synaptic transmission as well as activation of post-synaptic

potassium channels. I found that KOR agonism by U69,593 has a net inhibitory regulation of DRN neuronal excitability by inhibiting glutamatergic release probability pre-synaptically and activating GIRK channels post-synaptically. It was known that increases in dynorphin and decreases in serotonin in limbic regions both produced negative affect. This set of studies elucidates how these two neurochemicals interact with each to produce this change in emotional state. Moreover dynorphin-KOR regulation of the DRN may promote increased behavioral flexibility or activity by decreasing serotonin release and inactivating behavioral inhibition. Collectively this data supports a working model in which stressors elicit CRF release which then engages the dynorphin-KOR system to negatively regulate serotonergic neuronal firing and release into the forebrain.

Importantly, CRF is not only released in response to acute stressors[210,288], but also is released extra-hypothalamically in response to arousing stimuli that at face value do not seem inherently stressful or negative such as cues that are associated with palatable food[212,213]. Recently, it has been shown that when CRF is administered specifically into the nucleus accumbens, a region that is important for controlling motivated behavior, promotes appetitive behaviors including social pair bonding and responding to cued rewards[215,216]. Both of these behaviors are mediated by accumbal dopamine release[217,218]. While this may seem paradoxical given what we know about the behavioral consequences of the CRF i.c.v. administration, it is intuitive that arousing environmental stimuli may elicit appetitive or approach behaviors; this is how we are able to engage in the environment. Here we show that CRF acts at CRF receptors (CRF R1 and R2) on dopamine terminals within the nucleus accumbens to increase

dopamine release and that this cellular action causes a place preference (i.e. promotes approach to environment in which the animal has experience CRF-elicited dopamine release). Importantly, we also show that endogenous CRF release causes animals to approach and interact with a novel object. Exposure to novelty has been characterized as inherently stressful or anxiogenic in rodents, yet also elicits exploratory and investigatory behavior by the animal. In other words, animals will overcome the anxiogenic component of novelty and explore the novel object. It is obvious how this mechanism would be adaptive for an organism as it would allow the organism to interrogate new environments in search of food and mates despite some inherent risk. As discussed above, a critical feature of accumbens dopamine release is that it allows organisms to overcome obstacles to obtain rewards (see Salamone et al. 2003). I would assert that CRF positive regulation of dopamine release in the nucleus accumbens is a mechanism by which an organism can engage in the environment and overcome obstacles that may have some risk associated with them.

The body of evidence presented in this thesis describes and characterizes how the stress-related neuropeptides CRF and dynorphin impinge on the serotonin and dopamine systems to regulate behavioral responding to environmental stimuli with varying saliencies and valences associated with them.

The impact of severe or repeated stress on neuropeptidergic regulation of monoamine systems

Even though behavioral responses to acute stressors are undoubtedly adaptive for an organism's survival, one could argue that even acute or mild stressors have an

inherent negative subjective quality. While these acute stress responses may protect against threat and motivate an individual to achieve long-term goals, they do have an intrinsic negative affective quality. It is, therefore, intuitive that normal stress responding is particularly fragile to dysregulation or adaptation following severe or repeated stress exposure. Following stress trauma, as Beck asserted in his initial theoretical framework of depression, there is a shift to a negative perceptual bias. To be more specific, there seems to be an amplification of the negative affective qualities of stressors and an ablation of the motivational qualities of stressors. In concert, this trauma leads to a shift in the organism's behavioral responding such that it now passively copes with environmental challenges (i.e. Dove mode) and withdraws from the environment.

Many of these studies examine behaviors during the actual stress challenge, but fewer go on to look at behavioral responding following stress exposure. Having established the manner in which CRF and dynorphin regulate the dopamine and serotonin systems respectively in naïve animals, we then exposed another group of mice to repeated swim stress and asked how this normal regulation is altered or becomes dysregulated. Here we report that normal dynorphin-KOR negative regulation of the DRN is blunted following repeated stressor exposure, specifically by reducing the effectiveness of KOR activation of post-synaptic GIRK currents. Interestingly, this neuroadaptation persists as long as the stress exposure is reinforced (i.e. the animal is given additional exposures to swim stress), but returns when the animals are given a 24 hour recovery period. Interestingly, the reduction in KOR activated GIRK current appears to be fairly moderate. Even though KOR activation of GIRK currents is reduced by half, it is still present and KOR inhibition of excitatory transmission pre-

synaptically remains intact. I were also struck at how dynamic the dynorphin-KOR system, at least in the DRN, appears to be in that its functionality can shift fairly rapidly depending on the specific temporal kinetics of the stress exposure, especially when contrasted to the effect of stress exposure on CRF signaling in the DRN. Swim stress exposure completely ablates CRF regulation of DRN neuronal excitability[154]. It is unknown if CRF signaling in the DRN ever recovers, but it is absent in stress vulnerable WKY rats suggesting that this adaptation does not necessarily require a severe stress exposure[88]. These findings suggest that the dynorphin-KOR system is a critical system to the animal's survival in that is fairly resilient and is in large part retained even after severe stress exposure. Indeed, I found another example of this when assessing KOR regulation of dopamine release in the nucleus accumbens. I found that stress exposure had absolutely no effect on KOR inhibition of dopamine release in the nucleus accumbens. Taken together, the data suggest that dynorphin-KOR inhibition of monoamine systems is preserved following severe repeated stress exposure and may underlie the amplification a negative perceptual bias. This notion is corroborated by our findings that stress exposure completely ablates CRF mediated potentiation of dopamine release which we have shown underlies appetitive or approach behavior.

As stated in the introduction, Korte and colleagues (2005) have asserted that it is likely that severe or chronic stress exposure produces of number of neuroadaptations. How do we know what adaptations are compensatory and which are at the root of maladaptive behavioral responses that lead to the actual pathology. One hint may come from the actual diagnostic criterion for MDD. Part of the diagnostic criterion for MDD is that the individual must experience a preponderance of systems nearly every

day for a period of two weeks[4]. Moreover, in a study examining the protracted course of MDD, Coryell and colleagues (1994) found that only 25% of patients recovered from a depressive episode within two months of the initiation of the episode and even more alarming, 20% of patients had not significantly recovered after two years[231]. Additionally, with an 80% reoccurrence rate, it is very likely that patients suffer from sub-threshold depressive symptoms even after remission from a major depressive episode. With all that in mind, what has seemed to be relatively lacking in pre-clinical research modeling stress-induced depression is investigation of the persistence of stress-induced neuroadaptations. Most studies have not assessed the recovery or persistence of stress-induced neuroadaptations passed 24 hours after the final stress exposure and no studies assess these adaptations similar to a time course that maps onto a protracted course of depression. We were struck by the persistence of the stress-induced ablation of the CRF potentiation of dopamine release in the nucleus accumbens. We found that even 90 days after the swim stress, CRF regulation of dopamine release was still ablated. Moreover, this long-lasting cellular dysregulation was accompanied by an ablation of the CRF induced place preference when assessed at 7 and 90 days following stress exposure. Similarly, endogenous CRF release no longer promoted exploratory behavior. In animals tested at 7 days, there was not only an ablation of the CRF place preference, but there was a switch to a CRF aversion. Unlike many other neuroadaptations that are precipitated by stress exposure, this alteration does not recover and therefore, may be a critical locus for the etiology of protracted depression.

Future directions

Epigenetics

The stress-induced ablation of CRF potentiation of dopamine release was mediated by glucocorticoid receptors. Glucocorticoid signaling has been shown to have genomic repressive effects of the CRF system[56], in particular it causes the downregulation of both CRF R1[148] and CRF R2[149] mRNA. This finding suggests that stress-induced long-lasting changes in brain function may due to epigenetic and transcriptional regulation. Recently it has been shown that glucocorticoid-mediated potentiation of anxiety behavior as well as glucocorticoid-induced alterations in DRN neuronal function were dependent on glucocorticoid-mediated activation of the cytoplasmic lysine deacetylase HDAC6, which controls Hsp90 acetylation[85]. One direction that research on stress-induced psychiatric diseases is heading in is to further examine stress-induced epigenetic changes[289]. Long-lasting changes in brain function that lead to chronic recurring or unremitting depression may be highly dependent on stress-induced epigenetic changes (i.e. histone modifications and DNA methylation). A recent set of studies has demonstrated a role for DNA methytransferases (Dmnts) (catalyzes DNA methylation which is inversely related to gene expression)[290] and histone methyltransferases (promotes increased gene expression)[291] in mediating pro-depressive- and pro-addictive-like behaviors produced by chronic social defeat stress. The main technological limitation in studying the causal relationship between epigenetic modifications and depression is the lack of specificity of our current tools. There are very few, if any pharmacological tools that are selective for specific HDACs or Dmnts. Development of virally mediated overexpression techniques as well as transgenic tools

have improved the specificity of the manipulation of the epigenetic effector, yet has not addressed the lack the specificity of the epigenetic substrate. In other words, if we wanted to test hypothesis that glucocorticoids cause epigenetic changes in DNA transcription that leads to long-term downregulation of CRF R1 in dopaminergic neurons, we could not because there is currently no way to examine that specific interaction. Rather, with our current tools, we can only assess the epigenetic actions of glucocorticoids in the VTA on all of its downstream targets, not CRF R1 specifically. Glucocorticoid receptors have both genomic and non-genomic actions that are incredibly diverse and must select for different repressive or activating effects on the large number of substrates that are regulated by GRs[56]. Certainly for enzymes like Dmnts there are recognition sites along the DNA strand (i.e. C-6 position of adenines), but does this mean that when a stress-related receptor activates Dmnts there is a global repression of gene transcription or is there an additional “zip code” or different protein complex formations that allows Dmnts to target specific adenines that would lead to one gene being repressed over another? Further study needs to be done to understand if these “zip codes” exist and how they work, which in turn will aid in better pharmacological and genetic tools to understand behavioral questions.

Temporal dynamics of neuropeptide release and regulation

One of the major gaps in our understanding of neuropeptide signaling is the temporal dynamics of neuropeptide release and regulation. There have been very few studies that examine the release of endogenous neuropeptides at all and even fewer that have done so in awake behaving animals. Moreover, until recently the only tool available to directly measure extracellular neuropeptides is microdialysis with HPLC.

The limitation with microdialysis is that it has relatively slow temporal dynamics (on the order of 10s and 20s of minutes) and causes substantial damage to the surrounding tissue due to the size of the probe. However, work from Robert Kennedy's lab revolutionized old microdialysis techniques by resolving both of these pitfalls. Using a combination of microfluidics/capillary segmentation and mass spectrometry, the Kennedy group has been able to resolve neurochemical measurements, even in awake behaving animals to seconds for small molecules[292-295]. Using a method called capillary liquid chromatography-triple-stage mass spectrometry, they have been able to measure endogenous opioid peptides, including dynorphin (1-8), in the extracellular space on the order of minutes and have also immensely improved the feasibility of measuring these peptides at all[296,297]. Very large peptides such as CRF(41 aa) still pose technical challenges due to its impermeability of the microdialysis probe membrane. However, these new techniques will promote more *in vivo* monitoring of the neurochemicals with temporal and spatial resolution and is the start of a very exciting and new direction.

Accompanying this technique is the development of neuropeptide-cre transgenic animals. In 2010, Martin and colleagues developed and published a CRF-Cre transgenic mouse [298]. Like other Cre-lines, this new transgenic tool opens up a huge amount of new research on the dynamics of the CRF system. With this tool, investigators can electrophysiologically characterize CRF containing neurons, genetically remove proteins from these neurons and also manipulate the firing of these neurons with both optogenentic techniques and virally-expressed designer receptors (i.e. DREDDs). This will allow investigators to really assess the temporal relationship between the engagement of the CRF system within discrete brain regions and

subsequent behavioral consequences. Along the same line there has been the development of floxed CRF R1 mice in which CRF R1 is excised from different neuronal populations. (Floxed KOR mice are also being developed, but have not been published yet). Refojo and colleagues (2010) demonstrated that CRF R1 excision from glutamatergic neurons produced anxiolysis whereas CRF R1 excision from dopamine neurons produced anxiety-like behaviors in mice[241]. These data support one of the main assertions from our own body of work that neuropeptide regulation of disparate neurochemical systems leads mediates different behavioral responses and that CRF does not act globally to produce one behavioral or emotional consequence. Similar mouse lines that interrogate the dynorphin-KOR system are currently in development.

Clearly we have come a long way from the eras of Freud, Selye and Beck in understanding the biological basis of psychiatric pathology, though their contributions cannot be overstated. However, even with the advancement of new tools, the real goal for the next 50 years of research is to take everything we know about the biology of depression and use it, not to treat MDD, but to treat *individuals* suffering from depression. The words “treatment-resistance” and “unremitting” are perhaps the two most alarming realities about the current state of MDD. If we continue to use more sophisticated and higher resolution tools to interrogate depression-like behavior using the same behavioral models we are not going to advance as far as we could. In the future we have to create better animal models that model different constellations of symptoms that fall into the larger diagnostic criterion of the disease so that we can learn how to better tailor treatments.

References

1 Kessler, R. C. *et al.* Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Archives of general psychiatry* **62**, 593-602 (2005).

2 Samuels, B. A. *et al.* Modeling treatment-resistant depression. *Neuropharmacology* **61**, 408-413, doi:10.1016/j.neuropharm.2011.02.017 (2011).

3 Fava, M. & Kendler, K. S. Major depressive disorder. *Neuron* **28**, 335-341 (2000).

4 American Psychiatric Association. *Diagnostic criteria from DSM-IV-TR*. (American Psychiatric Association, 2000).

5 Vohringer, P. A. & Ghaemi, S. N. Solving the antidepressant efficacy question: effect sizes in major depressive disorder. *Clinical therapeutics* **33**, B49-61, doi:10.1016/j.clinthera.2011.11.019 (2011).

6 Beck, A. T. Thinking and Depression. I. Idiosyncratic Content and Cognitive Distortions. *Archives of general psychiatry* **9**, 324-333 (1963).

7 Beck, A. T. Thinking and Depression. Ii. Theory and Therapy. *Archives of general psychiatry* **10**, 561-571 (1964).

8 Beck, A. T. The evolution of the cognitive model of depression and its neurobiological correlates. *The American journal of psychiatry* **165**, 969-977 (2008).

9 Beck, A. T. & Dozois, D. J. Cognitive therapy: current status and future directions. *Annual review of medicine* **62**, 397-409, doi:10.1146/annurev-med-052209-100032 (2011).

10 Seligman, M. E. Learned helplessness. *Annual review of medicine* **23**, 407-412, doi:10.1146/annurev.me.23.020172.002203 (1972).

11 Chourbaji, S. *et al.* Learned helplessness: validity and reliability of depressive-like states in mice. *Brain Res Brain Res Protoc* **16**, 70-78 (2005).

12 Henkel, V., Bussfeld, P., Moller, H. J. & Hegerl, U. Cognitive-behavioural theories of helplessness/hopelessness: valid models of depression? *Eur Arch Psychiatry Clin Neurosci* **252**, 240-249 (2002).

13 Maier, S. F. & Watkins, L. R. Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neuroscience and biobehavioral reviews* **29**, 829-841, doi:10.1016/j.neubiorev.2005.03.021 (2005).

14 Charney, D. S. Monoamine dysfunction and the pathophysiology and treatment of depression. *The Journal of clinical psychiatry* **59 Suppl 14**, 11-14 (1998).

15 Graeff, F. G., Guimaraes, F. S., De Andrade, T. G. & Deakin, J. F. Role of 5-HT in stress, anxiety, and depression. *Pharmacology, biochemistry, and behavior* **54**, 129-141 (1996).

16 Carlsson, A. The contribution of drug research to investigating the nature of endogenous depression. *Pharmakopsychiatrie, Neuro-Psychopharmakologie* **9**, 2-10 (1976).

17 Owens, M. J. & Nemeroff, C. B. The serotonin transporter and depression. *Depression and anxiety* **8 Suppl 1**, 5-12 (1998).

18 Delgado, P. L. Depression: the case for a monoamine deficiency. *The Journal of clinical psychiatry* **61 Suppl 6**, 7-11 (2000).

19 Delgado, P. L. & Moreno, F. A. Role of norepinephrine in depression. *The Journal of clinical psychiatry* **61 Suppl 1**, 5-12 (2000).
20 Heninger, G. R., Delgado, P. L. & Charney, D. S. The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. *Pharmacopsychiatry* **29**, 2-11, doi:10.1055/s-2007-979535 (1996).
21 Moreno, F. A., Heninger, G. R., McGahuey, C. A. & Delgado, P. L. Tryptophan depletion and risk of depression relapse: a prospective study of tryptophan depletion as a potential predictor of depressive episodes. *Biological psychiatry* **48**, 327-329 (2000).
22 Moreno, F. A. *et al.* Tryptophan depletion selectively reduces CSF 5-HT metabolites in healthy young men: results from single lumbar puncture sampling technique. *Int J Neuropsychopharmacol* **3**, 277-283, doi:10.1017/S1461145700002133 (2000).
23 Bale, T. L. Stress sensitivity and the development of affective disorders. *Horm Behav* **50**, 529-533 (2006).
24 Selye, H. Stress and the general adaptation syndrome. *British medical journal* **1**, 1383-1392 (1950).
25 Selye, H. & Fortier, C. Adaptive reaction to stress. *Psychosomatic medicine* **12**, 149-157 (1950).
26 Selye, H. Stress and distress. *Comprehensive therapy* **1**, 9-13 (1975).
27 Selye, H. Implications of stress concept. *New York state journal of medicine* **75**, 2139-2145 (1975).
28 Selye, H. Confusion and controversy in the stress field. *Journal of human stress* **1**, 37-44, doi:10.1080/0097840X.1975.9940406 (1975).
29 McEwen, B. S. & Sapolsky, R. M. Stress and cognitive function. *Curr Opin Neurobiol* **5**, 205-216 (1995).
30 Korte, S. M., Koolhaas, J. M., Wingfield, J. C. & McEwen, B. S. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neuroscience and biobehavioral reviews* **29**, 3-38 (2005).
31 Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neuroscience and biobehavioral reviews*.
32 Maier, S. F. Stressor controllability and stress-induced analgesia. *Annals of the New York Academy of Sciences* **467**, 55-72 (1986).
33 Porsolt, R. D., Le Pichon, M. & Jalfre, M. Depression: a new animal model sensitive to antidepressant treatments. *Nature* **266**, 730-732 (1977).
34 Cryan, J. F., Valentino, R. J. & Lucki, I. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neuroscience and biobehavioral reviews* **29**, 547-569 (2005).
35 Nestler, E. J. *et al.* Preclinical models: status of basic research in depression. *Biological psychiatry* **52**, 503-528 (2002).
36 Willner, P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology* **134**, 319-329 (1997).

37 Willner, P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* **52**, 90-110 (2005).

38 Willner, P. & Mitchell, P. J. The validity of animal models of predisposition to depression. *Behav Pharmacol* **13**, 169-188 (2002).

39 Bjorkqvist, K. Social defeat as a stressor in humans. *Physiol Behav* **73**, 435-442 (2001).

40 Huhman, K. L. Social conflict models: can they inform us about human psychopathology? *Horm Behav* **50**, 640-646 (2006).

41 Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34**, 13-25 (2002).

42 Kalsbeek, A. *et al.* Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Molecular and cellular endocrinology* **349**, 20-29, doi:10.1016/j.mce.2011.06.042 (2012).

43 Papadimitriou, A. & Priftis, K. N. Regulation of the hypothalamic-pituitary-adrenal axis. *Neuroimmunomodulation* **16**, 265-271, doi:10.1159/000216184 (2009).

44 Spiess, J., Rivier, J., Rivier, C. & Vale, W. Primary structure of corticotropin-releasing factor from ovine hypothalamus. *Proc Natl Acad Sci U S A* **78**, 6517-6521 (1981).

45 Sapolsky, R. M. Stress and plasticity in the limbic system. *Neurochem Res* **28**, 1735-1742 (2003).

46 Sapolsky, R. M., Krey, L. C. & McEwen, B. S. The adrenocortical axis in the aged rat: impaired sensitivity to both fast and delayed feedback inhibition. *Neurobiology of aging* **7**, 331-335 (1986).

47 Sapolsky, R. M., Krey, L. C. & McEwen, B. S. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. *Endocrine reviews* **7**, 284-301 (1986).

48 McEwen, B. S. Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. *Metabolism: clinical and experimental* **54**, 20-23 (2005).

49 Gold, P. W., Licinio, J., Wong, M. L. & Chrousos, G. P. Corticotropin releasing hormone in the pathophysiology of melancholic and atypical depression and in the mechanism of action of antidepressant drugs. *Annals of the New York Academy of Sciences* **771**, 716-729 (1995).

50 Rittenhouse, P. A., Lopez-Rubalcava, C., Stanwood, G. D. & Lucki, I. Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat. *Psychoneuroendocrinology* **27**, 303-318 (2002).

51 Scott, L. V. & Dinan, T. G. Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. *Life sciences* **62**, 1985-1998 (1998).

52 Scott, L. V., Medbak, S. & Dinan, T. G. Blunted adrenocorticotropin and cortisol responses to corticotropin-releasing hormone stimulation in chronic fatigue syndrome. *Acta psychiatrica Scandinavica* **97**, 450-457 (1998).

53 Young, E. A., Akana, S. & Dallman, M. F. Decreased sensitivity to glucocorticoid fast feedback in chronically stressed rats. *Neuroendocrinology* **51**, 536-542 (1990).

54 Young, E. A., Haskett, R. F., Murphy-Weinberg, V., Watson, S. J. & Akil, H. Loss of glucocorticoid fast feedback in depression. *Archives of general psychiatry* **48**, 693-699 (1991).
55 Chrousos, G. P. & Kino, T. Glucocorticoid signaling in the cell. Expanding clinical implications to complex human behavioral and somatic disorders. *Annals of the New York Academy of Sciences* **1179**, 153-166, doi:10.1111/j.1749-6632.2009.04988.x (2009).
56 Revollo, J. R. & Cidlowski, J. A. Mechanisms generating diversity in glucocorticoid receptor signaling. *Annals of the New York Academy of Sciences* **1179**, 167-178, doi:10.1111/j.1749-6632.2009.04986.x (2009).
57 Aston-Jones, G. & Bloom, F. E. Norepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. *J Neurosci* **1**, 887-900 (1981).
58 Aston-Jones, G. & Bloom, F. E. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *J Neurosci* **1**, 876-886 (1981).
59 Aston-Jones, G., Rajkowski, J. & Cohen, J. Role of locus coeruleus in attention and behavioral flexibility. *Biological psychiatry* **46**, 1309-1320 (1999).
60 Usher, M., Cohen, J. D., Servan-Schreiber, D., Rajkowski, J. & Aston-Jones, G. The role of locus coeruleus in the regulation of cognitive performance. *Science* **283**, 549-554 (1999).
61 Foote, S. L., Berridge, C. W., Adams, L. M. & Pineda, J. A. Electrophysiological evidence for the involvement of the locus coeruleus in alerting, orienting, and attending. *Prog Brain Res* **88**, 521-532 (1991).
62 Page, M. E., Berridge, C. W., Foote, S. L. & Valentino, R. J. Corticotropin-releasing factor in the locus coeruleus mediates EEG activation associated with hypotensive stress. *Neuroscience letters* **164**, 81-84 (1993).
63 Funkenstein, D. H. & Meade, L. W. Norepinephrine-like and epinephrine-like substances and the elevation of blood pressure during acute stress. *The Journal of nervous and mental disease* **119**, 380-397 (1954).
64 Silverberg, A. B., Shah, S. D., Haymond, M. W. & Cryer, P. E. Norepinephrine: hormone and neurotransmitter in man. *The American journal of physiology* **234**, E252-256 (1978).
65 Bremner, J. D., Krystal, J. H., Southwick, S. M. & Charney, D. S. Noradrenergic mechanisms in stress and anxiety: II. Clinical studies. *Synapse* **23**, 39-51, doi:10.1002/(SICI)1098-2396(199605)23:1<39::AID-SYN5>3.0.CO;2-I (1996).
66 Bremner, J. D., Krystal, J. H., Southwick, S. M. & Charney, D. S. Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse* **23**, 28-38, doi:10.1002/(SICI)1098-2396(199605)23:1<28::AID-SYN4>3.0.CO;2-J (1996).
67 Gresch, P. J., Sved, A. F., Zigmond, M. J. & Finlay, J. M. Stress-induced sensitization of dopamine and norepinephrine efflux in medial prefrontal cortex of the rat. *Journal of neurochemistry* **63**, 575-583 (1994).

68 Loughlin, S. E., Foote, S. L. & Bloom, F. E. Efferent projections of nucleus locus coeruleus: topographic organization of cells of origin demonstrated by three-dimensional reconstruction. *Neuroscience* **18**, 291-306 (1986).

69 Loughlin, S. E., Foote, S. L. & Fallon, J. H. Locus coeruleus projections to cortex: topography, morphology and collateralization. *Brain research bulletin* **9**, 287-294 (1982).

70 Aston-Jones, G., Delfs, J. M., Druhan, J. & Zhu, Y. The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Annals of the New York Academy of Sciences* **877**, 486-498 (1999).

71 Barnes, N. M. & Sharp, T. A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152 (1999).

72 Cools, R., Roberts, A. C. & Robbins, T. W. Serotoninergic regulation of emotional and behavioural control processes. *Trends in cognitive sciences* **12**, 31-40, doi:10.1016/j.tics.2007.10.011 (2008).

73 Lucki, I. The spectrum of behaviors influenced by serotonin. *Biological psychiatry* **44**, 151-162 (1998).

74 Deakin, J. F. & Graeff, F. G. 5-HT and mechanisms of defence. *J Psychopharmacol* **5**, 305-315, doi:10.1177/026988119100500414 (1991).

75 Miyazaki, K., Miyazaki, K. W. & Doya, K. Activation of dorsal raphe serotonin neurons underlies waiting for delayed rewards. *J Neurosci* **31**, 469-479, doi:10.1523/JNEUROSCI.3714-10.2011 (2011).

76 Miyazaki, K. W., Miyazaki, K. & Doya, K. Activation of the central serotonergic system in response to delayed but not omitted rewards. *The European journal of neuroscience* **33**, 153-160, doi:10.1111/j.1460-9568.2010.07480.x (2011).

77 Kirby, L. G., Allen, A. R. & Lucki, I. Regional differences in the effects of forced swimming on extracellular levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. *Brain Res* **682**, 189-196 (1995).

78 Neumeister, A., Young, T. & Stastny, J. Implications of genetic research on the role of the serotonin in depression: emphasis on the serotonin type 1A receptor and the serotonin transporter. *Psychopharmacology* **174**, 512-524, doi:10.1007/s00213-004-1950-3 (2004).

79 Fallon, J. H. & Loughlin, S. E. Monoamine innervation of the forebrain: collateralization. *Brain research bulletin* **9**, 295-307 (1982).

80 Reneric, J. P. & Lucki, I. Antidepressant behavioral effects by dual inhibition of monoamine reuptake in the rat forced swimming test. *Psychopharmacology* **136**, 190-197 (1998).

81 Owens, M. J. & Nemeroff, C. B. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clinical chemistry* **40**, 288-295 (1994).

82 Samuels, B. A. & Hen, R. Neurogenesis and affective disorders. *The European journal of neuroscience* **33**, 1152-1159, doi:10.1111/j.1460-9568.2011.07614.x (2011).

83 Sahay, A. & Hen, R. Adult hippocampal neurogenesis in depression. *Nature neuroscience* **10**, 1110-1115, doi:10.1038/nn1969 (2007).

84 Albert, P. R., Le Francois, B. & Millar, A. M. Transcriptional dysregulation of 5-HT1A autoreceptors in mental illness. *Molecular brain* **4**, 21, doi:10.1186/1756-6606-4-21 (2011).
85 Espallergues, J. *et al.* HDAC6 regulates glucocorticoid receptor signaling in serotonin pathways with critical impact on stress resilience. *J Neurosci* **32**, 4400-4416, doi:10.1523/JNEUROSCI.5634-11.2012 (2012).
86 Kirby, L. G. *et al.* Cellular effects of swim stress in the dorsal raphe nucleus. *Psychoneuroendocrinology* **32**, 712-723, doi:10.1016/j.psyneuen.2007.05.001 (2007).
87 Le Francois, B., Czesak, M., Steubl, D. & Albert, P. R. Transcriptional regulation at a HTR1A polymorphism associated with mental illness. *Neuropharmacology* **55**, 977-985, doi:10.1016/j.neuropharm.2008.06.046 (2008).
88 Lemos, J. C. *et al.* Stress-hyperresponsive WKY rats demonstrate depressed dorsal raphe neuronal excitability and dysregulated CRF-mediated responses. *Neuropsychopharmacology* **36**, 721-734, doi:10.1038/npp.2010.200 (2011).
89 Canli, T. & Lesch, K. P. Long story short: the serotonin transporter in emotion regulation and social cognition. *Nature neuroscience* **10**, 1103-1109, doi:10.1038/nn1964 (2007).
90 Beaver, K. M., Vaughn, M. G., Wright, J. P. & Delisi, M. An interaction between perceived stress and 5HTTLPR genotype in the prediction of stable depressive symptomatology. *The American journal of orthopsychiatry* **82**, 260-266, doi:10.1111/j.1939-0025.2012.01148.x (2012).
91 Nestler, E. J. & Carlezon, W. A., Jr. The mesolimbic dopamine reward circuit in depression. *Biological psychiatry* **59**, 1151-1159, doi:10.1016/j.biopsych.2005.09.018 (2006).
92 Glimcher, P. W. *Neuroeconomics : decision making and the brain*. 1st edn, (Academic Press, 2009).
93 Yokel, R. A. & Wise, R. A. Increased lever pressing for amphetamine after pimozide in rats: implications for a dopamine theory of reward. *Science* **187**, 547-549 (1975).
94 Wise, R. A., Spindler, J., deWit, H. & Gerberg, G. J. Neuroleptic-induced "anhedonia" in rats: pimozide blocks reward quality of food. *Science* **201**, 262-264 (1978).
95 Wise, R. A. & Bozarth, M. A. Brain mechanisms of drug reward and euphoria. *Psychiatric medicine* **3**, 445-460 (1985).
96 Wise, R. A., Bauco, P., Carlezon, W. A., Jr. & Trojniar, W. Self-stimulation and drug reward mechanisms. *Annals of the New York Academy of Sciences* **654**, 192-198 (1992).
97 Schultz, W., Dayan, P. & Montague, P. R. A neural substrate of prediction and reward. *Science* **275**, 1593-1599 (1997).
98 Clark, J. J. *et al.* Chronic microsensors for longitudinal, subsecond dopamine detection in behaving animals. *Nature methods* **7**, 126-129, doi:10.1038/nmeth.1412 (2010).
99 Gan, J. O., Walton, M. E. & Phillips, P. E. Dissociable cost and benefit encoding of future rewards by mesolimbic dopamine. *Nature neuroscience* **13**, 25-27, doi:10.1038/nn.2460 (2010).

100 Tobler, P. N., Fiorillo, C. D. & Schultz, W. Adaptive coding of reward value by dopamine neurons. *Science* **307**, 1642-1645, doi:10.1126/science.1105370 (2005).

101 Salamone, J. D., Correa, M., Mingote, S. & Weber, S. M. Nucleus accumbens dopamine and the regulation of effort in food-seeking behavior: implications for studies of natural motivation, psychiatry, and drug abuse. *The Journal of pharmacology and experimental therapeutics* **305**, 1-8, doi:10.1124/jpet.102.035063 (2003).

102 Phillips, P. E., Walton, M. E. & Jhou, T. C. Calculating utility: preclinical evidence for cost-benefit analysis by mesolimbic dopamine. *Psychopharmacology* **191**, 483-495, doi:10.1007/s00213-006-0626-6 (2007).

103 Piazza, P. V. & Le Moal, M. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Res Brain Res Rev* **25**, 359-372 (1997).

104 Piazza, P. V. *et al.* Glucocorticoids have state-dependent stimulant effects on the mesencephalic dopaminergic transmission. *Proc Natl Acad Sci U S A* **93**, 8716-8720 (1996).

105 Rouge-Pont, F., Deroche, V., Le Moal, M. & Piazza, P. V. Individual differences in stress-induced dopamine release in the nucleus accumbens are influenced by corticosterone. *The European journal of neuroscience* **10**, 3903-3907 (1998).

106 Jan, L. Y. & Jan, Y. N. Role of an LHRH-like peptide as a neurotransmitter is sympathetic ganglia of the frog. *Federation proceedings* **40**, 2560-2564 (1981).

107 Jan, L. Y. & Jan, Y. N. Peptidergic transmission in sympathetic ganglia of the frog. *J Physiol* **327**, 219-246 (1982).

108 Jan, L. Y., Jan, Y. N. & Brownfield, M. S. Peptidergic transmitters in synaptic boutons of sympathetic ganglia. *Nature* **288**, 380-382 (1980).

109 Jan, Y. N., Jan, L. Y. & Kuffler, S. W. A peptide as a possible transmitter in sympathetic ganglia of the frog. *Proc Natl Acad Sci U S A* **76**, 1501-1505 (1979).

110 Jan, Y. N., Jan, L. Y. & Kuffler, S. W. Further evidence for peptidergic transmission in sympathetic ganglia. *Proc Natl Acad Sci U S A* **77**, 5008-5012 (1980).

111 Wagner, J. J., Caudle, R. M. & Chavkin, C. Kappa-opioids decrease excitatory transmission in the dentate gyrus of the guinea pig hippocampus. *J Neurosci* **12**, 132-141 (1992).

112 Wagner, J. J., Evans, C. J. & Chavkin, C. Focal stimulation of the mossy fibers releases endogenous dynorphins that bind kappa 1-opioid receptors in guinea pig hippocampus. *Journal of neurochemistry* **57**, 333-343 (1991).

113 Wagner, J. J., Terman, G. W. & Chavkin, C. Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature* **363**, 451-454 (1993).

114 Castillo, P. E., Salin, P. A., Weisskopf, M. G. & Nicoll, R. A. Characterizing the site and mode of action of dynorphin at hippocampal mossy fiber synapses in the guinea pig. *J Neurosci* **16**, 5942-5950 (1996).

115 Weisskopf, M. G., Zalutsky, R. A. & Nicoll, R. A. The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. *Nature* **362**, 423-427 (1993).

116 Akil, H., Mayer, D. J. & Liebeskind, J. C. Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science* **191**, 961-962 (1976).
117 Akil, H., Shiomi, H. & Matthews, J. Induction of the intermediate pituitary by stress: synthesis and release of a nonopioid form of beta-endorphin. *Science* **227**, 424-426 (1985).
118 Turnbull, A. V. *et al.* CRF type I receptor-deficient mice exhibit a pronounced pituitary-adrenal response to local inflammation. *Endocrinology* **140**, 1013-1017 (1999).
119 Bale, T. L. *et al.* Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nature genetics* **24**, 410-414 (2000).
120 Dautzenberg, F. M. & Hauger, R. L. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol Sci* **23**, 71-77 (2002).
121 Milan-Lobo, L. *et al.* Subtype-specific differences in corticotropin-releasing factor receptor complexes detected by fluorescence spectroscopy. *Mol Pharmacol* **76**, 1196-1210, doi:10.1124/mol.109.059139 (2009).
122 Merchenthaler, I. Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. *Peptides* **5 Suppl 1**, 53-69 (1984).
123 Bale, T. L. & Vale, W. W. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* **44**, 525-557 (2004).
124 Millan, M. A., Jacobowitz, D. M., Hauger, R. L., Catt, K. J. & Aguilera, G. Distribution of corticotropin-releasing factor receptors in primate brain. *Proc Natl Acad Sci U S A* **83**, 1921-1925 (1986).
125 Steckler, T. & Holsboer, F. Corticotropin-releasing hormone receptor subtypes and emotion. *Biological psychiatry* **46**, 1480-1508 (1999).
126 Van Pett, K. *et al.* Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *The Journal of comparative neurology* **428**, 191-212 (2000).
127 Zorrilla, E. P., Valdez, G. R., Nozulak, J., Koob, G. F. & Markou, A. Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. *Brain Res* **952**, 188-199 (2002).
128 Deak, T. *et al.* The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. *Endocrinology* **140**, 79-86 (1999).
129 Swerdlow, N. R. & Koob, G. F. Separate neural substrates of the locomotor-activating properties of amphetamine, heroin, caffeine and corticotropin releasing factor (CRF) in the rat. *Pharmacology, biochemistry, and behavior* **23**, 303-307 (1985).
130 Kalivas, P. W., Duffy, P. & Latimer, L. G. Neurochemical and behavioral effects of corticotropin-releasing factor in the ventral tegmental area of the rat. *The Journal of pharmacology and experimental therapeutics* **242**, 757-763 (1987).
131 Cador, M., Ahmed, S. H., Koob, G. F., Le Moal, M. & Stinus, L. Corticotropin-releasing factor induces a place aversion independent of its neuroendocrine role. *Brain Res* **597**, 304-309 (1992).

132 Shalev, U., Erb, S. & Shaham, Y. Role of CRF and other neuropeptides in stress-induced reinstatement of drug seeking. *Brain Res* **1314**, 15-28, doi:10.1016/j.brainres.2009.07.028 (2010).
133 Koob, G. F. The role of CRF and CRF-related peptides in the dark side of addiction. *Brain Res* **1314**, 3-14, doi:10.1016/j.brainres.2009.11.008 (2010).
134 Liu, J. *et al.* Corticotropin-releasing factor and Urocortin I modulate excitatory glutamatergic synaptic transmission. *J Neurosci* **24**, 4020-4029, doi:10.1523/JNEUROSCI.5531-03.2004 (2004).
135 Kirby, L. G. *et al.* Corticotropin-releasing factor increases GABA synaptic activity and induces inward current in 5-hydroxytryptamine dorsal raphe neurons. *J Neurosci* **28**, 12927-12937, doi:10.1523/JNEUROSCI.2887-08.2008 (2008).
136 Kirby, L. G., Rice, K. C. & Valentino, R. J. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology* **22**, 148-162 (2000).
137 Curtis, A. L., Lechner, S. M., Pavcovich, L. A. & Valentino, R. J. Activation of the locus coeruleus noradrenergic system by intracoerulear microinfusion of corticotropin-releasing factor: effects on discharge rate, cortical norepinephrine levels and cortical electroencephalographic activity. *The Journal of pharmacology and experimental therapeutics* **281**, 163-172 (1997).
138 Page, M. E. & Valentino, R. J. Locus coeruleus activation by physiological challenges. *Brain research bulletin* **35**, 557-560 (1994).
139 Valentino, R. J., Foote, S. L. & Aston-Jones, G. Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus. *Brain Res* **270**, 363-367 (1983).
140 Valentino, R. J. & Van Bockstaele, E. Convergent regulation of locus coeruleus activity as an adaptive response to stress. *European journal of pharmacology* **583**, 194-203, doi:10.1016/j.ejphar.2007.11.062 (2008).
141 Snyder, K., Wang, W. W., Han, R., McFadden, K. & Valentino, R. J. Corticotropin-releasing factor in the norepinephrine nucleus, locus coeruleus, facilitates behavioral flexibility. *Neuropsychopharmacology* **37**, 520-530, doi:10.1038/npp.2011.218 (2012).
142 Clark, M. S., McDevitt, R. A., Hoplight, B. J. & Neumaier, J. F. Chronic low dose ovine corticotropin releasing factor or urocortin II into the rostral dorsal raphe alters exploratory behavior and serotonergic gene expression in specific subregions of the dorsal raphe. *Neuroscience* **146**, 1888-1905, doi:10.1016/j.neuroscience.2007.03.032 (2007).
143 Lukkes, J., Vuong, S., Scholl, J., Oliver, H. & Forster, G. Corticotropin-releasing factor receptor antagonism within the dorsal raphe nucleus reduces social anxiety-like behavior after early-life social isolation. *J Neurosci* **29**, 9955-9960, doi:10.1523/JNEUROSCI.0854-09.2009 (2009).
144 Wanat, M. J., Hopf, F. W., Stuber, G. D., Phillips, P. E. & Bonci, A. Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of Ih. *J Physiol* **586**, 2157-2170 (2008).

145 Beckstead, M. J. *et al.* CRF enhancement of GIRK channel-mediated transmission in dopamine neurons. *Neuropsychopharmacology* **34**, 1926-1935 (2009).
146 Ungless, M. A. *et al.* Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* **39**, 401-407 (2003).
147 Riegel, A. C. & Williams, J. T. CRF facilitates calcium release from intracellular stores in midbrain dopamine neurons. *Neuron* **57**, 559-570 (2008).
148 Iredale, P. A. & Duman, R. S. Glucocorticoid regulation of corticotropin-releasing factor1 receptor expression in pituitary-derived AtT-20 cells. *Mol Pharmacol* **51**, 794-799 (1997).
149 Chen, A. *et al.* Mouse corticotropin-releasing factor receptor type 2alpha gene: isolation, distribution, pharmacological characterization and regulation by stress and glucocorticoids. *Molecular endocrinology (Baltimore, Md* **19**, 441-458 (2005).
150 Reyes, B. A., Fox, K., Valentino, R. J. & Van Bockstaele, E. J. Agonist-induced internalization of corticotropin-releasing factor receptors in noradrenergic neurons of the rat locus coeruleus. *The European journal of neuroscience* **23**, 2991-2998, doi:10.1111/j.1460-9568.2006.04820.x (2006).
151 Reyes, B. A., Valentino, R. J. & Van Bockstaele, E. J. Stress-induced intracellular trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons. *Endocrinology* **149**, 122-130, doi:10.1210/en.2007-0705 (2008).
152 Curtis, A. L., Pavcovich, L. A. & Valentino, R. J. Long-term regulation of locus ceruleus sensitivity to corticotropin-releasing factor by swim stress. *The Journal of pharmacology and experimental therapeutics* **289**, 1211-1219 (1999).
153 Waselus, M., Nazzaro, C., Valentino, R. J. & Van Bockstaele, E. J. Stress-induced redistribution of corticotropin-releasing factor receptor subtypes in the dorsal raphe nucleus. *Biological psychiatry* **66**, 76-83 (2009).
154 Lamy, C. M. & Beck, S. G. Swim stress differentially blocks CRF receptor mediated responses in dorsal raphe nucleus. *Psychoneuroendocrinology* **35**, 1321-1332, doi:10.1016/j.psyneuen.2010.03.003 (2010).
155 McEuen, J. G., Beck, S. G. & Bale, T. L. Failure to mount adaptive responses to stress results in dysregulation and cell death in the midbrain raphe. *J Neurosci* **28**, 8169-8177, doi:10.1523/JNEUROSCI.0004-08.2008 (2008).
156 Liu, J. *et al.* Chronic cocaine administration switches corticotropin-releasing factor2 receptor-mediated depression to facilitation of glutamatergic transmission in the lateral septum. *J Neurosci* **25**, 577-583 (2005).
157 Orozco-Cabal, L., Pollandt, S., Liu, J., Shinnick-Gallagher, P. & Gallagher, J. P. Regulation of synaptic transmission by CRF receptors. *Reviews in the neurosciences* **17**, 279-307 (2006).
158 Pollandt, S. *et al.* Cocaine withdrawal enhances long-term potentiation induced by corticotropin-releasing factor at central amygdala glutamatergic synapses via CRF, NMDA receptors and PKA. *The European journal of neuroscience* **24**, 1733-1743 (2006).
159 Hahn, J., Hopf, F. W. & Bonci, A. Chronic cocaine enhances corticotropin-releasing factor-dependent potentiation of excitatory transmission in ventral tegmental area dopamine neurons. *J Neurosci* **29**, 6535-6544 (2009).

160 Xu, G. P., Van Bockstaele, E., Reyes, B., Bethea, T. & Valentino, R. J. Chronic morphine sensitizes the brain norepinephrine system to corticotropin-releasing factor and stress. *J Neurosci* **24**, 8193-8197, doi:10.1523/JNEUROSCI.1657-04.2004 (2004).

161 Chavkin, C., James, I. F. & Goldstein, A. Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* **215**, 413-415 (1982).

162 Chavkin, C. & Goldstein, A. Specific receptor for the opioid peptide dynorphin: structure--activity relationships. *Proc Natl Acad Sci U S A* **78**, 6543-6547 (1981).

163 Schwarzer, C. 30 years of dynorphins--new insights on their functions in neuropsychiatric diseases. *Pharmacol Ther* **123**, 353-370, doi:10.1016/j.pharmthera.2009.05.006 (2009).

164 Wittmann, W. *et al.* Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. *Neuropsychopharmacology* **34**, 775-785, doi:10.1038/npp.2008.142 (2009).

165 Pfeiffer, A., Brantl, V., Herz, A. & Emrich, H. M. Psychotomimesis mediated by kappa opiate receptors. *Science* **233**, 774-776 (1986).

166 Sheffler, D. J. & Roth, B. L. Salvinorin A: the "magic mint" hallucinogen finds a molecular target in the kappa opioid receptor. *Trends Pharmacol Sci* **24**, 107-109 (2003).

167 Bruchas, M. R., Land, B. B., Lemos, J. C. & Chavkin, C. CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. *PloS one* **4**, e8528, doi:10.1371/journal.pone.0008528 (2009).

168 McLaughlin, J. P., Land, B. B., Li, S., Pintar, J. E. & Chavkin, C. Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. *Neuropsychopharmacology* **31**, 787-794 (2006).

169 McLaughlin, J. P., Li, S., Valdez, J., Chavkin, T. A. & Chavkin, C. Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system. *Neuropsychopharmacology* **31**, 1241-1248 (2006).

170 McLaughlin, J. P., Marton-Popovici, M. & Chavkin, C. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci* **23**, 5674-5683 (2003).

171 Redila, V. A. & Chavkin, C. Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. *Psychopharmacology* **200**, 59-70, doi:10.1007/s00213-008-1122-y (2008).

172 Schindler, A. G., Li, S. & Chavkin, C. Behavioral stress may increase the rewarding valence of cocaine-associated cues through a dynorphin/kappa-opioid receptor-mediated mechanism without affecting associative learning or memory retrieval mechanisms. *Neuropsychopharmacology* **35**, 1932-1942, doi:10.1038/npp.2010.67 (2010).

173 Beardsley, P. M., Howard, J. L., Shelton, K. L. & Carroll, F. I. Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. *Psychopharmacology* **183**, 118-126 (2005).

174 Hjelmstad, G. O. & Fields, H. L. Kappa opioid receptor activation in the nucleus accumbens inhibits glutamate and GABA release through different mechanisms. *J Neurophysiol* **89**, 2389-2395 (2003).
175 Castillo, P. E., Weisskopf, M. G. & Nicoll, R. A. The role of Ca2+ channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. *Neuron* **12**, 261-269 (1994).
176 Terman, G. W., Wagner, J. J. & Chavkin, C. Kappa opioids inhibit induction of long-term potentiation in the dentate gyrus of the guinea pig hippocampus. *J Neurosci* **14**, 4740-4747 (1994).
177 Henry, D. J., Grandy, D. K., Lester, H. A., Davidson, N. & Chavkin, C. Kappa-opioid receptors couple to inwardly rectifying potassium channels when coexpressed by Xenopus oocytes. *Mol Pharmacol* **47**, 551-557 (1995).
178 Ulens, C., Daenens, P. & Tytgat, J. The dual modulation of GIRK1/GIRK2 channels by opioid receptor ligands. *European journal of pharmacology* **385**, 239-245 (1999).
179 Torrecilla, M. *et al.* G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. *J Neurosci* **22**, 4328-4334 (2002).
180 Costa, A. C., Stasko, M. R., Stoffel, M. & Scott-McKean, J. J. G-protein-gated potassium (GIRK) channels containing the GIRK2 subunit are control hubs for pharmacologically induced hypothermic responses. *J Neurosci* **25**, 7801-7804 (2005).
181 Ippolito, D. L., Xu, M., Bruchas, M. R., Wickman, K. & Chavkin, C. Tyrosine phosphorylation of K(ir)3.1 in spinal cord is induced by acute inflammation, chronic neuropathic pain, and behavioral stress. *J Biol Chem* **280**, 41683-41693 (2005).
182 Madamba, S. G., Schweitzer, P. & Siggins, G. R. Dynorphin selectively augments the M-current in hippocampal CA1 neurons by an opiate receptor mechanism. *J Neurophysiol* **82**, 1768-1775 (1999).
183 Bausch, S. B., Esteb, T. M., Terman, G. W. & Chavkin, C. Administered and endogenously released kappa opioids decrease pilocarpine-induced seizures and seizure-induced histopathology. *The Journal of pharmacology and experimental therapeutics* **284**, 1147-1155 (1998).
184 Loacker, S., Sayyah, M., Wittmann, W., Herzog, H. & Schwarzer, C. Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. *Brain* **130**, 1017-1028 (2007).
185 de Lanerolle, N. C. *et al.* Dynorphin and the kappa 1 ligand [3H]U69,593 binding in the human epileptogenic hippocampus. *Epilepsy Res* **28**, 189-205 (1997).
186 Houser, C. R. *et al.* Altered patterns of dynorphin immunoreactivity suggest mossy fiber reorganization in human hippocampal epilepsy. *J Neurosci* **10**, 267-282 (1990).
187 De Sarro, G. B. & De Sarro, A. Anticonvulsant properties of non-competitive antagonists of the N-methyl-D-aspartate receptor in genetically epilepsy-prone rats: comparison with CPPene. *Neuropharmacology* **32**, 51-58 (1993).
188 McGinty, J. F., Henriksen, S. J., Goldstein, A., Terenius, L. & Bloom, F. E. Dynorphin is contained within hippocampal mossy fibers: immunochemical

alterations after kainic acid administration and colchicine-induced neurotoxicity. *Proc Natl Acad Sci U S A* **80**, 589-593 (1983).
189 Romualdi, P. *et al.* Early changes in prodynorphin mRNA and ir-dynorphin A levels after kindled seizures in the rat. *The European journal of neuroscience* **7**, 1850-1856 (1995).
190 Iadarola, M. J., Shin, C., McNamara, J. O. & Yang, H. Y. Changes in dynorphin, enkephalin and cholecystokinin content of hippocampus and substantia nigra after amygdala kindling. *Brain Res* **365**, 185-191 (1986).
191 Stogmann, E. *et al.* A functional polymorphism in the prodynorphin gene promotor is associated with temporal lobe epilepsy. *Ann Neurol* **51**, 260-263 (2002).
192 Gambardella, A. *et al.* Prodynorphin gene promoter polymorphism and temporal lobe epilepsy. *Epilepsia* **44**, 1255-1256 (2003).
193 Mathieu-Kia, A. M., Fan, L. Q., Kreek, M. J., Simon, E. J. & Hiller, J. M. Mu-, delta- and kappa-opioid receptor populations are differentially altered in distinct areas of postmortem brains of Alzheimer's disease patients. *Brain Res* **893**, 121-134 (2001).
194 Daumas, S. *et al.* Transient activation of the CA3 Kappa opioid system in the dorsal hippocampus modulates complex memory processing in mice. *Neurobiol Learn Mem* **88**, 94-103 (2007).
195 Carlezon, W. A., Jr. *et al.* Regulation of cocaine reward by CREB. *Science* **282**, 2272-2275 (1998).
196 Knoll, A. T. & Carlezon, W. A., Jr. Dynorphin, stress, and depression. *Brain Res* **1314**, 56-73, doi:10.1016/j.brainres.2009.09.074 (2010).
197 Sweatt, J. D. Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr Opin Neurobiol* **14**, 311-317 (2004).
198 Thomas, G. M. & Huganir, R. L. MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* **5**, 173-183 (2004).
199 Zhu, J. J., Qin, Y., Zhao, M., Van Aelst, L. & Malinow, R. Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110**, 443-455 (2002).
200 Rumbaugh, G., Adams, J. P., Kim, J. H. & Huganir, R. L. SynGAP regulates synaptic strength and mitogen-activated protein kinases in cultured neurons. *Proc Natl Acad Sci U S A* **103**, 4344-4351 (2006).
201 Hudmon, A. *et al.* Phosphorylation of sodium channel Na(v)1.8 by p38 mitogen-activated protein kinase increases current density in dorsal root ganglion neurons. *J Neurosci* **28**, 3190-3201 (2008).
202 Bruchas, M. R. *et al.* Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. *J Neurosci* **27**, 11614-11623 (2007).
203 Contarino, A. & Papaleo, F. The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal. *Proc Natl Acad Sci U S A* **102**, 18649-18654 (2005).
204 Ingallinesi, M., Rouibi, K., Le Moine, C., Papaleo, F. & Contarino, A. CRF(2) receptor-deficiency eliminates opiate withdrawal distress without impairing stress coping. *Molecular psychiatry*, doi:10.1038/mp.2011.119 (2011).

205 Land, B. B. *et al.* The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J Neurosci* **28**, 407-414 (2008).

206 McLaughlin, J. P., Xu, M., Mackie, K. & Chavkin, C. Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. *J Biol Chem* **278**, 34631-34640 (2003).

207 Xu, M. *et al.* Neuropathic pain activates the endogenous kappa opioid system in mouse spinal cord and induces opioid receptor tolerance. *J Neurosci* **24**, 4576-4584 (2004).

208 Sharifi, N., Diehl, N., Yaswen, L., Brennan, M. B. & Hochgeschwender, U. Generation of dynorphin knockout mice. *Brain research. Molecular brain research* **86**, 70-75 (2001).

209 Clark, D. A. & Beck, A. T. Cognitive theory and therapy of anxiety and depression: convergence with neurobiological findings. *Trends in cognitive sciences* **14**, 418-424.

210 Wang, B. *et al.* Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. *J Neurosci* **25**, 5389-5396 (2005).

211 Richter, R. M. & Weiss, F. In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. *Synapse* **32**, 254-261 (1999).

212 Merali, Z., McIntosh, J. & Anisman, H. Anticipatory cues differentially provoke in vivo peptidergic and monoaminergic release at the medial prefrontal cortex. *Neuropsychopharmacology* **29**, 1409-1418 (2004).

213 Ohmura, Y. *et al.* Corticotropin releasing factor enhances attentional function as assessed by the five-choice serial reaction time task in rats. *Behavioural brain research* **198**, 429-433 (2009).

214 Gallagher, J. P., Orozco-Cabal, L. F., Liu, J. & Shinnick-Gallagher, P. Synaptic physiology of central CRH system. *European journal of pharmacology* **583**, 215-225 (2008).

215 Pecina, S., Schulkin, J. & Berridge, K. C. Nucleus accumbens corticotropin-releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? *BMC biology* **4**, 8 (2006).

216 Lim, M. M. *et al.* CRF receptors in the nucleus accumbens modulate partner preference in prairie voles. *Horm Behav* **51**, 508-515 (2007).

217 Aragona, B. J. *et al.* Nucleus accumbens dopamine differentially mediates the formation and maintenance of monogamous pair bonds. *Nature neuroscience* **9**, 133-139 (2006).

218 Lex, A. & Hauber, W. Dopamine D1 and D2 receptors in the nucleus accumbens core and shell mediate Pavlovian-instrumental transfer. *Learning & memory (Cold Spring Harbor, N.Y* **15**, 483-491 (2008).

219 Hnasko, T. S. *et al.* Cre recombinase-mediated restoration of nigrostriatal dopamine in dopamine-deficient mice reverses hypophagia and bradykinesia. *Proc Natl Acad Sci U S A* **103**, 8858-8863, doi:10.1073/pnas.0603081103 (2006).

220 Timpl, P. *et al.* Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nature genetics* **19**, 162-166 (1998).

221 Tzschentke, T. M. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Progress in neurobiology* **56**, 613-672 (1998).

222 Carvalho-Netto, E. F., Litvin, Y., Nunes-de-Souza, R. L., Blanchard, D. C. & Blanchard, R. J. Effects of intra-PAG infusion of ovine CRF on defensive behaviors in Swiss-Webster mice. *Behavioural brain research* **176**, 222-229, doi:10.1016/j.bbr.2006.10.003 (2007).

223 Holahan, M. R., Kalin, N. H. & Kelley, A. E. Microinfusion of corticotropin-releasing factor into the nucleus accumbens shell results in increased behavioral arousal and oral motor activity. *Psychopharmacology* **130**, 189-196 (1997).

224 Miguel, T. T. & Nunes-de-Souza, R. L. Anxiogenic and antinociceptive effects induced by corticotropin-releasing factor (CRF) injections into the periaqueductal gray are modulated by CRF1 receptor in mice. *Hormones and behavior* **60**, 292-300, doi:10.1016/j.yhbeh.2011.06.004 (2011).

225 Sahuque, L. L. *et al.* Anxiogenic and aversive effects of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis in the rat: role of CRF receptor subtypes. *Psychopharmacology* **186**, 122-132, doi:10.1007/s00213-006-0362-y (2006).

226 Donatti, A. F. & Leite-Panissi, C. R. Activation of corticotropin-releasing factor receptors from the basolateral or central amygdala increases the tonic immobility response in guinea pigs: an innate fear behavior. *Behavioural brain research* **225**, 23-30, doi:10.1016/j.bbr.2011.06.027 (2011).

227 Zieba, B. *et al.* The behavioural and electrophysiological effects of CRF in rat frontal cortex. *Neuropeptides* **42**, 513-523, doi:10.1016/j.npep.2008.05.004 (2008).

228 Oldfield, E. H. *et al.* Active clearance of corticotropin-releasing factor from the cerebrospinal fluid. *Neuroendocrinology* **40**, 84-87 (1985).

229 Fink, J. S. & Smith, G. P. Mesolimbicocortical dopamine terminal fields are necessary for normal locomotor and investigatory exploration in rats. *Brain Res* **199**, 359-384 (1980).

230 Fink, J. S. & Smith, G. P. Mesolimbic and mesocortical dopaminergic neurons are necessary for normal exploratory behavior in rats. *Neuroscience letters* **17**, 61-65 (1980).

231 Coryell, W. *et al.* The time course of nonchronic major depressive disorder. Uniformity across episodes and samples. National Institute of Mental Health Collaborative Program on the Psychobiology of Depression--Clinical Studies. *Archives of general psychiatry* **51**, 405-410 (1994).

232 Torres, G., Horowitz, J. M., Laflamme, N. & Rivest, S. Fluoxetine induces the transcription of genes encoding c-fos, corticotropin-releasing factor and its type 1 receptor in rat brain. *Neuroscience* **87**, 463-477 (1998).

233 Wang, H. T., Han, F. & Shi, Y. X. Activity of the 5-HT1A receptor is involved in the alteration of glucocorticoid receptor in hippocampus and corticotropin-

releasing factor in hypothalamus in SPS rats. *International journal of molecular medicine* **24**, 227-231 (2009).
234 Bruchas, M. R. & Chavkin, C. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology* **210**, 137-147, doi:10.1007/s00213-010-1806-y (2010).
235 Dautzenberg, F. M., Braun, S. & Hauger, R. L. GRK3 mediates desensitization of CRF1 receptors: a potential mechanism regulating stress adaptation. *American journal of physiology. Regulatory, integrative and comparative physiology* **280**, R935-946 (2001).
236 Shirayama, Y. *et al.* Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. *Journal of neurochemistry* **90**, 1258-1268, doi:10.1111/j.1471-4159.2004.02589.x (2004).
237 Gelenberg, A. J. Depression symptomatology and neurobiology. *The Journal of clinical psychiatry* **71**, e02.
238 Beninger, R. J., Hoffman, D. C. & Mazurski, E. J. Receptor subtype-specific dopaminergic agents and conditioned behavior. *Neuroscience and biobehavioral reviews* **13**, 113-122 (1989).
239 Hoffman, D. C. & Beninger, R. J. Selective D1 and D2 dopamine agonists produce opposing effects in place conditioning but not in conditioned taste aversion learning. *Pharmacology, biochemistry, and behavior* **31**, 1-8 (1988).
240 Hoffman, D. C. & Beninger, R. J. The effects of selective dopamine D1 or D2 receptor antagonists on the establishment of agonist-induced place conditioning in rats. *Pharmacology, biochemistry, and behavior* **33**, 273-279 (1989).
241 Refojo, D. *et al.* Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. *Science* **333**, 1903-1907, doi:10.1126/science.1202107 (2011).
242 Chaki, S. *et al.* Anxiolytic- and antidepressant-like profile of a new CRF1 receptor antagonist, R278995/CRA0450. *European journal of pharmacology* **485**, 145-158 (2004).
243 Cryan, J. F. & Mombereau, C. In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Molecular psychiatry* **9**, 326-357, doi:10.1038/sj.mp.4001457 (2004).
244 Van Bockstaele, E. J., Sesack, S. R. & Pickel, V. M. Dynorphin-immunoreactive terminals in the rat nucleus accumbens: cellular sites for modulation of target neurons and interactions with catecholamine afferents. *The Journal of comparative neurology* **341**, 1-15 (1994).
245 Peters, A., Palay, S. L. & Webster, H. d. *The Fine Structure of the Nervous System*. (Oxford University Press, 1991).
246 Peters, A. & Palay, S. L. The morphology of synapses. *J Neurocytol* **25**, 687-700 (1996).
247 Ford, C. P., Gantz, S. C., Phillips, P. E. & Williams, J. T. Control of extracellular dopamine at dendrite and axon terminals. *J Neurosci* **30**, 6975-6983.
248 Panerai, A. E., Bianchi, M., Brini, A. & Sacerdote, P. Endogenous opioids and their receptors in stress-induced analgesia. *Polish journal of pharmacology and pharmacy* **39**, 597-607 (1987).

249 Wee, S. & Koob, G. F. The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology* **210**, 121-135, doi:10.1007/s00213-010-1825-8 (2010).

250 Bruchas, M. R., Land, B. B. & Chavkin, C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res* **1314**, 44-55, doi:10.1016/j.brainres.2009.08.062 (2010).

251 Xu, M., Bruchas, M. R., Ippolito, D. L., Gendron, L. & Chavkin, C. Sciatic nerve ligation-induced proliferation of spinal cord astrocytes is mediated by kappa opioid activation of p38 mitogen-activated protein kinase. *J Neurosci* **27**, 2570-2581 (2007).

252 Bruchas, M. R., Macey, T. A., Lowe, J. D. & Chavkin, C. Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. *J Biol Chem* **281**, 18081-18089 (2006).

253 Bruchas, M. R. *et al.* Selective p38alpha MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. *Neuron* **71**, 498-511, doi:10.1016/j.neuron.2011.06.011 (2011).

254 Land, B. B. *et al.* Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proc Natl Acad Sci U S A* **106**, 19168-19173, doi:10.1073/pnas.0910705106 (2009).

255 Azmitia, E. C. & Gannon, P. J. The primate serotonergic system: a review of human and animal studies and a report on Macaca fascicularis. *Advances in neurology* **43**, 407-468 (1986).

256 Kosofsky, B. E. & Molliver, M. E. The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. *Synapse* **1**, 153-168, doi:10.1002/syn.890010204 (1987).

257 O'Hearn, E. & Molliver, M. E. Organization of raphe-cortical projections in rat: a quantitative retrograde study. *Brain research bulletin* **13**, 709-726 (1984).

258 Vasudeva, R. K., Lin, R. C., Simpson, K. L. & Waterhouse, B. D. Functional organization of the dorsal raphe efferent system with special consideration of nitrergic cell groups. *Journal of chemical neuroanatomy* **41**, 281-293, doi:10.1016/j.jchemneu.2011.05.008 (2011).

259 Adell, A., Casanovas, J. M. & Artigas, F. Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. *Neuropharmacology* **36**, 735-741 (1997).

260 Roche, M., Commons, K. G., Peoples, A. & Valentino, R. J. Circuitry underlying regulation of the serotonergic system by swim stress. *J Neurosci* **23**, 970-977 (2003).

261 Melief, E. J., Miyatake, M., Bruchas, M. R. & Chavkin, C. Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc Natl Acad Sci U S A* **107**, 11608-11613, doi:10.1073/pnas.1000751107 (2010).

262 Vaught, J. L. & Takemori, A. E. Differential effects of leucine and methionine enkephalin on morphine-induced analgesia, acute tolerance and dependence. *The Journal of pharmacology and experimental therapeutics* **208**, 86-90 (1979).

263 Clayton, C. C., Xu, M. & Chavkin, C. Tyrosine phosphorylation of Kir3 following kappa-opioid receptor activation of p38 MAPK causes heterologous desensitization. *J Biol Chem* **284**, 31872-31881 (2009).

264 Land, B. B. *et al.* The Dysphoric Component of Stress Is Encoded by Activation of the Dynorphin {kappa}-Opioid System. *J Neurosci* **28**, 407-414 (2008).
265 Lemos, J. C. *et al.* Selective 5-HT receptor inhibition of glutamatergic and GABAergic synaptic activity in the rat dorsal and median raphe. *The European journal of neuroscience* **24**, 3415-3430, doi:10.1111/j.1460-9568.2006.05222.x (2006).
266 Williams, J. T., Colmers, W. F. & Pan, Z. Z. Voltage- and ligand-activated inwardly rectifying currents in dorsal raphe neurons in vitro. *J Neurosci* **8**, 3499-3506 (1988).
267 Kirby, L. G., Pernar, L., Valentino, R. J. & Beck, S. G. Distinguishing characteristics of serotonin and non-serotonin-containing cells in the dorsal raphe nucleus: electrophysiological and immunohistochemical studies. *Neuroscience* **116**, 669-683 (2003).
268 Halasy, K., Racz, B. & Maderspach, K. Kappa opioid receptors are expressed by interneurons in the CA1 area of the rat hippocampus: a correlated light and electron microscopic immunocytochemical study. *Journal of chemical neuroanatomy* **19**, 233-241 (2000).
269 Hjelmstad, G. O. & Fields, H. L. Kappa opioid receptor inhibition of glutamatergic transmission in the nucleus accumbens shell. *J Neurophysiol* **85**, 1153-1158 (2001).
270 Grudt, T. J. & Williams, J. T. kappa-Opioid receptors also increase potassium conductance. *Proc Natl Acad Sci U S A* **90**, 11429-11432 (1993).
271 Mague, S. D. *et al.* Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. *J Pharmacol Exp Ther* **305**, 323-330 (2003).
272 Ippolito, D. L., Temkin, P. A., Rogalski, S. L. & Chavkin, C. N-terminal tyrosine residues within the potassium channel Kir3 modulate GTPase activity of Galphai. *J Biol Chem* **277**, 32692-32696 (2002).
273 McLaughlin, J. P. *et al.* Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J Biol Chem* **279**, 1810-1818 (2004).
274 Rogalski, S. L., Appleyard, S. M., Pattillo, A., Terman, G. W. & Chavkin, C. TrkB activation by brain-derived neurotrophic factor inhibits the G protein-gated inward rectifier Kir3 by tyrosine phosphorylation of the channel. *J Biol Chem* **275**, 25082-25088, doi:10.1074/jbc.M000183200 (2000).
275 Simmons, M. L. & Chavkin, C. k-Opioid receptor activation of a dendrotoxin-sensitive potassium channel mediates presynaptic inhibition of mossy fiber neurotransmitter release. *Mol Pharmacol* **50**, 80-85 (1996).
276 Pinnock, R. D. Activation of kappa-opioid receptors depresses electrically evoked excitatory postsynaptic potentials on 5-HT-sensitive neurones in the rat dorsal raphe nucleus in vitro. *Brain Res* **583**, 237-246 (1992).
277 Waselus, M., Valentino, R. J. & Van Bockstaele, E. J. Collateralized dorsal raphe nucleus projections: a mechanism for the integration of diverse functions during stress. *Journal of chemical neuroanatomy* **41**, 266-280, doi:10.1016/j.jchemneu.2011.05.011 (2011).

278 Hjorth, S. *et al.* Serotonin autoreceptor function and antidepressant drug action. *J Psychopharmacol* **14**, 177-185 (2000).
279 Sanchez, C. & Meier, E. Behavioral profiles of SSRIs in animal models of depression, anxiety and aggression. Are they all alike? *Psychopharmacology* **129**, 197-205 (1997).
280 Bambico, F. R., Nguyen, N. T. & Gobbi, G. Decline in serotonergic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **19**, 215-228, doi:10.1016/j.euroneuro.2008.11.005 (2009).
281 Rozeske, R. R. *et al.* Uncontrollable, but not controllable, stress desensitizes 5-HT1A receptors in the dorsal raphe nucleus. *J Neurosci* **31**, 14107-14115, doi:10.1523/JNEUROSCI.3095-11.2011 (2011).
282 Bruchas, M. R. *et al.* Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. *J Biol Chem* **282**, 29803-29811 (2007).
283 Melief, E. J. *et al.* Duration of action of a broad range of selective kappa-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. *Mol Pharmacol* **80**, 920-929, doi:10.1124/mol.111.074195 (2011).
284 Richardson-Jones, J. W. *et al.* 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. *Neuron* **65**, 40-52, doi:10.1016/j.neuron.2009.12.003 (2010).
285 Laaris, N., Le Poul, E., Hamon, M. & Lanfumey, L. Stress-induced alterations of somatodendritic 5-HT1A autoreceptor sensitivity in the rat dorsal raphe nucleus--in vitro electrophysiological evidence. *Fundamental & clinical pharmacology* **11**, 206-214 (1997).
286 Stahl, S. M. Mechanism of action of serotonin selective reuptake inhibitors. Serotonin receptors and pathways mediate therapeutic effects and side effects. *Journal of affective disorders* **51**, 215-235 (1998).
287 Le Poul, E., Laaris, N., Hamon, M. & Lanfumey, L. Fluoxetine-induced desensitization of somatodendritic 5-HT1A autoreceptors is independent of glucocorticoid(s). *Synapse* **27**, 303-312, doi:10.1002/(SICI)1098-2396(199712)27:4<303::AID-SYN4>3.0.CO;2-G (1997).
288 Wang, B., You, Z. B., Rice, K. C. & Wise, R. A. Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat. *Psychopharmacology* **193**, 283-294 (2007).
289 Heim, C. & Binder, E. B. Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics. *Experimental neurology* **233**, 102-111, doi:10.1016/j.expneurol.2011.10.032 (2012).
290 LaPlant, Q. *et al.* Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nature neuroscience* **13**, 1137-1143, doi:10.1038/nn.2619 (2010).
291 Covington, H. E., 3rd *et al.* A role for repressive histone methylation in cocaine-induced vulnerability to stress. *Neuron* **71**, 656-670, doi:10.1016/j.neuron.2011.06.007 (2011).

292 Wang, M., Hershey, N. D., Mabrouk, O. S. & Kennedy, R. T. Collection, storage, and electrophoretic analysis of nanoliter microdialysis samples collected from awake animals in vivo. *Analytical and bioanalytical chemistry* **400**, 2013-2023, doi:10.1007/s00216-011-4956-9 (2011).

293 Wang, M., Roman, G. T., Perry, M. L. & Kennedy, R. T. Microfluidic chip for high efficiency electrophoretic analysis of segmented flow from a microdialysis probe and in vivo chemical monitoring. *Analytical chemistry* **81**, 9072-9078, doi:10.1021/ac901731v (2009).

294 Wang, M., Roman, G. T., Schultz, K., Jennings, C. & Kennedy, R. T. Improved temporal resolution for in vivo microdialysis by using segmented flow. *Analytical chemistry* **80**, 5607-5615, doi:10.1021/ac800622s (2008).

295 Wang, M., Slaney, T., Mabrouk, O. & Kennedy, R. T. Collection of nanoliter microdialysate fractions in plugs for off-line in vivo chemical monitoring with up to 2 s temporal resolution. *Journal of neuroscience methods* **190**, 39-48, doi:10.1016/j.jneumeth.2010.04.023 (2010).

296 Li, Q., Zubieta, J. K. & Kennedy, R. T. Practical aspects of in vivo detection of neuropeptides by microdialysis coupled off-line to capillary LC with multistage MS. *Analytical chemistry* **81**, 2242-2250, doi:10.1021/ac802391b (2009).

297 Mabrouk, O. S., Li, Q., Song, P. & Kennedy, R. T. Microdialysis and mass spectrometric monitoring of dopamine and enkephalins in the globus pallidus reveal reciprocal interactions that regulate movement. *Journal of neurochemistry* **118**, 24-33, doi:10.1111/j.1471-4159.2011.07293.x (2011).

298 Martin, E. I. *et al.* A novel transgenic mouse for gene-targeting within cells that express corticotropin-releasing factor. *Biological psychiatry* **67**, 1212-1216, doi:10.1016/j.biopsych.2010.01.026 (2010).

Julia Cristine Lemos
Ph.D. 2012
Program in Neurobiology and Behavior

Mailing Address
Department of Pharmacology
University of Washington School of Medicine
Box 357280
1959 NE Pacific Street
Seattle, WA 98195-7280

Home Address
504 E. Denny Way
Apt. 309
Seattle, WA 98122

jlemos27@u.washington.edu
267.253.0461 (cell)
206.543.1127 (lab)
206.685.3822 (fax)

Education

Year	University	DEGREE	FIELD OF STUDY
2006-2012	University of Washington	Ph.D.	Neurobiology & Behavior
2000-2004	University of Pennsylvania	B.A. (with distinction)	Biological Basis of Behavior

Honors & Awards

2012	Irwin J. Kopin Traveling Fellowship for Excellence in Catecholamine research
2010	Serotonin Club Conference Travel Awardee
2009-2011	Ruth L. Kirschstein National Research Service Award (F31MH086269): *Cellular mechanisms underlying kappa opioid regulation of stress responses*
2008-2011	National Scholars Program Awardee, Society for Neuroscience
2008	International Narcotics Research Council Pre-doctoral Travel Awardee
2007	Carl Storm Underrepresented Minority (URM) Fellowship
2006-2009	Institutional training grant for Neurobiology (5T32GM007108-33)
2002-2004	University of Pennsylvania Dean’s list

Research Experience

2006-2012

Ph.D., University of Washington
Regulation of monoamingergic systems by stress-related neuropeptides**:** Investigation of the cellular actions and interactions of stress-related neuropeptides such as corticotropin releasing factor and dynorphin on the excitability of monoaminergic systems/limbic brain regions. Using a combination of behavior, fluorescent immunohistochemistry and *in vitro* slice electrophysiology and voltammetry I investigate the ability of stress-related neuropeptides to adaptively regulate monoamine (serotonin and dopamine) function. Furthermore, I investigate the dysregulation of these systems following exposure to severe or repeated stressor exposure.
Advisors: Charles Chavkin Ph.D. & Paul Phillips Ph.D.

2004-2006

Research Technician, Children's Hospital of Philadelphia
Regulation of serotonergic raphe nuclei excitability in Sprague Dawley and WKY rats. Investigation of modulatory effects of 5-HT_{1B} activation, benzodiazapines and CRF on excitatory and inhibitory synaptic currents in 5-HT and non 5-HT containing neurons of the dorsal and median raphe nuclei using whole cell voltage clamp techniques in an *in vitro* slice preparation. This research focused on differences in neuromodulation of synaptic currents between control Sprague-Dawley rats and stress hyperresponsive Wistar-Kyoto rats.
Advisor: Sheryl Beck Ph.D.

2003-2004

Senior Honors Thesis Student/Research Assistant, University of Pennsylvania
$Ankyrin_G$ dependent localization of KCNQ2/3 to axon initial segment**:** Examination of subcellular localization of KCNQ2/3 channels in WT and $Ankyrin_G$ site specific (cerebellum) -/- mouse. Primarily using immunohistochemical techniques, this research examined co-localization of KCNQ2/3 and NaV to the axon initial segment in an $Ankyrin_G$ dependent fashion. Changes in subcellular localization of KCNQ2/3 through development and in pilocarpine treated animals were also investigated.
Advisor: Edward Cooper, M.D., Ph.D.

Fall 2003

Independent Study Student, University of Pennsylvania
Neural correlates of semantic memory: My independent study examined top-down control of semantic retrieval and storage by the

left inferior prefrontal cortex (BA 44/45) using fMRI and behavioral techniques.
Advisor: Sharon Thompson-Schill Ph.D.

Summer 2003 **Independent Study Student,** Estacion Biologica Monteverde, Costa Rica
Feeding behavior in the predatory tiger beetle: My independent study looked at differences in predatory feeding behavior of *Pseudoxychila tarsalis* in relation to prey distribution and composition.
Advisors: Mauricio Garcia Ph.D. & Carlos Guindon Ph.D.

2001-2002 **Research Assistant** University of Pennsylvania
Efficacy of Cognitive-Behavioral Therapy for Drug Resistant Depression: Assisted in data collection, session transcription, literature searches and organization of study subjects.
Advisor: Karla Moras Ph.D.

Publications

Published works

Lemos JC, Roth CA, Messinger DI, Gill HK, Phillips PEM, Chavkin C. Repeated stress dysregulates kappa opioid receptor signaling in the dorsal raphe through a p38 MAPK dependent mechanism. ***Accepted at J Neuroscience*.**

Bruchas MR, Schindler AG, Shankar H, Messinger DI, Miyatake M, Land BB, **Lemos JC**, Hagan CE, Neumaier JF, Quintana A, Palmiter RD, Chavkin C. (2011). Selective p38α MAPK Deletion in Serotonergic Neurons Produces Stress Resilience in Models of Depression and Addiction. **Neuron**, Aug 11;71(3):498-511

Calizo LH, Ma X, PanY**,** Akanwa A, **Lemos JC,** Craige C, Heemstra L, Beck SG. Raphe serotonin neurons are not homogenous: Electrophysiological, morphological and neurochemical evidence. **Neuropharmacology**, Sep;61(3):524-43. Epub 2011 Apr 16.

Lemos JC, Roth C, Chavkin C. (2011). Signaling events initiated by kappa opioid receptor activation: quantification and immunolocalization using phospho-selective KOR, p38-MAPK and K_{IR} 3.1 antibodies. Methods Mol Biol. 717:197-219.

Lemos JC, Chavkin C. (2011). “Functional analysis of Kappa receptor circuits” In **Opioid Receptors, 2nd Edition.** Eds. G. Pasternak.

Lemos JC, Zhang G, Walsh T, Kirby LG, Akanwa AC, Brooks-Kayal A, Beck SG. (2011). Stress hyperresponsive WKY rats demonstrate depressed dorsal raphe neuronal excitability and dysregulated CRF mediated responses. ***Neuropsychopharmacology.*** Mar;36(4):721-34. Epub 2010 Dec 15.

Bruchas MR, Land BB, **Lemos JC**, Chavkin C. (2009). CRF1-R activation of the Dynorphin/Kappa Opioid system in the mouse basolateral amygdale mediates anxiety-like behavior. ***PLOS One.*** 4(12): 1-9.

Kirby LG, Freedman-Daniels E, **Lemos JC**, Nunan JD, Akanwa AC, Beck SG. (2008). Corticotropin-releasing factor increases GABA synaptic activity and induces inward current in 5-hydroxytryptamine dorsal raphe neurons. ***J Neurosci.*** 28: 12927-12937,

Land BB, Bruchas MR**, Lemos JC**, Melief EJ, Xu M, Chavkin C. (2008) Stress-induced Dysphoria is Encoded by the Dynorphin-Kappa Opioid System. ***J Neurosci.*** 28:407-414.

Lemos JC, Pan YZ, Lamy C, Akanwa AC, Beck, SG. (2006) Selective 5-HT_{1B} receptor inhibition of glutamatergic and GABAergic synaptic activity in rat dorsal and median raphe. ***European J Neurosci***. 24: 3415-3430.

Pan Z, Kao TC, Horvath, Z, **Lemos J**, Sul JY, Cranstoun SD, Bennett V, Scherer SS, Cooper EC. (2006) A common Ankyrin-G-based mechanism retains KCNQ and Na_V channels at electrically active domains of the axon. ***J Neurosci.*** 26(10): 2599-2613.

Works in progress

Lemos JC, Wanat MJ, Smith JS, Reyes BAS, Hollon NG, Van Bockstaele EJ, Chavkin C, Phillips PEM. Stress exposure produces a switch from appetitive to aversive signaling by CRF in the nucleus accumbens . *In revision at Nature*.

Wang W, Nitulescu I, **Lemos JC**, Phillips PEM, Bamford IJ, Posielski NM, Bamford NS. Prenatal cocaine exposure causes synaptic plasticity at corticostriatal synapses. *Submitted at Annals of Neurology.*

Schindler AG, Messinger DI, Smith JS, Shankar H, Gustin RM, Schattauer SS, **Lemos JC**, Chavkin NW, Hagan CE, Neumaier JF, Chavkin C. Stress produces aversion and potentiates cocaine reward by releasing endogenous dynorphins in the ventral striatum to locally stimulate serotonin reuptake. *Submitted at J Neuroscience*.

Abstracts/Poster Presentations

Lemos JC, Reyes BAS, Van Bockstaele EJ, Chavkin C, Phillips PE. (2011) Repeated stress causes persistent disruption of CRF mediated increases in dopamine in the nucleus accumbens. Winter Conference on Brain Research, Keystone, CO.

Lemos JC, Phillips PE, Chavkin C. (2010). Stress-induced dysregulation of kappa opioid modulation of serotonergic DRN neuronal excitability. Program No. 905.13. Abstract Viewer/Itinerary Planner. San Diego, CA. Society for Neuroscience. Online.

Phillips PE, Chavkin C, **Lemos JC**. (2010). Corticotropin releasing factor (CRF) increases mesolimbic dopamine release in naïve, but not stressor exposed mice. Program No. 711.9. Abstract Viewer/Itinerary Planner. San Diego, CA. Society for Neuroscience. Online.

Lemos JC, Chavkin C, Phillips, PEM. (2010). Corticotropin releasing factor (CRF) increases mesolimbic dopamine release in naïve, but not stressor exposed mice. Winter Conference on Brain Research. Breckenridge, CO.

Lemos JC, Roth C, Phillips PE, Chavkin C. (2009). Stress-induced dysregulation of Kappa opioid modulation of serotonergic DRN neuronal excitability. Program No. 469.2. Abstract Viewer/Itinerary Planner. Chicago, IL. Society for Neuroscience. Online.

Lemos JC, Phillips PE, Chavkin C. (2009). KOR modulation of Dorsal Raphe Nucleus (DRN) neuronal excitability. International Narcotics Research Conference. Portland, OR

Hart AS, Wanat M, **Lemos JC**, Phillips PEM. (2007). Stress, drugs, dopamine and risk taking. Society for Neuroeconomics, Hull, MA.

Land BB, Bruchas MR, **Lemos JC**, Melief EJ, Chavkin C. (2007). The dynorphin-kappa opioid system mediates the aversive properties of CRF. *International Narcotics Research Conference*, Berlin, Germany.

Lemos JC, Zhang G, Akanwa AC, Beck SG. (2006). GABA-A synaptic activity and its modulation by EtOH are altered in 5-HT neurons of the dorsal raphe of stress hyperresponsive WKY rats. Program No. 58.5. Abstract Viewer/Itinerary Planner. Atlanta, GA. Society for Neuroscience. Online.

Lemos JC, Walsh T, Nunan J, Kirby LG, Beck SG (2005). Biphasic modification of CRF effects on GABA-A synaptic activity in median and dorsal raphe neurons in WKY rats. Program No. 186.4. Abstract Viewer/Itinerary Planner. Washington D.C. Society for Neuroscience. Online.

Lamy CM, **Lemos JC**. Akanwa AC, Nunan J, Kirby LG, Beck SG. (2005). Swim stress alters GABAergic synaptic activity in dorsal raphe serotoninergic neurons. Program No. 185.12. Abstract Viewer/Itinerary Planner. Washington, D.C. Society for Neuroscience. Online.

Pan ZM, **Lemos J**, Kao TC, Horvath Z, Scherer SS, Bennett V, Cooper EC. (2005). A common sequence motif mediates the concentration of voltage-gated Na and KCNQ 2/3 K channels at axon initial segments. Society for Biophysics, Long Beach, CA.

Hirshorn EA, **Lemos JC**, Thompson-Schill SL. (2004). Role of the left inferior frontal gyrus in covert word retrieval: Neural correlates of switching during verbal fluency. No. TH 45. Human Brain Mapping Conference Abstract, Budapest, Hungary.

Techniques

- *In vitro* electrophysiology (visualized whole cell voltage/current patch clamp) in acute slice preparations (expertise in dorsal/median raphe, nucleus accumbens and hippocampus). I also have some experience in blind whole-cell patching.

- *In vitro* and *in vivo* voltammetry. My expertise is in *in vitro* slice voltammetry in the dorsal and ventral striatum, however I also have some experience in *in vivo* voltammetry using chronic electrodes.

- Fluorescent immunohistochemistry and neuroanatomy

- Epifluorescent and confocal microscopy

- Stereotaxic intra-cranial survival surgeries. I have moderate experience with i.c.v. and intra-region cannula implantation as well microinjections.

- Behavior. I have experience with various stress paradigms including swim stress and social defeat stress. I have some minimal experience with operant/pavlovian conditioning and conditioned place preference assays.

Service

2006-2009 Co-chair, Neurobiology and Behavior Seminar Series Organizing Committee

2006-present Brain awareness week volunteer

Membership in Professional Organizations

2010-present Serotonin Club

2007-2010 International Narcotics Research Council

2005-present Society for Neuroscience

Teaching

Autumn 2010 University of Washington – Guest lecturer – Molecular Basis of Drug addiction, Charles Chavkin Ph.D. “Cellular mechanisms of stress-induced relapse to addiction”

Winter 2007 University of Washington- TA- Undergraduate Introduction to Neurbiology (NBio 301), William Moody Ph.D). This course

included a weekly lab portion that included both intracellular and extracellular recording protocols.