

A Neurobiological Pathway That Mediates Stress-Induced Drug Use

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A NEUROBIOLOGICAL PATHWAY THAT MEDIATES STRESS-INDUCED DRUG
USE

by

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Marquette University,
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ABSTRACT
A NEUROBIOLOGICAL PATHWAY THAT MEDIATES STRESS-INDUCED DRUG
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Oliver Vranjkovic B.S.

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Cocaine addiction represents a tremendous health and financial burden on our society and the high rate of relapse to cocaine use in abstinent addicts represents a major barrier to effective therapy. Thus, understanding the factors that contribute to relapse and the underlying neurobiological processes is important for guiding the development of treatment for addiction. Stressful life events often trigger drug use in recovering addicts. The contribution of stress to drug use is problematic due to the unpredictable and often uncontrollable nature of stress. A growing literature indicates that norepinephrine and corticotropin releasing factor (CRF) in the brain play key roles in stress interactions with motivational neurocircuitry that mediate stress-induced drug seeking. Previous work from our lab has demonstrated that activation of the CRFR1 receptor within the ventral tegmental area (VTA) is both necessary and sufficient for drug-seeking behavior during periods of stress. However, the afferent CRF projection into the VTA, and how CRF affects the neurocircuitry of VTA to evoke stress-induced relapse are poorly understood.

We report that stress-induced cocaine use involves a beta-2 adrenergic receptor-regulated CRF pathway from the ventral bed nucleus of the stria terminalis to the VTA and a CRFR1 receptor-regulated dopaminergic pathway to the prelimbic cortex. It is hypothesized that dopamine released into the prelimbic cortex activates dopamine D1 receptors on pyramidal neurons that comprise a glutamatergic projection to the nucleus accumbens core that is critical for relapse to drug use in abstinent cocaine addicts. It is also reported that the ability of stressors to trigger drug use is determined by the amount and pattern of prior drug use. Findings suggesting that excessive cocaine use establishes susceptibility to stress-induced relapse by recruiting CRF regulation of this key stressor-responsive mesocortical dopaminergic pathway through increased CRFR1 expression are described.

This dissertation defines a key pathway through which stress can promote relapse and describes its recruitment as a result of repeated excessive drug use. Understanding the processes through which stress contributes to cocaine seeking in these rodent models should facilitate translational work aimed at targeting these mechanisms clinically and therefore the development of new medications or approaches for managing addiction.

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Chapter I

General Introduction

Risk factors for cocaine addiction

The total cost of drug abuse and addiction due to use of tobacco, alcohol, and illegal drugs is estimated at \$524 billion a year, while illicit drug use alone, such as cocaine, accounts for \$181 billion in health care, productivity loss, crime, incarceration and drug enforcement (NIDA). Addiction to cocaine and other illicit drugs, is a chronically relapsing disorder in which drug use progresses from its initial stages of limited, non-dependent intake to later stages of uncontrolled abuse (Koob & Volkow 2010, Volkow et al 2003). The major risk factors for developing cocaine abuse disorders or relapse to cocaine are bipolar disorder, schizophrenia, antisocial personality disorder, and other substance abuse disorders (DSM-V). Cocaine use amongst youth can be attributed to prenatal cocaine exposure, postnatal cocaine use by parents, and exposure to community violence during childhood (DSM-V).

Cellular mechanisms of cocaine

The positive reinforcing value of drugs drives the initial periods of drug use, while later stages of addiction are driven, in part, by negative reinforcement such as the relief of withdrawal which is induced by negative affective states (Koob & Volkow 2010). The primary reinforcing effects of drugs such as cocaine are thought to occur by increased dopamine (DA) signaling that leads to enhanced activity of the brain's pleasure pathway which in turn leads to increased craving. Cocaine physically inhibits the

dopamine transporters within the synapse and therefore increases extracellular dopamine levels due to decreased reuptake into the presynaptic terminal. Chronic drug use and over activation of this pathway is thought to devalue natural rewards, diminish cognitive control of behaviors, and increase salience of drug-related stimuli (Edwards & Koob 2010, Volkow et al 2003). One major factor that plagues drug users is the high rate of relapse, as 70-90% of people have reported periods of relapse.

Stress promotes drug use and relapse

Numerous studies suggest that stress plays a crucial role in addiction. Specifically, stress has long been considered an important factor contributing to drug craving and relapse of drug use in humans (Kreek & Koob 1998). Interestingly, clinical reports indicate that individuals with a history of drug use attribute episodes of relapse to stressful life events (Sinha 2001, Wang et al 2001). Consistent with these results, controlled clinical experiments in cocaine- and alcohol-dependent patients report a significant increase in drug craving following exposure to a personalized stressor (Fox et al 2009, Sinha et al 1999). Furthermore, population-based and clinical studies support a strong correlation between addiction, chronic distress, and negative affect (Sinha 2008). These studies have shown that stressors such as physical or sexual abuse, parental neglect and poor family structure increase the likelihood of substance abuse, or relapse of drug use (Sinha 2008). While stress and drug use are strongly correlated, the precise mechanism by which stress contributes to drug use remains unclear, and in many cases involves an interaction between stressful life events, cocaine-related cues, and the effect of cocaine itself. In addition, the effects of drug use may vary across different

subpopulations of addicts (Preston and Epstein, 2011), making it clear that management and treatment of stress-related drug use may need to be individualized.

In addition, anxiety and depression are often cited by recovering addicts as key contributors of drug craving and relapse (Sinha 2007). Intriguingly, epidemiological studies have shown that cocaine dependence is highly correlated with numerous stress-related disorders such as depression, anxiety, and post-traumatic stress disorder (Rounsaville et al 1991). Lifetime cocaine use is strongly correlated with PTSD and suicide risk; depressive symptoms with suicidal connotations are generally thought of the most serious problems (Narvaez et al 2014). However, studies have also indicated that the severity of traumatic events may predate cocaine use (Back et al 2001, Brady et al 2001). While it is clear that stress-related disorders and cocaine use (the initiation, escalation or relapse) go hand in hand, developing and treating stress-related cocaine use remains difficult because the appearance of stressors is unpredictable and in most cases unavoidable. Therefore, understanding the neurobiological pathways that mediate stress-related drug use remains an important goal for preventing relapse, the development of therapeutics for the management of drug use, and the prevention of stress-related drug seeking. To investigate the factors that contribute to stress-induced relapse observed in clinical studies, preclinical animal models of relapse, or reinstatement procedures, have been developed.

Methods in studying stress-induced cocaine use: Conditioned Place Preference

Several different animal models of addiction have been developed to better understand the changes in neuronal chemistry, physiology, and behavior that lead to drug

seeking during periods of stress. In general, these methods can be grouped into two categories based on the method of drug delivery. Non-contingent models involve the delivery of drugs by the experimenter; an example of a non-contingent model is conditioned place preference. Conditioned place preference (CPP) examines the ability of drugs of abuse to establish positive reinforcement with learned contextual cues; these cues can acquire additional appetitive properties when paired with cocaine or other drugs of abuse (Tzschentke 2007). In the CPP model, different compartments of a two, or three compartment chamber are paired with either cocaine or saline. CPP first consists of determining natural preference, termed preconditioning. To define preconditioning preference, the experimenter evaluates the natural value to each compartment by placing the rodent in the center compartment of the chamber and allow free access to all three compartments for 30 min in the absence of cocaine or saline and the time spent in each compartment is compared, and one compartment is designated as the cocaine compartment, and the other as the saline compartment. Three methods are used to determine preference: in a biased approach, the compartment that was not preferred in the preconditioning test is paired with the drug, while an un-biased protocol randomly pairs the compartment with the drug, in particular when animals have no general preference during preconditioning. Finally, a balanced protocol assigns the chamber so that no group as a whole has a preference for a particular chamber (Aguilar et al 2009). After preference has been established, the rodent enters the conditioning phase. During the 8-day conditioning phase of the experiment, mice receive cocaine (15 mg/kg, i.p.) on the odd days and saline injections on the even days. Immediately after the injection, mice confined (guillotine door closed) to the drug-appropriate compartment for 30 min. A day

after the final conditioning session, the mice are tested for the expression of cocaine-induced CPP by placing them in the center compartment and allowing them full access to the apparatus for 30 min. A conditioned place preference is defined as the change in time spent (in seconds) in the cocaine-paired compartment after conditioning compared with the initial preconditioning session. Mice are determined to exhibit a conditioned place preference if they spend more time in the cocaine-paired compartment during the post-conditioning session compared to the preconditioning session.

Next, animals are allowed to explore the CPP chamber freely in order to measure, and induce extinction behavior, using an approach that involves assessment of the incentive motivation properties of the drug-paired environment, until there is no significant difference between the times spent in the drug-paired compartment during preconditioning. This usually occurs for an average of five to eight days at which, once extinction criterion is met, animals undergo reinstatement testing.

Methods in studying stress-induced cocaine use: Self-Administration

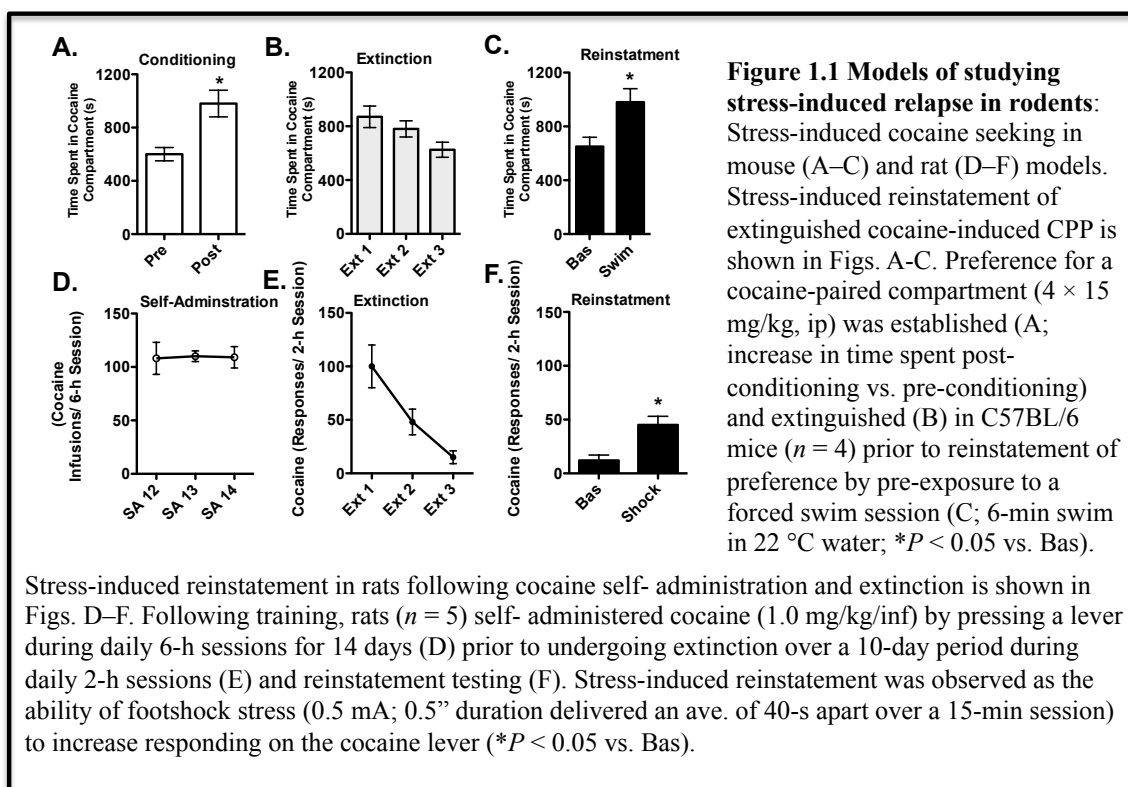
Another model of addiction is a contingent model in which the animal requires an operant response, such as activating a lever, or activating photobeams through a nose-poke to receive an intravenous infusion of cocaine; this is coined the self-administration model (SA). To study the neurobiological mechanisms involved in reinstatement, studies have utilized the reinstatement of drug seeking model following SA. In this model, rodents are trained to self-administer a drug of abuse (such as cocaine), followed by an extinction phase in which the cocaine is replaced with saline, or they are forced into abstinence in which the rodents have no more exposure to the drug of abuse and the

environment paired with drug use, and are sequestered in their home cage. If a rodent undergoes abstinence, exposure to the drug-paired environment will trigger a robust drug-seeking behavior phenotype from days to months after the last drug-administration session (Conrad et al 2008). In the case of extinction, lever responding will progressively diminish. Following extinction training, rats may be exposed to either a cue, non-contingent infusion of the drug of abuse, or a stressor (such as a mild-non noxious footshock) to elicit previous drug seeking behavior, and therein overcoming the extinction training and increasing lever responding. The SA model allows for the analysis of the neurobiological mechanism in drug-seeking behavior because it directly measures intake behavior.

Stress as a trigger for relapse

Since stress has been cited as a major factor that contributes to drug use in humans, studies examining the neurobiological mechanism involved have been developed; these studies involve the assessment of stress-induced reinstatement. Stress-induced reinstatement may involve different types of stressors such as a cold forced swim (Conrad et al 2010), or food restriction (Chen et al 2014, Shalev et al 2003); the most common type of a stressor utilized for self-administration experiments is an uncontrollable intermittent electric-footshock delivered throughout the steel floors of the self-administration chamber (Ahmed & Koob 1997, Erb et al 1996). Reinstatement testing, more specifically stress-induced reinstatement, is most commonly tested in the CPP model by either a forced-swim stressor (**Figure 1.1 A-C**-(Mantsch et al 2010)). Stress-induced reinstatement in most cases significantly increases the time spent in the

cocaine-paired compartment compared to the previous extinction day, and the preconditioning day. Once stress-induced reinstatement has been established, different neuropharmacological agents may be employed in order to prevent, or in some cases evoke, stress-induced drug seeking, allowing for a better understanding of the different neurobiological mechanisms that contribute to drug seeking during periods of stress. This is important because stress is known to influence the rewarding effects of drugs of abuse (Cleck & Blendy 2008, Der-Avakian et al 2005), and stress has a prominent role in relapse to drugs of abuse (Sinha 2001).



In the case of SA, the conditions under which stress evokes reinstatement involves animals with a specific history of self-administration; animals that undergo self-administration for 2 hours will not show reinstatement to an electric footshock stressor,

while animals that undergo self-administration for 6 hours (**Figure 1.1 D-F**) or more will show reinstatement (Mantsch et al 2008a, Mantsch et al 2008b). Preliminary studies have indicated that elevated glucocorticoids during 6-hr SA are involved in establishing susceptibility to stress-induced drug seeking (Mantsch et al 2008b) though more research needs to be conducted to better understand the neurobiological mechanism involved in this relapse susceptibility. The Mantsch lab has shown that animals with 6hr access to cocaine also show an escalating pattern of cocaine self-administration. In addition, these animals are more susceptible to reinstatement by cocaine, footshock, or intra-cerebroventricular injections of corticotropin releasing factor -(Mantsch et al., 2008a). In addition, surgical adrenalectomy, which eliminates corticosterone production, slows the escalation, and prevents the establishment of stress-induced reinstatement when conducted prior to but not after repeated daily 6-hr cocaine self-administration (Mantsch et al., 2008b). This suggests that corticosterone, and other adrenal hormones, may induce neuroplastic changes, possibly in the mesocortical system to make rats more susceptible to stress-induced relapse. These neuroplastic changes have been proposed to promote drug use during periods of stress through actions of corticotropin releasing factor (an important peptide in stress-induced reinstatement) or its receptor which may be up-regulated by drug use. Both factors are explored in this body of work.

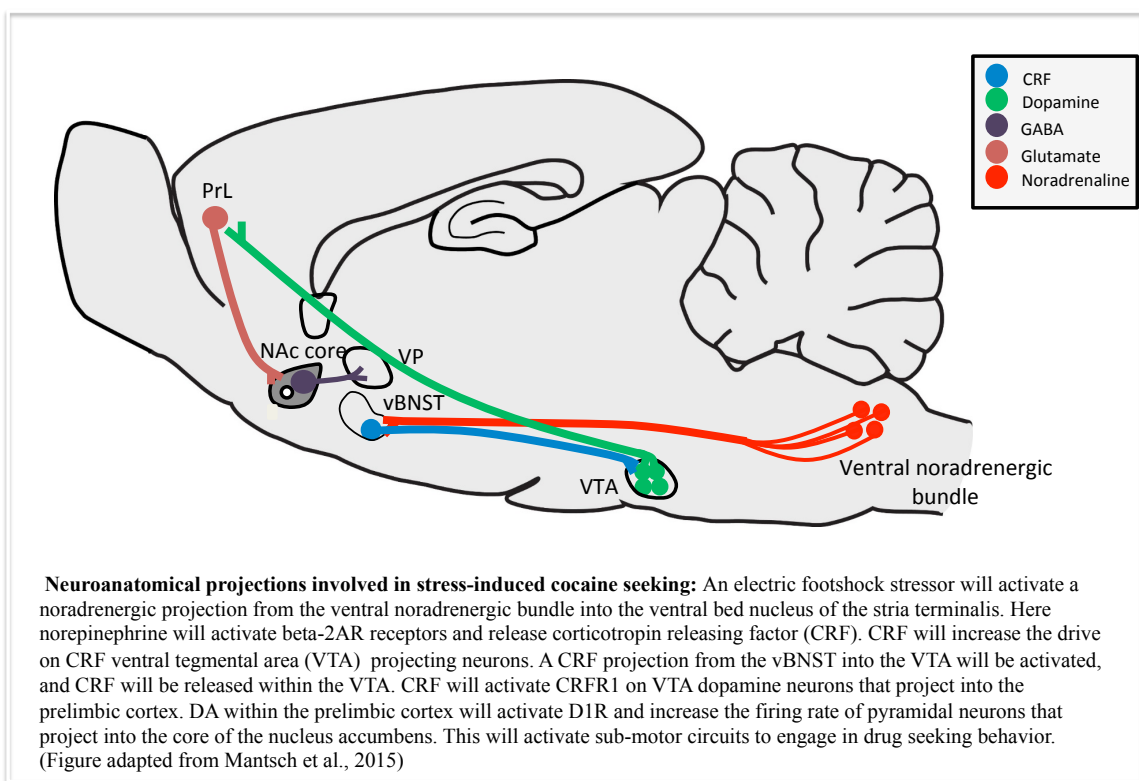
Using the approaches mentioned above has allowed us to better understand the neurobiological mechanisms that contribute-to drug use during stress. The CPP model has allowed us to explore which noradrenergic receptors mediate this process; this is important because clonidine, an agonist at the alpha-2 adrenergic receptor (AR) has been shown to decrease drug craving during periods of stress (Jobes et al 2011, Shaham et al

2000b). Our studies have also shown that the beta-2 AR is both necessary and sufficient in promoting drug use during periods of stress (Mantsch et al. 2010, Vranjkovic et al. 2012). Using this information, we moved our studies into the SA model and showed that activation of the beta-2 AR within the ventral bed nucleus of the stria terminalis mobilizes a corticotropin releasing factor pathway projection into the ventral tegmental area (Vranjkovic 2014; this data is presented in this dissertation).

Neurocircuitry involved in stress-induced cocaine seeking

Using the self-administration/reinstatement approach in rats, we have focused on defining the neurocircuitry (**Figure 1.2**) involved in stress-evoked drug seeking. Particularly, stress-induced reinstatement appears to involve a glutamatergic projection from the prelimbic cortex to the nucleus accumbens core (Kalivas and McFarland, 2003), which likely regulates motor outputs to produce drug-seeking behavior. This pathway may be regulated by glutamate release from the prefrontal cortex, because studies have shown that inhibition of the nucleus accumbens core with GABA receptor agonist cocktail blocks footshock-induced reinstatement in rats (McFarland et al., 2004) while other groups have found that dopamine release within the prelimbic cortex is required for stress-induced drug seeking (Capriles et al., 2003, McFarland et al., 2004). Specifically, administration of fluphenazine into the prelimbic, but not the core of the nucleus accumbens blocked footshock-induced reinstatement; the same study showed that a footshock stressor increased both glutamate and dopamine release within the prelimbic cortex, however, only glutamate but not dopamine was increased within the nucleus accumbens core (McFarland et al., 2004). In addition studies have shown that AMPA

administration within the core induced reinstatement while an AMPA antagonist was able to block cocaine primed reinstatement (Cornish and Kalivas., 2000) . This dopamine likely acts on pyramidal D1 receptors to increase the excitability of pyramidal neurons that specifically project to the nucleus accumbens core (Lewis and O'Donnell, 2000; Seamans et al., 2001).



The aim of this body of work is to identify the stress-related neurocircuitry that acts up-stream from the cortical-accumbens pathway. Here we will examine the actions of the neuropeptide corticotropin releasing factor (CRF) in different stress-related brain nuclei, most notably the bed nucleus of the stria terminalis; specifically we will examine its interaction with the noradrenergic system within the bed nucleus. We will also

examine its actions within the VTA and how it engages CRFR1 to promote drug seeking by activating a dopaminergic projection to the prefrontal cortex.

Bed Nucleus of the Stria Terminalis: General Introduction and anatomy

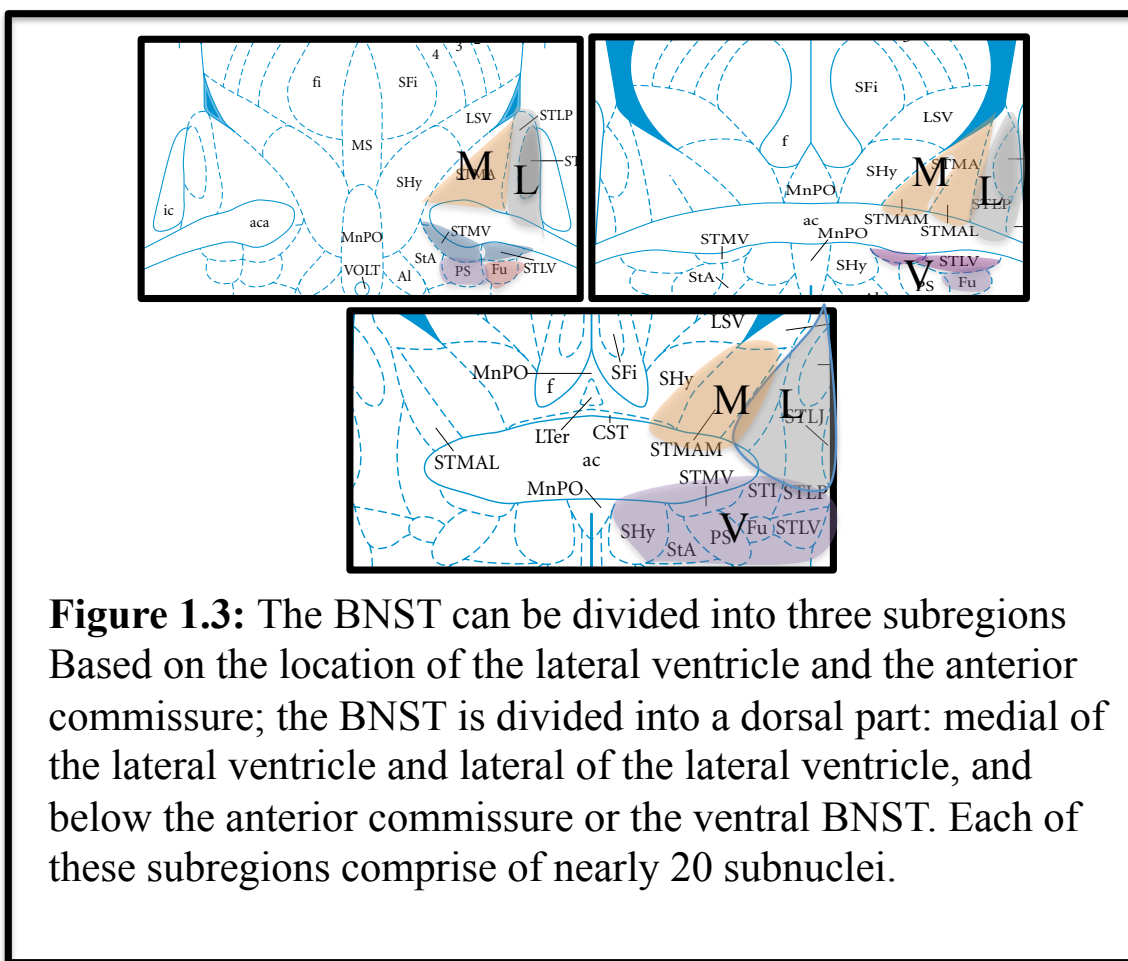


Figure 1.3: The BNST can be divided into three subregions Based on the location of the lateral ventricle and the anterior commissure; the BNST is divided into a dorsal part: medial of the lateral ventricle and lateral of the lateral ventricle, and below the anterior commissure or the ventral BNST. Each of these subregions comprise of nearly 20 subnuclei.

The bed nucleus of the stria terminalis (BNST) is considered the central coordinator between stress-responsive regions such as the amygdala, the paraventricular nucleus of the hypothalamus, and the brainstem and motivation systems such as the ventral tegmental area (VTA), and the nucleus accumbens (Georges & Aston-Jones 2001, Jalabert et al 2009). Structurally, the BNST is a rostral forebrain structure that is enclosed

by the lateral ventricles, lateral septum, fornix, the nucleus accumbens, the preoptic area, and the hypothalamus (Crestani et al 2013).

It is made up of 20 different subregions based on cyto- and chemo-architectonic studies; the BNST has been subdivided along an anterior to posterior axis. The anterior BNST is involved in autonomic control, while the posterior division is involved in controlling neuroendocrine responses (Bayer 1987, Ju & Swanson 1989, Ju et al 1989). In addition, the BNST can be divided into the dorsal medial, dorsal lateral, and ventral subregions (figure 1.3) though some early anatomical studies have suggested that the dorsal lateral and ventral portions of the BNST are a continuous structure (Swanson 1989). Recently, studies have shown that both the dorsal and ventral lateral divisions of the BNST are critical in bridging the pathway between stress and reward. The role of the dorsal BNST in stress-related drug use has been extensively studied by the Winder group (Flavin & Winder 2013, McElligott & Winder 2009). The current body of work will examine the ventral portion because studies have suggested that it has a direct involvement in stress-induced drug seeking as inhibition of the ventral BNST by a GABA agonist cocktail has been shown to prevent footshock-induced drug seeking (McFarland et al., 2004).

Bed Nucleus of the Stria Terminalis: Stress-Related Connectivity

The BNST is a critical modulator of stress-induced relapse of drug seeking (Aston-Jones et al 1999, Aston-Jones & Druhan 1999, Leri et al 2002, Rodaros et al 2007) partly due to its connectivity with different limbic brain structures. The BNST receives dense glutamatergic projections from the infralimbic cortex, prelimbic cortex,

insular cortex, entorhinal cortex, and the orbital frontal cortex (McDonald 1998) and a dense GABAergic projection from the amygdaloid nuclei (Dong & Swanson 2006). Interestingly, the BNST sends both GABAergic and glutamatergic projections to the VTA, a region critical in motivated behavior. These projections synapse in part onto putative VTA GABA neurons, indicating that the BNST can directly modulate VTA firing (Dumont & Williams 2004, Jalabert et al 2009, Kudo et al 2012, Silberman et al 2013). Specifically, recent data has shown that stimulation of the ventral portion of the BNST (vBNST) potently and consistently activates VTA dopamine neurons (Georges & Aston-Jones 2001, Massi et al 2008). This may indicate that dopamine firing within the VTA is, in part, dependent upon BNST-mediated inhibition of local VTA GABA neurons. In addition, other studies have shown that activation of the ventral BNST have an excitatory input on to VTA DA neurons (Georges and Aston Jones 2002). It is also important to note that both the ventral pallidum (Mahler et al 2014), and the tail of the VTA (tVTA or RMTg) have been shown to send GABA inputs to the VTA that are prominent during reinstatement behaviors (Barrot et al 2012, Sanchez-Catalan et al 2014). This indicates that multiple GABAergic inputs into the VTA, and possibly glutamatergic inputs, may regulate dopamine release in terminal regions. In this body of work, we have focused on the interaction between the vBNST and the VTA.

The Role of Norepinephrine in stress-related drug use

Norepinephrine is an important stress-related neuromodulator that is released within the BNST in response to aversive stimuli (Park et al 2012), and during periods of stress (Pacak et al., 1995) and has been suggested to promote drug use during times of

stress (Brown et al., 2009). Central noradrenergic (NE) neurons are activated by stress and play an important role in the stress response. Noradrenergic projections in the brain originate from seven distinctive cell bodies in the brainstem (A1-A7) (Dahlstrom & Fuxe 1964). These projections are divided into the dorsal noradrenergic bundle, which provides sole NE projections to the cortex, hippocampus, thalamus, and cerebellum; or the ventral noradrenergic bundle, which provides sole NE projections to the hypothalamus, and regions of the limbic forebrain such as the amygdala and the BNST. These projections are thought to be largely ipsilateral, and innervate a majority of the CNS (Moore & Bloom 1979). Importantly, the ventral forebrain bundles sends NE from the A2 region into the BNST (Wang et al 2001). Interestingly, it was determined that the ventral, but not the dorsal, NE pathway is responsible for footshock-induced reinstatement. For example, animals were given 6-hydroxydopamine (6-OHDA; a neurotoxin that enters the neuron through monoamine transporters) lesions in either the dorsal or ventral forebrain bundle and subsequently tested for footshock-induced reinstatement of heroin seeking; only lesions in the ventral NE pathway significantly attenuated the effects of footshock on reinstatement for heroin (Shaham et al 2000b), morphine (Wang et al 2001), and opioids (Aston-Jones et al 1999). Taken together, this suggests that the innervation of NE from the ventral forebrain bundle to the BNST is crucial in mediating reinstatement to drugs during times of stress.

In addition to the ventral forebrain bundle, the locus coeruleus (A6 group) is another major NE input into limbic forebrain structures such as the prefrontal cortex, and the BNST. The locus coeruleus has a major role in mediating the sleep-wake cycle, attention and memory, cognitive flexibility, and stress responses (Schwartz & Roth

2008). Recent studies have suggested that the kappa-opioid system interacts with the LC to contribute to drug-induced cocaine seeking (Al-Hasani et al 2013, McCall et al 2015, Siuda et al 2015).

Noradrenergic receptors and their involvement in stress-related drug use: alpha-2 Adrenergic receptors

Norepinephrine can activate five distinct adrenoceptor (-AR) subtypes: alpha-1 AR (G_q), alpha-2 AR (G_i), beta-1 AR, beta-2 AR, and beta-3 AR (all G_s), all of which are coupled to a G-protein and activate unique secondary messenger signals. All of the receptors are distributed throughout the central nervous system (Asanuma et al 1991, Bing et al 1992, Levin 1982, Young & Kuhar 1980). Both alpha-1 AR and the beta-ARs are largely located on post-synaptic cells; whereas alpha-2 ARs mainly assert their effects on the presynaptic cell to serve as auto/heteroreceptor: to function as a negative feedback system for neurotransmitter release. However, it has also become evident that many alpha-2 ARs are postsynaptic and may regulate brain function in a manner that can contribute to drug-seeking behavior (see e.g., (Zhang et al 2009)). Importantly, many studies examining the role of norepinephrine in stress have used the alpha-2 AR agonist clonidine and related drugs to prevent further NE release, and therefore block stress-induced reinstatement; importantly, clonidine has been shown to block stress-induced cocaine seeking in humans (Jobes et al 2011). The alpha-2 AR antagonist yohimbine and related agents, which stimulate NE release, have been used to mimic a stressor in preclinical settings and has been shown to induce cocaine-seeking behavior (Mantsch et al 2010, Shepard et al 2004).

Stress-induced reinstatement studies show that activation of noradrenergic receptors plays a crucial role in either forced swim or footshock stress induced reinstatement of drug seeking. For example, we have used the alpha-2 AR antagonists yohimbine, and BRL4408 (Kiss et al 1995) to induce reinstatement of extinguished cocaine CPP (Mantsch et al 2010) and yohimbine has been shown to reinstate drug seeking following self-administration (Shepard et al 2004). Systemic injections of the alpha-2 AR agonist clonidine have been shown to block footshock-induced reinstatement of cocaine seeking (Erb et al 2000), and forced swim-induced reinstatement of CPP (Mantsch et al 2010). In addition, central injections of clonidine into the lateral or fourth ventricles blocks footshock-induced reinstatement of heroin seeking (Shaham et al 2000b). While using these drugs has implied that the noradrenergic system plays a major role in stress-induced drug seeking, their use has some limitations. For example, yohimbine has other targets besides the alpha-2 AR; yohimbine has been shown to enhance turnover of dopamine and serotonin (Millan et al 2000), so this promiscuous effect of yohimbine makes it difficult to pinpoint its mechanism of action. However, work from the Erb lab has shown that central injections of norepinephrine induced reinstatement of cocaine seeking (Brown et al 2011), suggesting that norepinephrine does indeed have a prominent role in promoting drug seeking by ARs in the brain.

Noradrenergic receptors and their involvement in stress-related drug use: alpha-1 adrenergic receptors

Studies have examined the role of alpha-1 ARs on stress-induced reinstatement. These studies have produced mixed results: some studies found that the alpha-1 AR antagonist prazosin can block yohimbine-induced reinstatement of food, and alcohol

seeking, as well as footshock-induced reinstatement of alcohol seeking (Le et al 2011), whereas we observed a failure of prazosin to block forced-swim induced reinstatement of cocaine CPP (Mantsch et al 2010). More studies need to be performed to fully understand the role of alpha-1 ARs in stress-induced reinstatement, in part because it may have indirect effects through modulation of BNST activity. For example, application of an alpha-1 AR agonist induced a long-term depression in excitatory neurons of the BNST (McElligott & Winder 2008), which might be involved in promoting mood disorders and could regulate drug seeking. However, research on the role of the alpha-1 ARs in stress-related drug use has been overshadowed by the positive results with the beta-ARs.

Noradrenergic receptors and their involvement in stress-related drug use: beta adrenergic receptors

Studies have indicated that the beta-ARs play a crucial role in stress-induced reinstatement. Studies from our lab have found that pretreatment with the nonspecific beta-AR antagonist propranolol blocks stress-, but not cocaine-induced reinstatement (Mantsch et al 2010). Furthermore, we have shown this to be specific to beta-2 ARs as the beta-2 AR antagonist ICI 118,551, and not the beta-1 AR antagonist betaxolol, blocked forced swim-induced reinstatement (Mantsch et al 2010). Further studies from our lab have shown that beta-ARs, specifically beta-2 ARs, are both necessary and sufficient for stress-induced reinstatement because 1) the non-specific beta-AR agonist isoproterenol is sufficient to induce reinstatement; 2) beta-AR knockout mice failed to reinstate following forced swim; 3) the beta-2 AR agonist clenbuterol is sufficient to induce reinstatement (Vranjkovic et al 2012). Interestingly, other studies have indicated that beta-ARs are necessary for footshock-induced reinstatement in both the central

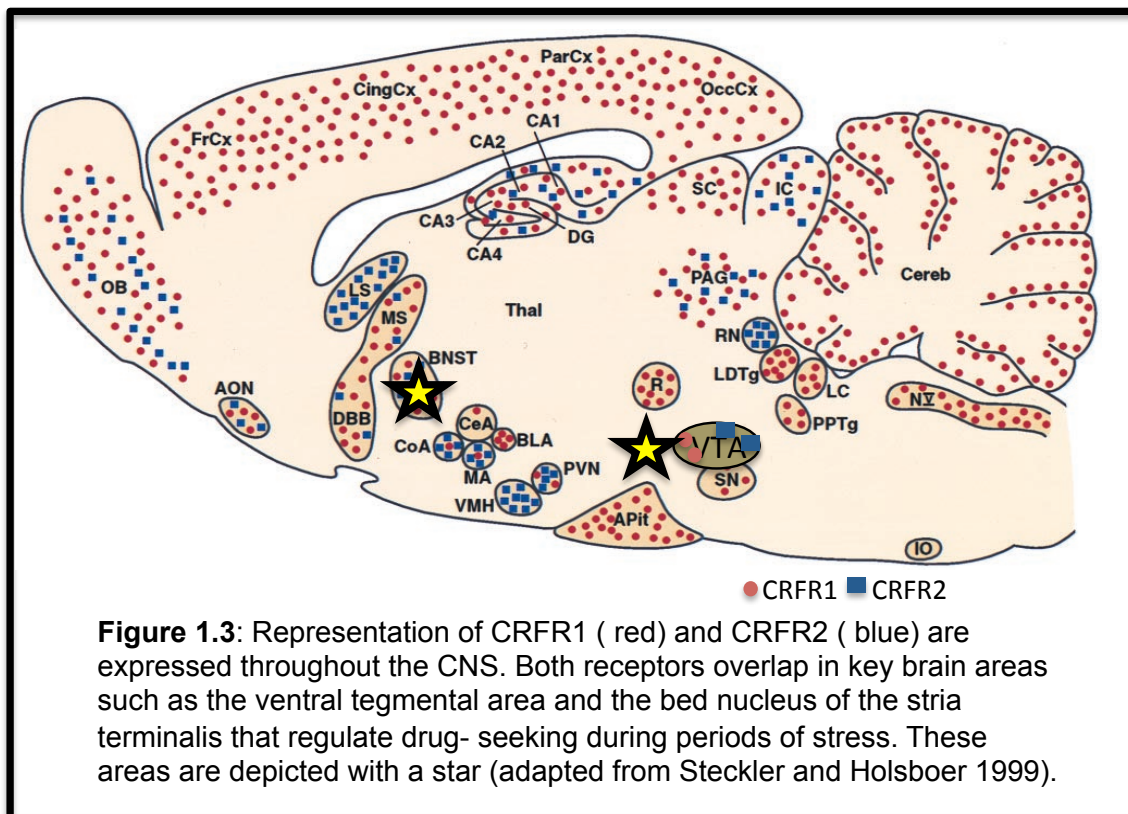
nucleus of the amygdala, and the vBNST, as a cocktail of betaxolol and ICI 118,551 (beta-1 AR, and beta-2 AR antagonist respectively) prevented reinstatement when injected into either of the two structures (Leri et al 2002). Taken together, these studies indicate that beta-AR activation within the central nucleus of the amygdala and the vBNST might be a key contributor to stress-induced reinstatement. Furthermore, studies have suggested that the noradrenergic system directly interacts with, and may stimulate the release of corticotropin releasing factor to induce cocaine seeking because central injections of NE causes reinstatement, and this is blocked by pretreatment with a nonspecific CRF receptor antagonist (Brown et al 2009).

Introduction to Corticotropin Releasing Factor

Corticotropin Releasing Factor (CRF) is a 41-amino acid peptide that has been found to play a critical role in the behavioral and neuroendocrine responses to stress. CRF binds to the 7-domain transmembrane receptors CRFR1 and CRFR2. CRFR1 and CRFR2 share approximately 70% amino acid identity, however, CRF has a greater affinity for CRFR1 compared to CRFR2 (Bale & Vale 2004). In addition, CRF interacts with the CRF-binding protein (CRF-BP). The CRF-BP has a high affinity for CRF, and it most likely prevents CRF from activating its receptors by directly binding CRF, and promoting its clearance and degradation (Woods et al 1999).

Genetically, CRFR1 and CRFR2 are produced from distinct genes with several splice variants (Bale and Vale, 2004). Both CRFR1 and CRFR2 are expressed throughout the central nervous system; CRFR1 is expressed more abundantly than CRFR2 (Steckler & Holsboer 1999). There is a 70% sequence homology between CRFR1 and CRFR2,

with an 8% homology at the transmembrane and intracellular domain levels (Lovenberg et al., 1995).



CRF: Endocrine Function

In its endocrine role, CRF initiates a cascade of events that activates the hypothalamic-pituitary-adrenocortical axis (HPA). Specifically, CRF is released from parvocellular neurosecretory cells in the paraventricular nucleus (PVN) of the hypothalamus via the median eminence in response to α -stressors. CRF travels to the anterior pituitary gland where it acts on CRFR1 to initiate the production and release of adrenocorticotrophic hormone (ACTH), which travels into the circulation and in turn promotes the release of corticosterone from the adrenal cortex. In addition to its

endocrine role, CRF acts within the brain. Within the central nervous systems, CRF, and other neuropeptides in general, have a very unique role.

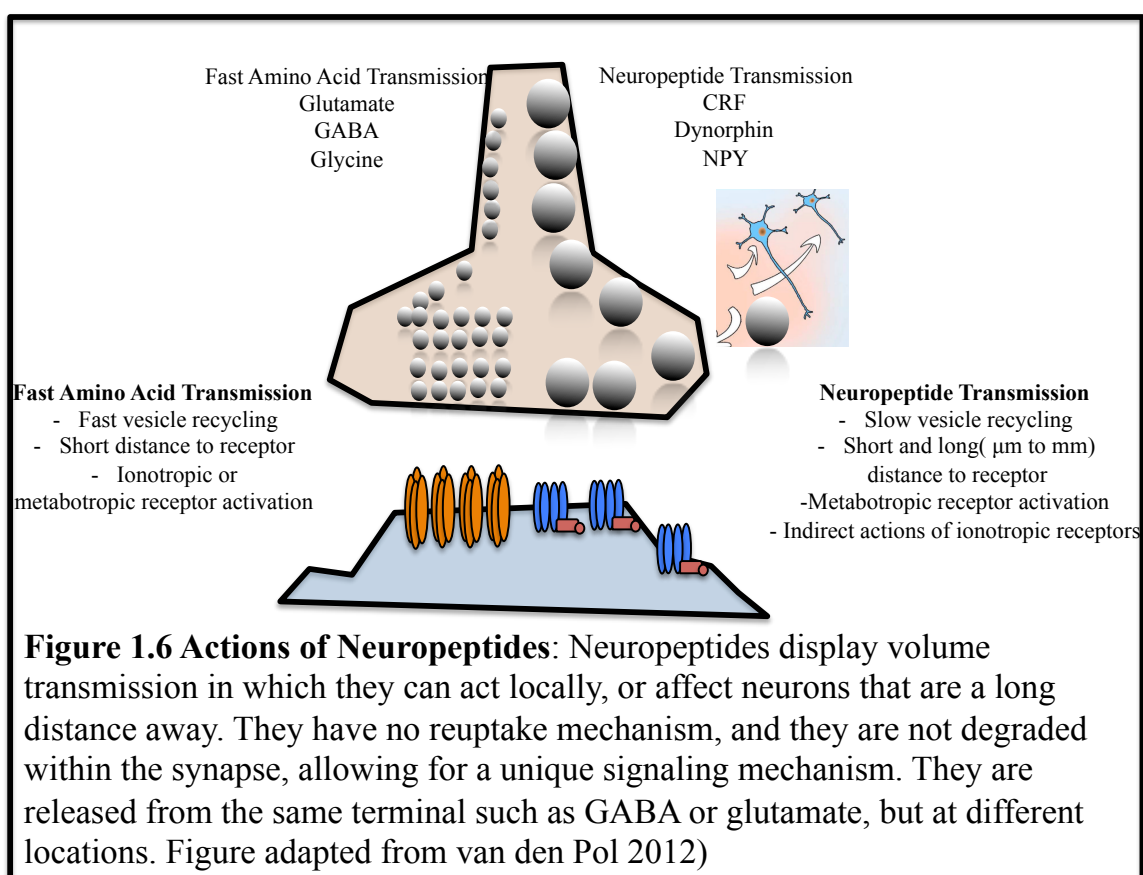
Neuropeptide Signaling

Neuropeptides act differently compared to small amino acids transmitters such as glutamate, GABA, or glycine. In general, these neurotransmitters (i.e., glutamate, GABA, and glycine) are rapidly released at a presynaptic active zone; they diffuse across a synapse, which is only a few nanometers in length, and rapidly activate their receptors, and are then rapidly degraded or transported within an astrocyte or the presynaptic terminal (van den Pol 2012). In contrast, neuropeptides are contained in dense-core vesicles and may be released at multiple sites along the neuron. Once released, the neuropeptide can travel a significantly greater distance (in the order of microns; volume transmission) and therefore can diffuse throughout a specific brain area. Neuropeptides are not degraded quickly so they remain within a synapse longer and diffuse vast distances (Nassel 2009, Palkovits 1995). Binding proteins may interact with the neuropeptides to inhibit their actions (Kormos & Gaszner 2013).

Classical neurotransmitters are often synthesized within the presynaptic terminal. The enzymes required to convert the precursors for classical neurotransmitters are synthesized in the cell body; they travel down the axon and convert the precursor molecule to the proper neurotransmitter, which is then loaded into the vesicle at the presynaptic site. In contrast, neuropeptides are synthesized as pre-propeptides and are modified within the endoplasmic reticulum to pro-peptides. Subsequently, they travel to the Golgi apparatus and are packaged into dense-core vesicles. Within the vesicles, pro-

peptides are modified by hydrolysis, glycosylation, phosphorylation, and disulfide bond formation to produce the final product (van den Pol 2012).

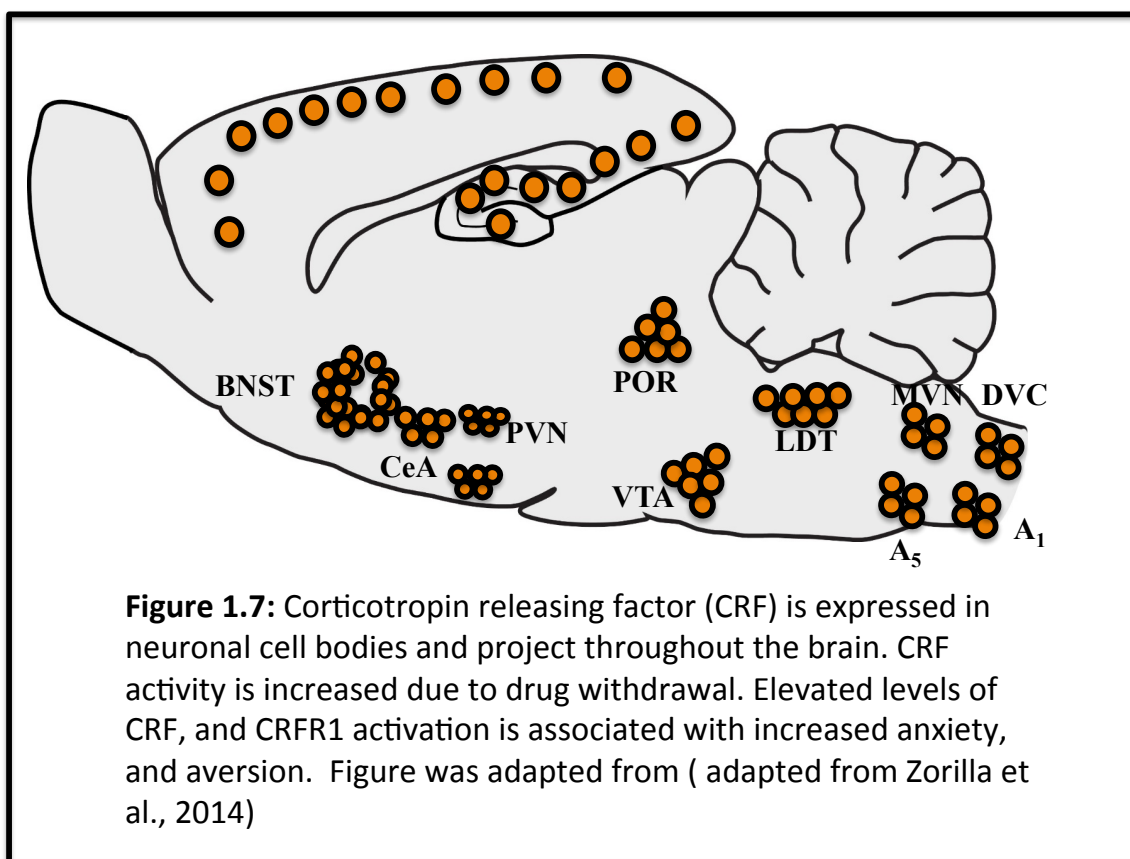
In addition, compared to classical neurotransmitters, neuropeptides exclusively activate G-protein coupled receptors; these G-protein receptors usually have a high affinity for their peptides (it would take nanomoles to activate CRFR1 by CRF, as compared to micromoles of glutamate for NMDA receptors).



Furthermore, all neuropeptides are thought to be co-released with classical neurotransmitters or neuromodulators. G-protein activation of neuropeptides will change many aspects of the cell's physiology; importantly, they have been shown to modulate receptor trafficking, and alter the conductance of different ion channels (van den Pol

2012). Importantly, neuropeptides are produced throughout the central nervous system, where they can act locally, or project vast distances. These characteristics allow for neuropeptides such as CRF to act on multiple different synapses to both promote, and inhibit the activity of the same cell population.

The role of Corticotropin Releasing Factor in stress-induced reinstatement of drug seeking



In addition to the PVN, other brain nuclei produce CRF as a neuromodulator in response to various physiological and emotional stressors. Accordingly, CRF cell bodies and receptors are widely distributed throughout the brain (Chen et al 2015, De Souza et al 1985, Perrin & Vale 1999). Importantly, CRF has been found in the BNST, both the

dorsal and the ventral subdivision (Chen et al 2015), and expression of CRFR1 and CRFR2 have been reported within the BNST and VTA (Justice et al 2008, Sawchenko et al 1993).

Given the importance of CRF in stress, early neurobiological investigations of stress-induced reinstatement focused on the role of CRF. Experiments found that intracerebroventricular pretreatment with the non-selective CRF receptor antagonists D-Phe CRF, and alpha-helical CRF₉₋₄₁, significantly attenuates, or blocks, footshock-induced reinstatement of heroin, cocaine, and alcohol seeking (Erb et al 1998, Le et al 2000, Liu & Weiss 2002, Zislis et al 2007). Conversely, central injections of CRF itself reinstate drug seeking (Erb & Brown 2006, Mantsch et al 2008a). These studies strongly suggest that CRF plays an important role in reinstatement of drug seeking during periods of stress. However, the site of action of CRF and the receptor that CRF acts on was just recently discovered.

The effects of CRF on footshock-induced reinstatement have been attributed to actions at the CRFR1 receptor. Administration of the selective CRFR1 receptor antagonist, CP-154, 526, prior to footshock stress blocked reinstatement of cocaine and heroin seeking (Blacktop et al 2011, Gehlert et al 2007, Shaham et al 2000a). In contrast, pretreatment with the selective CRFR2 receptor antagonist, antisauvagine failed to block footshock-induced reinstatement of extinguished nicotine seeking (Gehlert et al 2007, Lu et al 2000), or footshock-induced reinstatement of extinguished cocaine seeking (Blacktop et al., 2011). Interestingly, the actions of CRF on CRFR1 have been localized in part to the VTA. For example, studies have shown that CRF is released transiently in the VTA and that CRF release is enhanced during electric footshock (Wang et al 2005).

Recent data suggest that activation of CRFR1 in the VTA during electric footshock mediates stress-induced reinstatement of cocaine, heroin, and nicotine seeking (Blacktop et al 2011, Grieder et al 2014, Wang et al 2006). Therefore, from these studies, it can be concluded that CRFR1 activation within the VTA is an important contributing factor in the neurobiology of stress-mediated relapse. However, other studies have suggested that the CRFR2 receptor and not the CRFR1 receptor is involved in stress-induced drug seeking (Wise and Morales, 2010). Additionally, several different brain areas may contribute to stress-related drug use in conjunction with CRF including the locus coeruleus (Al-Hasani et al 2013, McCall et al 2015), the central nucleus of the amygdala (Erb et al 2001b), and the dorsal raphe nucleus (Zorrilla et al 2012). The source of the CRF input into the VTA is not yet known, though the BNST has been placed as a likely candidate.

Convergence of the noradrenergic and CRF systems within the BNST

The BNST has two stress-related noradrenergic inputs that modulate its activity and may lead to drug seeking during periods of stress. The first stress-related input is into the ventral BNST (vBNST); it receives arguably the strongest NE projections from the ventral forebrain bundle (Forsay & Gysling 2004). In addition, NE release within the vBNST has been shown to contribute to stress-induced reinstatement through the actions of the beta-adrenergic receptors (Leri et al 2002). Signaling through beta-ARs can increase both GABAergic and glutamatergic transmission through presynaptic mechanisms; beta-AR transmission increase IPSC frequency within the BNST (Dumont & Williams 2004, Egli et al 2005, McElligott et al 2010, Nobis et al 2011). Specifically,

NE can enhance, or decrease glutamate transmission in the dBNST, while its effects in the vBNST are less clear. NE, via a beta-AR mechanism, has been reported to inhibit glutamate transmission in the vBNST (Egli et al., 2005). However, studies have shown that beta-1 AR activation within the BNST produces a CRF-dependent increase of excitatory transmission; specifically, activation of beta-AR receptors causes an increase in spontaneous EPSC. However, in the presence of a CRFR1 antagonist, this effect is not observed, indicating that beta-AR dependent EPSCs are mediated by activation of CRFR1 (Nobis et al. 2011). This suggests that beta-ARs within the BNST may contribute to stress-dependent reinstatement, since stress-dependent norepinephrine release modulates BNST activity, most likely activating excitatory signaling through CRF-dependent actions of beta-AR within the BNST. Based on our work, we believe that activation of the beta-2 AR (Mantsch et al 2010, Vranjkovic et al 2014) within the BNST modulates stress-responsive signaling in a CRF-dependent manner.

The second stress-related input to the BNST is CRF. The BNST contains a local pool of CRF-positive cells that not only act within the BNST but also send CRF to other putative brain regions (Sakanaka et al 1986, Sink et al 2013, Wang et al 2011). Notably, stress exposure increases *crf* mRNA in the BNST (Funk et al 2006). For example, footshock has been shown to increase *crf* mRNA within the dorsal, but not ventral, BNST of withdrawn heroin animals (Shalev et al 2001). This suggests that CRF within the dBNST might act on other targets such as the vBNST to initiate reinstatement. Furthermore, injections of CRF within the BNST reinstates drug-seeking behavior, whereas CRF injections within the amygdala fail to promote the same response (Erb &

Stewart 1999). This further indicates that CRF signaling within the BNST is crucial for reinstatement.

Neuroanatomical evidence has shown that ventral NE projections, which are critical to stress-induced reinstatement, make direct synaptic contact with CRF cells within the ventral BNST (Hornby & Piekut 1989, Phelix et al 1994). Interestingly, within the BNST, CRF enhances beta-AR transmission through activation of CRFR1 (Nobis et al 2011). In addition, activation of the beta-AR depolarizes BNST CRF neurons, and CRF enhances the frequency of spontaneous EPSCs in BNST neurons that project to the VTA (Silberman et al 2013). These findings suggest that beta-AR activation may enhance CRF release from local BNST CRF sources, leading to enhancement of excitatory neurotransmission on VTA-projecting neurons by the activation of either CRFR1 on VTA-projecting cells, or the activation of CRFR1 on glutamate cells that interact with BNST cells projecting to the VTA. These findings place the BNST as a main candidate for a CRF projection to the VTA. However, no studies have shown that a direct BNST to VTA CRF projection mediates stress-induced reinstatement. In addition, no studies have analyzed the role of interactions of CRF and NE within the BNST in mediating stress-induced reinstatement.

CRF acts within the VTA to promote drug seeking during periods of stress

The VTA has been implicated as a key area where a number of pathways converge to regulate motivated behavior and reward to promote drug-seeking behavior when faced with a stressor. The VTA is the main source of dopamine within the central nervous system and via its two primary spokes, the mesocortical, and mesolimbic

systems, the VTA can coordinate drug-seeking behavior. Specifically, ~70% of VTA neurons are dopaminergic, ~30% of neurons are GABAergic, and about ~1-2% are glutamatergic (Nair-Roberts et al 2008, Walsh & Han 2014, Yamaguchi et al 2015).

Within the VTA, drug-dependent neuroplasticity can make an addict more susceptible to relapse when faced with a stressor (Mantsch et al., 2014). In order to develop therapeutics for the treatment of stress-dependent drug seeking, we need to better understand the neurobiological mechanisms through which stressors trigger drug seeking.

Actions of CRF within the VTA have been shown to promote drug use during periods of stress. Studies have shown that CRF administration into the VTA is sufficient to cause drug-seeking behavior (Blacktop et al 2011, Wang et al 2005, Wang et al 2007), and that the actions of CRFR1 (Blacktop et al 2011, Chen et al 2014), CRFR2 (Wang et al 2005, Wang et al 2007) and the CRF binding protein (Wang et al 2007) are necessary for stress-induced drug, and food (Chen et al 2014) seeking following cocaine self-administration. CRF inputs into the VTA have been shown to come from the oval bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus, and the central nucleus of the amygdala (Rodaros et al 2007); however, only the CRF input from the oval bed nucleus has been shown to be involved in stress-related drug-seeking behavior (Erb et al 2001a, Erb & Stewart 1999, Rodaros et al 2007). Studies have shown footshock stress promotes CRF release within the VTA in both drug-naïve, and drug-experienced rodents (Wang et al 2005).

Interestingly, a recent study has suggested that chronic nicotine self-administration upregulates local VTA CRF production, and that this CRF gets released in the posterior VTA in an autocrine fashion to activate CRFR1 on DA cells to promote

stress-induced nicotine relapse (Grieder et al 2014). This suggests that within the VTA, CRF may be released from terminals that originate from the amygdala, bed nucleus, or the paraventricular nucleus of the hypothalamus, or from VTA cell bodies during periods of stress where it most likely acts on CRFR1 to modulate VTA activity.

CRF receptor subtypes within the VTA

Polymorphisms in genes that encode CRF receptors are associated with exacerbated stress responses and propensity to develop drug addiction (Blomeyer et al 2008, Clarke & Schumann 2009, De Luca et al 2007, Enoch et al 2008, Treutlein et al 2006) indicating that CRF receptor function can contribute to substance abuse/addiction. CRF, and both of its receptors (CRFR1 and CRFR2) are expressed within the VTA (Ungless et al., 2003, Korotkova et al., 2006, (Grieder et al 2014). CRFR1 is expressed at much lower levels in VTA compared to other brain areas and is localized to both dopaminergic and GABAergic cells. Repeated drug use may increase the expression of CRFR1, partly; CRF binding is increased within the VTA due to chronic cocaine administration as assessed through receptor autoradiography (Goeders et al., 1990).

Stress seems drive DA activation within the VTA through a CRF-dependent mechanism. We have shown that CRFR1 receptor activation is both necessary and sufficient for stress-induced drug seeking (Blacktop et al., 2011). In addition, deletion of CRFR1 within VTA DA neurons has been shown to be anxiogenic, and decrease DA release within the prelimbic cortex (Refojo et al., 2011). Moreover, a VTA viral-mediated knockdown of CRFR1 decreased food deprivation stress-induced reinstatement of cocaine seeking in mice (Chen et al., 2014). We hypothesize that stress induces an

activation of VTA DA neurons that project into the prelimbic cortex (Deutch et al., 1991) and therefore increases the release of DA within the prelimbic cortex (Thierry et al., 1976; Sorg and Kalivas, 1993). While some studies have suggested this is a CRFR1 dependent process (Refojo et al., 2011), no studies have directly shown this to be the case as it relates to stress-induced drug seeking

Traditional *in situ* hybridization methods have failed to detect CRFR2 within the VTA (Van Pett et al., 2000); this may indicate that CRFR2 are localized on projecting presynaptic terminals within the VTA. In addition, other studies have suggested that both CRFR1 and CRFR2 within the VTA mediated EPSC potentiation and attenuation respectively (Williams et al., 2014). In addition, the same study showed that CRFR2 activation facilitated GABA release within the VTA (Williams et al., 2014). These studies have indicated that while CRFR1 within the VTA may directly mediate stress-induced drug seeking, it may do so in coordination with the CRFR2 receptors in a poorly understood fashion.

Actions of CRF on VTA neurons

The cellular effects of CRF within the VTA are diverse and have been shown to include regulation of both glutamatergic and GABAergic inputs; CRF has been hypothesized to directly modulate VTA DA firing at presynaptic, postsynaptic, and extrasynaptic sites. Studies have indicated that aversive stimuli may differently act upon VTA DA projections to either the prelimbic cortex or the nucleus accumbens. Specifically many studies have indicated that aversive stimuli (through actions of CRF within the VTA) increase DA release within the prefrontal cortex (Abercrombie et al

1989, Lammel et al 2008, Lammel et al 2012, Refojo et al 2011, Wanat et al 2008), while within the nucleus accumbens, some aversive stimuli such as quinine, an aversive tastant, may decrease DA release (Twining et al 2015). This may be attributed to differential actions of CRF on VTA DA-projecting neurons; CRF may enhance DA signaling into the prelimbic cortex, while have differential effects on, possibly attenuating, the DA projection to the nucleus accumbens.

Specifically, studies have shown that CRF, through activation of CRFR1 within the VTA, increases VTA DA I_h currents and dopamine neuron excitability through a PKC-dependent mechanism (Wanat et al., 2008). Other studies have shown that chronic cocaine use potentiates the magnitude and duration of CRF-induced NMDA currents, and that only mice treated with cocaine showed a CRF-dependent enhancement of AMPA transmission (Hahn et al., 2009). In addition, other studies have suggested that the potentiation of the NMDA receptor-mediated signaling is attributable to CRFR1 activation (Sparta et al., 2013). These studies suggest that CRF within the VTA enhances excitatory transmission, in part through regulation of NMDA and AMPA receptors.

Other studies have suggested that activation of CRFR1 within the VTA decreases VTA DA cell excitability (Beckstead et al., 2009), and dopamine release within the nucleus accumbens (Wanat et al., 2013; Twining et al., 2015). Specifically, Beckstead et al showed that CRF facilitated GIRK currents and reduced DA neuron excitability via activation of CRFR1. Moreover, CRF administration into the VTA has been reported to cause a decrease in basal nucleus accumbens DA (Wanat et al., 2013), while a CRFR1 antagonist administered into the VTA blocked the drop in accumbens DA, which is observed with intra-oral quinine (Twining et al., 2015). Interestingly, intra-oral quinine

also induces reinstatement. Therefore, these studies suggest that, during periods of stress, CRF may act to decrease the VTA DA projection into the accumbens (Twining et al., 2015).

Glutamate-CRF actions within the VTA promote drug use

The VTA receives glutamatergic inputs from many brain regions (Geisler et al 2007). Glutamatergic inputs to VTA DA neurons have been found to originate from the medial prefrontal cortex, mesopontine tegmentum nucleus, lateral habenula, periaqueductal gray, and the dorsal raphe (Charara et al 1996, Clements & Grant 1990, Omelchenko et al 2009, Omelchenko & Sesack 2007, Omelchenko & Sesack 2010, Qi et al 2014, Sesack & Pickel 1992). However, it is important to note that some of these glutamatergic regions also project to non-DA neurons within the VTA, and that glutamatergic inputs and outputs to the VTA can manipulate behavior in different ways. For example, reward-mediating inputs can come from the laterodorsal tegmental area, while aversion-mediating inputs can originate from the lateral habenula (Lammel et al 2012). These observations are an important indication that isolating selective inputs to the VTA using current techniques, such as optogenetics, is critical in order to link specific behaviors to specific inputs. Therefore, using current advances in optogenetics and chemogenetics, specific inputs onto specific cell populations is an important future research objective.

CRF actions within the VTA have mainly focused on the interaction between CRF and glutamate. Studies have shown that CRF-containing neurons make morphologically asymmetric synapses with VTA DA neurons, suggesting that CRF-

releasing terminals are predominantly glutamatergic (Tagliaferro & Morales 2008). CRF directly excites both DA and non-DA neurons within the VTA (Korotkova et al 2006, Wanat et al 2008). In addition, CRF increases NMDA current that uses a CRFR2-dependent mechanism (Ungless et al 2003), while actions on CRFR1 have been shown to potentiate both NMDA and AMPA currents in cocaine-experienced mice (Hahn et al 2009). VTA glutamate levels are increased by the direct administration of CRF within the VTA (Wang et al 2005) while administration of the AMPA/NMDA receptor antagonist kynurenic acid into the VTA has been reported to prevent both footshock-induced reinstatement and the increase in VTA DA levels (Wang et al 2005, Wise & Morales 2010). This suggests that CRF enhances glutamatergic transmission on VTA DA cells. However, it is important to note that VTA DA neurons do not ubiquitously project to the same terminals; the balance between VTA DA projection activation and inhibition may coordinate a differential behavioral response based on the output of the VTA DA neurons; for example, studies have shown that laterodorsal tegmentum neurons preferentially synapse on DA neurons projecting into the nucleus accumbens lateral shell, while lateral habenula neurons synapse primarily on DA neurons that project to the medial prefrontal cortex, and GABAergic neurons located within the tail of the VTA (Lammel et al 2014, Lammel et al 2012). This is important because studies have shown that the lateral habenula is activated during aversive stimuli (Barrot et al 2012, Jhou et al 2013, Stamatakis & Stuber 2012) which suggests that the stress-dependent increase of DA release within the prefrontal cortex may be dependent on activation of VTA DA neurons by the lateral habenula.

GABA neurotransmission within the VTA during stress-induced relapse

Much research has focused on the role of excitatory neurotransmission within the VTA that leads to relapse during periods of stress. However, the inhibitory neurotransmitter γ -aminobutyric acid (GABA) may have diverse functions in controlling DA cell firing within the VTA. It is important to note that the VTA is not only comprised of DA neurons, but also a subset of GABAergic neurons that are interneurons (Cruz et al 2004, Steffensen et al 1998) or GABAergic-projecting neurons (Pupe & Wallen-Mackenzie 2015, Walsh & Han 2014). Specifically, ~70% of VTA neurons are dopaminergic, ~30% of neurons are GABAergic, and about ~1-2% are glutamatergic (Nair-Roberts et al 2008, Walsh & Han 2014, Yamaguchi et al 2015). Interestingly, very small subsets of neurons have been found to co-release DA along with either glutamate, or GABA in their terminal region (Stuber et al 2010, Sulzer et al 1998, Tritsch et al 2012). In addition, the VTA receives GABAergic input from various other brain areas such as: the nucleus accumbens (Bocklisch et al 2013, Yim & Mogenson 1980), the periaqueductal gray (Omelchenko & Sesack 2010, Zhang et al 2015), the bed nucleus of the stria terminalis (Kudo et al 2012), the lateral septum (Luo et al 2011), laterodorsal tegmentum (Omelchenko & Sesack 2005), and the rostromedial tegmental area (Barrot et al 2012, Zhou et al 2009). Once released, GABA may act on either GABA_A receptors, to open up a Cl⁻ channel to hyperpolarize the cell and thus decrease the chance of firing an action potential, or it can activate GABA_B receptors, which will also decrease the membrane potential by activating a potassium channel (GIRK) through a G-protein (G_i) that is coupled onto the GABA_B receptor (Johnston 1996, Kasten & Boehm 2015). Both GABA_A and GABA_B receptors are located on VTA DA neurons where they can modulate

DA activity (Creed et al 2014, Enoch 2008, Lomazzi et al 2008); the diversity of these VTA DA neurons can contribute to the complexity of how stress-related inputs regulate VTA DA neurons. For example, studies have shown that CRF modulates DA and GABA-mediated inhibition within the midbrain by activating inhibitory postsynaptic currents through GIRK channels on DA cells. Specifically, CRF enhances the amplitude and slows the kinetics of inhibitory postsynaptic current through interactions with GABA_B receptors and D₂ receptors (Beckstead et al 2009).

Studies have shown that stress may “turn off the brakes” on VTA DA cells by blocking long-term potentiation of GABAergic neurons that synapse onto DA cells (Graziane et al 2013). In addition, GABA release is increased within the VTA during periods of stress in animals that received daily non-contingent cocaine injections (Sotomayor-Zarate et al 2015). Some studies have suggested that CRF interacts with GABA neurons; studies have shown that CRF regulates GABA release through a pre-synaptic mechanism (Kasten & Boehm 2015, Williams et al 2014). This would suggest that GABA acts to disinhibit DA firing. However, other literature has suggested that GABA directly acts to decrease DA firing. Studies have shown that footshock excites VTA GABA neurons while concurrently inhibiting DA neurons (Tan et al 2012). In addition, footshock stressors have been shown to reduce the firing of VTA DA neurons (Ungless et al 2004). Interestingly, we have recently found that GABA_B receptors are necessary for both stress-, and CRF-induced drug seeking; our data indicates that GABA_B activation may be upstream from the actions of CRF (Blacktop et al., 2015). This demonstrates that VTA neurons may have a dual action in response to aversive stimuli; some neurons will increase their firing rate, while others will have a decrease in firing. It

is important to understand the projection areas of these differentially responding VTA neurons.

VTA projections to the PFC

Recent innovations in optogenetics and viral mediated-gene transfer have led to findings that DA neurons located in the medial posterior VTA selectively project to the medial prefrontal cortex (Lammel et al 2008). These neurons are very unique because they have the ability to fire at a very high frequency, have longer waveforms, and have significantly smaller after-hyperpolarization curves, indicating that these neurons have the potential to fire rapidly. Interestingly, these neurons express relatively low amounts of the DA transporter, and they lack somatodendritic D2 receptors and autoreceptor DA D2 receptors on their presynaptic terminals which interact with G-protein inward rectifying receptors, in addition, these pyramidal neurons have been shown to lack GIRK channels as well (Lammel et al 2008).

Studies have indicated that VTA DA neurons innervate pyramidal neurons (the principal glutamatergic neuron of the prefrontal cortex) located in layers II, and V/VI within the prelimbic cortex (Swanson 1982). Pyramidal neurons in this layer may be found in two types of state: a down-state in which the neurons display a negative resting membrane potential and therefore are less excitable, and an up-state which is characterized by a plateau depolarization and therefore are more excitable (Peters et al 2000). Electrical stimulation of VTA DA neurons have been shown to evoke a long-lasting transition of pyramidal neurons from the down-state to the up-state and this effect was blocked by administration of a D1 receptor antagonist (Lewis & O'Donnell 2000). The activation of D1 receptors within the prefrontal cortex is thought to increase current

through the NMDA receptor via a PKA-, and Ca^{+2} -dependent mechanism (Wang & O'Donnell 2001). In addition, studies have suggested that activation of D1 receptors on pyramidal neurons also inhibits G protein-dependent inward recertifying currents (Witkowski et al 2008). These results suggest that VTA DA neurons control the probability of prefrontal pyramidal firing and/or synchronize pyramidal neuron activity. This is very important because the prefrontal cortex is necessary for stress-induced drug seeking, particularly pyramidal neurons (McFarland et al 2004), and drug seeking during periods of stress may be mediated by the activation of D1 receptors in this region (Capriles et al 2003). However, no studies have directly shown that mesocortical DA neurons are recruited in a CRF-dependent manner to promote drug use during periods of stress.

The prefrontal cortex is involved in cocaine use during periods of stress

Drugs of abuse decrease the value of natural reward, diminish cognitive control, and enhance glutamatergic drive to drug-associated stimuli within the cortico-accumbens pathway (Kalivas & Volkow 2005). It is well accepted that the reinforcing properties of cocaine and other drugs of abuse involves the activation of the mesocorticolimbic reward pathway, which includes DA neurons originating from the VTA and projecting to the ventral striatum and PFC. The PFC is involved in driving cognitive processes, and executive functioning by processing information from vast sensory modalities to form memories, perception, and decision-making (Siddiqui et al 2008). Our early understanding of the PFC comes from Phineas Gage who was unfortunate enough to have a metal rod obliterate his left PFC in an explosion. Phineas Gage developed both memory

problems and a change in personality. However, our understanding of the role of the PFC in stress-related drug use remains unclear.

Preclinical studies have indicated that acute cocaine administration increases the activity of the PFC. Studies have shown that non-contingent cocaine administration increases blood flow to the dorsolateral PFC and self-administration of cocaine increases blood flow to both the dorsolateral PFC and the anterior cingulate cortex (Howell et al 2002, Howell et al 2010). Blood oxygen level-dependent (BOLD) responses are elevated in subregions of the PFC such as the dorsolateral prefrontal cortex, anterior cingulate, anterior orbital gyrus, orbitofrontal cortex, medial orbital gyrus, and frontopolar cortex (Goldstein & Volkow 2011). In addition, intracerebroventricular administration of cocaine resulted in a large fMRI response within the PFC, and other brain areas (Febo et al 2004). This increase in activity of the prefrontal cortex has been proposed to induce neuroplastic changes that transform drug habits from recreational to regular and even uncontrollable use (Goldstein & Volkow 2011). In addition, BOLD signals have indicated that the human PFC response is linked to both liking and wanting of cocaine because BOLD signals in most PFC regions are correlated with the “rush” ratings (Breiter et al 1997) and are increased when cocaine administration is expected (Kufahl et al 2008). These human studies make it very apparent that subregions of the PFC are highly involved in drug-seeking behavior.

Cocaine intake induces hypofrontality within the prefrontal cortex

It has been hypothesized that chronic cocaine intake may cause hypofrontality within the prefrontal cortex, which may be reversed when addicts are exposed to

environments that are previously associated with drug taking (Childress et al., 1999). It has been hypothesized that hypofrontality also occurs with repeated cocaine intake in rats; studies using rats trained to self-administer cocaine showed that overall firing and burst rates were significantly decreased after the first cocaine exposure relative to prior activity; these effects dissipated after 10 days of drug SA, and were replaced by a significant increase in burst duration and firing rate (Sun & Rebec 2006). Interestingly, imaging data has indicated that activity of the PFC in drug addicts is lower compared with controls (Volkow et al., 2003); these studies however cannot determine whether this hypofrontality is induced by cocaine or a pre-existing condition. In addition, BOLD signals cannot differentiate the activity between pyramidal neurons or interneurons. However, theories have proposed that while drug addiction devaluates natural rewards through hypofrontality, drugs, and drug-related cues, may enhance the activity of the prefrontal cortex (Volkow et al 2003). In addition, extensive drug use (such as our long-access self-administration model) has been hypothesized to enhance the activity of the cortico-striatal pathways by priming the system to triggers such as cues or exposure to a stressor (Sinha 2013). Furthermore, studies have indicated that magnitude of stress-induced craving correlates with levels of prior-drug use in humans (Fox et al 2005). This would suggest that a history of increased drug use might prime the prefrontal cortex to be more active when exposed to a stressor. However fMRI assessment of the PFC in cocaine addicts upon exposure to stress-related imagery has shown that BOLD signal is actually reduced rather than increased (Sinha et al., 2009).

Interestingly, studies have also suggested that chronic drug use alters CRF activity (Sinha 2013). Therefore, it is intriguing to speculate that a CRF-dependent mechanism

may control the activity of the prefrontal cortex during stressful stimuli that in a manner that elicits relapse to drug use.

Neuroanatomy of the rat prefrontal cortex

In the rodent, the prefrontal cortex is comprised of three different topographical regions (medial, orbital, and lateral parts (Ongur & Price 2000)). The medial PFC can be subdivided into four main structures: the medial granular, the anterior cingulate, the prelimbic cortex (PL), and the infralimbic cortex (IL; (Hoover & Vertes 2007)). The medial PFC can be divided into dorsal and ventral divisions; the dorsal medial PFC is involved in motor behaviors (medial granular), while the ventral medial PFC is involved in emotional, and cognitive processing (anterior cingulate, PL, and IL; (Heidbreder & Groenewegen 2003)). While this work focuses mainly on the VTA DA projection to the prelimbic cortex, this region does receive numerous projections from other areas. Notably, the main projections to the PL and IL are the orbital medial prefrontal, agranular insular, perirhinal and entorhinal cortices, the hippocampus, the claustrum, the medial basal forebrain, the basal nuclei of the amygdala, the midline thalamus, and the locus coeruleus of the brainstem (Hoover & Vertes 2007). Conversely, both the PL and IL cortices project to multiple brain areas, all of which may be reviewed by the works of Vertes; the important projections as it pertains to stress-evoked drug use, are the lateral septum, the bed nucleus of the stria terminalis, and the parabrachial nucleus of the brainstem for the IL and the nucleus accumbens, and mediodorsal nucleus of the thalamus for the PL (Vertes 2004). These projections suggest that the IL is involved, in part, in visceral/autonomic activity, while the PL is involved in cognitive function (Vertes

2004). The infralimbic cortex sends a glutamatergic projection to the shell of the nucleus accumbens that is thought to be involved in extinction learning (LaLumiere et al 2010, LaLumiere et al 2012). Furthermore, it has been shown that the glutamatergic projection from the IL to the shell of the accumbens exerts suppression of drug seeking (Peters et al 2009, Peters et al 2008). Specifically, inactivation of the infralimbic cortex, or the shell of the nucleus accumbens, reinstates drug seeking in the absence of drugs, cue, or stressful triggers (Peters et al 2008)

The prelimbic cortex is involved in stress-related cocaine use

While much attention has been placed on the role of the PFC as it pertains to drug taking, the PFC also has a prominent role in stress-related relapse to drug use. Stress-related drug use has been mimicked in the rodent using a mild-non-noxious footshock stressor. Interestingly, in 1976, Glowinski found that an electric-footshock stressor significantly increased DA, but not norepinephrine, release within the prefrontal cortex while DA release was not increased within the ventral striatum (Thierry et al 1976). In addition, retrograde tracing studies have shown that a restraint stressor increased Fos protein (a neuronal activity marker) immunoreactivity within VTA DA neurons that project to the PFC; however, Fos expression was not increased in any DA projections to the nucleus accumbens (Deutch et al 1991), in addition studies have shown that a tail-shock increased DA reactivity by 95% within the PFC; and to much lesser extents in the nucleus accumbens, and other terminal areas (Abercrombie et al 1989). These studies pioneered the idea that stress may selectively activate DA projections from midbrain DA neurons to the PFC, which likely precipitate drug use during periods of stress (Capriles et

al 2003, McFarland et al 2004). Many studies have shown that aversive stimuli, such as footshock, increase DA release in terminal regions of the mesocorticolimbic DA system (Abercrombie et al 1989, Gresch et al 1994, Kalivas & Duffy 1995). This is also seen with other triggers for relapse, such as exposure to cues previously associated with the drug, and the drug itself (see Mantsch et al., 2015 for review). The prefrontal cortex appears to process these different triggers to relapse by engaging the nucleus accumbens core. Electrophysiological data has suggested that prefrontal cortical DA activation is involved in cocaine seeking rather than the hedonic process of cocaine itself (Rebec & Sun 2005).

Topographical organization of the DA projection from the VTA to the PFC

As mentioned previously, dopamine originates from the VTA/substantia nigra (or A10) and projects to most limbic system structures, especially the prefrontal cortex (Swanson 1982). Most cells that project to the prefrontal cortex from the VTA are located in medial/caudal parts of the VTA (Lammel et al 2014) and are mostly topographically organized wherein dorsomedial parts of the VTA project to the anterior cingulate, while ventromedial parts of the VTA project to the dorsomedial prefrontal cortex (Swanson 1982).

Interestingly, roughly 11% of cells that project to the PFC cross the midline and 66% of cells that project to the PFC are dopaminergic (Swanson 1982). Most studies have examined the DA projection from the VTA to the PFC in mice using TH-cre/DAT-cre GFP mouse line. Combining this approach with retrograde tracers, studies have started to suggest that VTA DA projections differentially encode reward and aversion in

mice (Lammel et al., 2012) and non-human primates (Kormos & Gaszner 2013). While mouse and rodent brains are fairly similar, the lack of genetically modified rats has made it difficult to pinpoint VTA DA neurons that are 1) activated by aversive stimuli and 2) are topographically located within the VTA.

Activation of DA receptors

Once DA is released into the synaptic cleft, it can act on five major receptor subtypes, designated D₁-D₅. All of these receptors belong to the seven transmembrane domain G-protein coupled receptors. The DA receptors are commonly categorized as either D₁-like (D₁ and D₅) or D₂-like (D₂, D₃, and D₄) based on sequence homology and G-protein coupling. Activation of D₁-like receptors activates the G-protein G_{sa}, leading to activation of adenylyl cyclase, and an increase in second messengers such as cAMP, while activation of D₂-like receptors are inhibitory through actions of the G-protein G_{ai}.

Dopamine receptor location within the PFC

D₁-like and D₂-like receptors are found within the PFC, specifically within layers II-VI of the PFC with the highest density observed within layer V and VI of the prefrontal cortex; these layers also receive a prominent projection from the VTA as compared to layers II-III (Swanson 1982, Vincent et al 1993). Studies have suggested that D₁-like receptors are located post-synaptically (Levey et al 1993) on pyramidal neurons (Vincent et al 1993) while D₂-like receptors interface with the extrasynaptic space of the presynaptic projecting neurons where they act as autoreceptor to inhibit DA release (Goldstein et al 1990). However, some D₂-like receptors have been localized to GABAergic interneurons and some expression has been observed on astrocytes (Vincent

et al 1993). Interestingly, some studies have reported that D₁-like receptors are also present on parvalbumin-containing interneurons; these neurons control the synchronization of PFC pyramidal neurons (Muly et al 1998). This serves to show the complexity of the PFC, in that DA within the PFC can act to either increase or decrease the excitability of pyramidal neurons based the location of DA release and/or targeted receptor, and neuroplastic changes can occur in many different areas to shape the output of the prelimbic cortex. However, in the case of the projection from the VTA, D₁-like receptors are preferentially expressed postsynaptically on pyramidal neurons in the PFC (Boyson et al 1986). Therefore, DA will act on D₁-like receptors to increase the likelihood of firing rate of PFC pyramidal neurons (Lewis & O'Donnell 2000); these PFC neurons are thought to project to the core of the nucleus accumbens (NAc) whereby DA indirectly modulates the NAc by increasing the size of the NMDA component of an excitatory postsynaptic potential on these neurons (Lewis & O'Donnell 2000, Seamans et al 2001, Yang & Seamans 1996). The pyramidal neuron projections from the PFC to the NAc is glutamatergic. This suggests that during periods of stress, DA release from the VTA to the PFC is increased and activates D₁-like receptors on pyramidal neurons, leading to an increase in the firing rate of glutamatergic projection neurons to the NAc, and therefore promoting drug seeking.

Prelimbic interneurons regulate pyramidal neurons through actions of both D1 and D2 receptors

While mesocortical DA fibers innervate pyramidal neurons (Goldman-Rakic et al 1989, Verney et al 1990), they also innervate GABAergic interneurons (Sesack et al 1995, Vincent et al 1993). These GABAergic interneurons are important in initiating and

maintaining rhythm and synaptic kinetics within the prefrontal cortex (Gupta et al 2000) through their variable intrinsic firing rates (Markram et al. 2004). Parvalbumin interneurons constitute the majority of interneurons within cortical layer V (Markram et al. 2004) where they synapse close to pyramidal neurons and other local interneurons (Kawaguchi and Kubota 1997, 1998). DA can increase the excitability of interneurons, as evidenced by the finding that bath application of the D₂ agonist quinpirole or the D₁ agonist SK38393 induces an increase in the interneuron excitability (Tseng and O'Donnell 2007) and therefore may increase GABAergic synaptic transmission onto pyramidal neurons within the prefrontal cortex. At low DA levels, D₁ receptors are activated therefore increase interneuron excitability (Gorelova et al., 2002, Trantham-Davidson et al., 2004a, Trantham-Davidson 2004b) and D₁ agonists have been shown to enhance spontaneous (sIPSCs) inhibitory neurotransmission, indicating that the intrinsic excitability of interneurons is increased (Seamans et al., 2001). By contrast, activation of D₂ receptors during times of high DA concentrations has been shown to decrease cortical IPSCs via a postsynaptic mechanism on interneurons (Trantham-Davidson 2004b) and D₂ receptor agonists reduce the postsynaptic response to a GABA_A agonist, indicating that signaling through D₂ receptors may work via a postsynaptic mechanism to reduce the GABA tone of interneurons (Seamans et al., 2001). Other studies have shown that DA can decrease IPSCs in the prefrontal cortex by acting on presynaptic D₁ receptors likely located on interneuron terminals (Gonzalez-Islas and Hablitz et al, 2001). All of these studies suggest that dopamine interactions with interneurons within the prefrontal cortex is very complex, since it can control interneuron firing, and since these interneurons can

coordinate pyramidal neuron firing by either synchronizing them, or inhibiting other interneurons.

Evidence implicating DA in footshock-induced reinstatement of cocaine seeking comes from Capriles and colleagues (2003). This group showed that local administration of the D₁-like and not the D₂-like receptor antagonist into the PFC or the orbital frontal cortex blocked footshock-induced reinstatement (Capriles et al 2003). In addition, other findings have shown that either administration of fluphenazine (a D₁/D₂ like receptor antagonist) into the PFC (McFarland et al 2004) inhibits stress-induced reinstatement. The actions of D₁ receptors on pyramidal neurons are thought to increase the excitability of pyramidal neurons that project to the nucleus accumbens core. Within layer V of the prefrontal cortex, activation of D₁-like receptors has been shown to increase the size of the NMDA current (Seamans et al 2001). This suggests that VTA DA projections to the PFC are important in stress-induced reinstatement.

CRF may regulate the DA projection to the prelimbic cortex

Recent research suggests that stress, in part through the actions of CRF, activates the mesocortical DA system to induce reinstatement following footshock. This interaction between CRF and DA systems has been shown by both behavioral and neuroanatomical studies, which suggests that these systems converge in key brain regions known to play an important role in stress-evoked reinstatement. Early studies employed microdialysis to measure catecholamine release in the PFC and the medial hypothalamus during systemic and central administration of CRF. These studies demonstrated that intracerebroventricular CRF injections increase DA release in the PFC (Lavicky & Dunn

1993). Patch-clamp recordings from VTA slices demonstrated that CRF, through the actions of CRFR1, dose-dependently increased VTA dopamine firing (Wanat et al 2008). In addition, studies have reported that bath application of CRF to VTA DA neurons induces an increase in NMDA receptor-mediated excitatory postsynaptic potential (Ungless et al 2003). This effect is attributed to activation of CRFR2 receptors and the CRF-binding protein; this is interesting because many studies have suggested that drug seeking (Blacktop et al 2011, Boyson et al 2014, Grieder et al 2014), or food seeking during periods of stress (Chen et al 2014), is attributed to actions on CRFR1 and not CRFR2. However, some studies have suggested that CRFR2 and not CRFR1 is involved in drug-seeking behavior (Ungless et al 2003, Wang et al 2007). This difference may be attributed to different receptor localization, or it may be attributed to differences in prior drug-taking behavior (Mantsch et al 2015, Mantsch et al 2014). Interestingly, recent reports have shown that CRFR1 mediates DA release within the PFC and controls anxiety since a deletion of CRFR1 within midbrain DA neurons decreased DA release within the PFC, and increased anxiety levels in mice (Refojo et al 2011). CRF neurons that project to the VTA may contain, and therefore may co-release, glutamate onto VTA DA neurons. Electron microscopy work has shown that CRF terminals that synapse onto tyrosine hydroxylase-positive cells (TH; the precursor for DA) form asymmetric synapses and therefore most likely release glutamate (Tagliaferro & Morales 2008). This suggests that the presence of, and release of CRF during a footshock stressor may promote glutamatergic excitation of VTA DA neurons. However, as previously noted, many CRF-releasing inputs are likely GABAergic. It is not clear whether stress-induced reinstatement is dependent on DA cells that are activated by CRF to precipitate stress-

related drug use, since electron microscopy has only focused on CRF terminals that synapse onto DA neurons, and not GABAergic cells.

Dopamine within the nucleus accumbens during stress-induced reinstatement

In addition to the PFC, the nucleus accumbens receives VTA DA projections. Microdialysis studies have shown that stress can increase DA levels within the NAc shell (Kalivas & Duffy 1995, Sorg & Kalivas 1991). Temporary inactivation of the NAc core and shell with GABA receptor agonists prevents footshock-induced reinstatement (McFarland et al 2004). However, local infusion of fluphenazine (the non-specific DA antagonist) into the NAc core fails to block footshock-induced reinstatement, suggesting that activity at the DA receptors within the NAc core does not mediate relapse due to stress; however shock-induced reinstatement of heroin seeking has been reported to be blocked by a D1 receptor antagonist (Shaham et al., 1996). Moreover, it has been found that, in contrast to glutamate, DA levels in the NAc core are not changed during footshock stress-induced reinstatement of cocaine seeking (McFarland et al 2004). Taken together these studies suggest that stress-induced reinstatement may depend on activation of VTA DA neurons projecting to the PFC, which in turn stimulates glutamate release within the NAc, but not on elevated NAc DA.

Anatomy of the nucleus accumbens

Generally, the NAc, also termed the ventral striatum, is divided into two subregions, based on different cytoarchitecture, and unique functions. The first subregion is the nucleus accumbens core; this subdivision is considered an extension of the dorsal

striatum (motor pathway), which is involved in instrumental learning and reinstatement behavior, specifically during periods of stress (Cardinal & Everitt 2004, Ito et al 2000, Ito et al 2004). The NAc core receives glutamate input from the prelimbic cortex (PL); specifically layers V/VI (Gabbott et al 2005, Reynolds & Zahm 2005). This is thought to represent the final common pathway in mediating reinstatement of drug-seeking behavior in response to a cue, a stressor, or the drug itself (Kalivas & Volkow 2005). The second subregion is the nucleus accumbens shell, which is an extension of the extended amygdala; this part of the NAc is critical for the reinforcing/rewarding effects of drugs of abuse. In contrast to glutamatergic projections from the PFC to the core, PFC projections to the shell are thought to inhibit drug-seeking behavior, especially since inhibiting these projections can induce reinstatement without the presence of a cue, a stressor, or a drug (Kalivas 2005, Pierce & Kumaresan 2006). In addition, a recent study has indicated that the dorsomedial shell encodes reward, while the ventromedial shell encodes aversion in a dynorphin-dependent manner (Al-Hasani et al 2013). The shell receives glutamatergic input from the infralimbic cortex (Gabbott et al 2005, Reynolds & Zahm 2005).

Both the core and the shell have similar, but yet distinct output pathways. Both of these regions have a distinct topographical projection to the ventral pallidum and the entopeduncular nucleus (Groenewegen & Russchen 1984). The core projects to the dorsolateral part of the ventral pallidum while the shell projects to the medial part of the subcommissural ventral pallidum (Groenewegen et al 1993). The core has a midbrain projection to the substantia nigra pars reticulata (which sends input to the thalamus) (Deniau et al 1994), while the shell projects to the VTA (Ikemoto 2007). In addition, the core has some sparse projections to the lateral hypothalamus, while the shell projects

diffusely throughout the lateral hypothalamus, but mainly to the extended amygdala (Heimer et al 1991, Kelley 1999).

Medium spiny neurons within the nucleus accumbens are GABAergic and can be subdivided into either the direct or the indirect pathway based dopamine receptor expression. Although the presumed organization of NAc MSN outputs has been recently called into question (Kupchik et al., 2015), it has been suggested that one subpopulation of medium spiny neurons expresses D1R and sends projections to midbrain regions, most notably the substantia nigra, while another subset of MSNs express D2R and putatively sends projections to the ventral pallidum (although projections to the SN have been identified). It is believed that direct and indirect pathway have complementary, and possibly, opposing actions on behavior that are controlled by the cortico-accumbal pathway (Gerfen and Smeier 2011). Studies have shown that the D1R-regulated pathway increases conditioned place preference for cocaine, whereas activation of the D2R-regulated pathway decrease conditioned place preference for cocaine (Lobo et al., 2010). Studies have also shown that activating glutamatergic inputs onto subpopulations of MSNs in the NAc determine cocaine susceptibility and AMPA/NMDA ratios can be differentially altered in excitatory synapses on different MSN population in mice exposed to cocaine (Bock et al., 2012). It has been hypothesized that the activation of the “direct” (D1R) pathway causes an increase in the motivation to seek cocaine, while the “indirect” (D2R) pathway serves to decrease cocaine-seeking behavior when activated (Bock et al., 2012).

The NAc is an important brain region that serves as the gateway through which the limbic system (basolateral amygdala, VTA, PFC, and NAc) communicates with the

motor subcircuit (motor cortex, dorsal striatum, and the substantia nigra; (Groenewegen et al 1996, Kalivas 2009). Studies have found that the NAc can act to engage and implement adaptive behaviors for a desired outcome by priming the motor system (Doya 2008). For example, if a response such as activation of a lever leads to a positive outcome (such as delivery of a sucrose pellet) the NAc will engage the motor subcircuit to recognize this response. It has been proposed that as behavior becomes persistent/habitual, activation of the NAc diminishes, while the motor system becomes more organized to adapt to this behavior. However, if lever activation fails to yield an expected outcome, then the limbic subcircuit is strongly engaged, while the motor subcircuit becomes more disorganized (Kalivas 2009). This allows the limbic system to prime the motor system to maximize adaptive behavior through interactions of the NAc with the motor system. When it comes to relapse to cocaine use, the current thinking states “drug seeking arises from an impaired ability of the limbic subcircuit to effectively process and/or use the negative environmental contingencies associated with relapse” (Kalivas 2009); in other words, drug use can hijack the limbic system so that the learned behavior is never truly extinguished, and that environmental stimuli such as a footshock stressor may overcome the learned behavior of extinction and promote drug use through a mechanisms in which DA from the VTA potentiates PFC pyramidal neuron firing to activate the nucleus accumbens core.

The primary neurons of the NAc are medium spiny neurons (MSN) and they are critical for goal-directed behavior. These MSNs are mainly GABAergic. The MSNs of the NAc integrate emotional salience from the amygdala, contextual cues from the hippocampus, and executive or motor control from the PFC to produce a response by

influencing motor regions that execute motivated-behavior (Groenewegen et al 1999, Kelley 1999). Rodent models have shown that activation of NAc MSN by AMPA receptors is required for cocaine-seeking behavior, a process that is facilitated by neuroplastic changes that occur on AMPA containing synapses of MSNs with repeated cocaine use (Wolf 2010). Studies have indicated that prefrontal glutamatergic innervation of the NAc is changed with chronic drug use, in that the compulsivity of drug use in addicts becomes more desirable than natural rewards (Kalivas & Volkow 2005). This neuroadaptation to drug use is mediated by at least three different mechanisms, 1) changes in AMPA receptor expression within the NAc (e.g. an increase in Ca^{+2} permeable AMPA, the Glu-A2 lacking AMPA receptors); 2) impaired cysteine-glutamate exchange leading to a decrease in extracellular glutamate release and therefore decreased negative feedback through metabotropic glutamate receptors; 3) changes in the intrinsic excitability of MSNs (Wolf 2010). These adaptations serve to increase the excitability of MSNs. However the NAc also receives a distinct DA projection from the VTA, which may also effect the excitability of MSNs.

Dopamine in the nucleus accumbens is critical for the rewarding and stimulant effects of cocaine. It has been shown that DA infused into the NAc core will induce an increase in locomotion, which can be inhibited by inactivating the globus pallidus with injections of GABA (Jones & Mogenson 1980). Studies have shown that DA receptor agonists are self-administered by rodents, and non-human primates when given systemically, and when infused directly into the nucleus accumbens (Carlezon et al 1995, Woolverton et al 1984, Yokel & Wise 1978). Microdialysis studies have shown that both non-contingent, and contingent administration of cocaine (and other drugs of abuse)

increases dopamine levels within the NAc (Di Chiara & Imperato 1988, Di Ciano et al 1998, Di Ciano et al 1995, Imperato et al 1992, Kalivas & Duffy 1990, Kalivas & Duffy 1993, Wise et al 1995, Wise et al 2008). Interestingly, the same studies have shown that rats will administer cocaine in order to keep DA levels consistent within the NAc such that when DA levels start to decrease, the animal will self-administer more cocaine in order to keep the levels stable (Wise et al 1995). Therefore, these studies suggest that fluctuating DA levels within the NAc correlate with increased drug use.

Studies have also examined the role of the nucleus accumbens core during either cocaine self-administration, or following extinction training during reinstatement behavior. Since, intra- nucleus accumbens core administration of DA receptor antagonists increases cocaine self-administration suggesting that inhibition of DA receptors decreases the rewarding properties of cocaine (Caine et al 1995, Koob et al 1994). While administration of wither AMPA or NMDA within the nucleus accumbens during self-administration caused a significant leftward shift of cocaine responding which suggested enhancement of cocaine reward (Cornish et al 1999).

Dopamine in the NAc core does not appear to be necessary for cocaine seeking; however, glutamatergic neurotransmission in the NAc is critical (Cornish and Kalivas, 2000). Administration of either AMPA or NMDA following SA and extinction in rats reinstates drug seeking (Cornish et al 1999). At a cellular level, glutamatergic terminals originating from the prelimbic cortex, and dopamine originating from the VTA converge on the same medium spiny neurons; therefore the observation that intra-accumbens AMPA administration could reinstate cocaine-seeking behavior could be explained by AMPA directly activating MSN—Since numerous studies have shown that DA release is

increased within the prelimbic cortex during stress-related behaviors (McFarland et al 2004, Moghaddam 1994, Refojo et al 2011, Thierry et al 1976), and that this increase in DA modulates pyramidal neuron fringing into the nucleus accumbens it is hypothesized that increases in glutamatergic transmission in the nucleus accumbens driven in part by elevated DA in the prelimbic cortex is an important factor for initiating relapse to drug taking; this has been shown to be involved in drug-primed reinstatement since an AMPA antagonist, and not a DA antagonist infused into the nucleus accumbens prevents reinstatement (Cornish & Kalivas 2000).

Neurocircuitry involved in stress-induced drug seeking

The use of a rodent reinstatement model of drug relapse is extremely important in understanding the neurobiological pathways that mediate drug seeking during periods of stress. This is especially important since the vulnerability to relapse due to a stressor persists years after the cessation of drug use. This dissertation will explore different aspects of this pathway to understand how stress triggers relapse, and hopefully provide aid in the development of therapeutics that can better manage or prevent drug use that is stress-related. Chapter 2 will test the hypothesis that beta-2 ARs are required for stress-induced activation of CRF pathways responsible for reinstatement. Work done in our lab, and others, has suggested that noradrenergic receptors (Leri et al., 2002), specifically beta-2 ARs (Mantsch et al., 2010; Vranjkovic et al., 2012) may be critically involved in the interaction with the CRF system. Second, the self-administration model will be used to test the hypothesis that stress-induced reinstatement is dependent upon a beta-2 AR regulated vBNST-to-VTA CRF-releasing pathway, wherein stress-induced CRF will

activate CRFR1 on VTA neurons. Finally, this dissertation will explore how excessive cocaine use establishes susceptibility to stress-induced relapse by recruiting CRF regulation of a key stressor-responsive mesocortical dopaminergic pathway.

Chapter II

Beta-2 adrenergic receptors mediate stress-evoked reinstatement of cocaine-induced conditioned place preference and increases in CRF mRNA in the bed nucleus of the stria terminalis in mice

Abstract

Rationale: Understanding the mechanisms responsible for stress-induced relapse is important for guiding treatment strategies aimed at minimizing the contribution of stress to addiction. Evidence suggests that these mechanisms involve interactions between noradrenergic systems and the neuropeptide corticotropin-releasing factor (CRF).

Objectives: The interaction between beta-adrenergic receptors (ARs) and CRF as it relates to the reinstatement of cocaine-conditioned reward in response to a stressor was examined in mice. We hypothesized that beta 2-ARs are required for stress-induced activation of CRF pathways responsible for reinstatement.

Methods: Stress-induced relapse was examined based on the re-establishment of cocaine-induced conditioned place preference (CPP; 4×15 mg/kg cocaine, i.p.) after extinction using forced swim (6 min at 22 °C) or an injection of the beta 2-AR agonist, clenbuterol (4 mg/kg, i.p.). The CRF-R1 antagonist antalarmin (10 mg/kg, i.p.) or the beta 2-AR antagonist ICI- 118,551 (1 mg/kg, i.p.) were given 30 min prior to reinstating stimuli. Quantitative PCR was conducted in dissected bed nucleus of the stria terminalis (BNST) and amygdala, putative sources of CRF that contribute to reinstatement, to examine the effects of ICI-118,551 on swim-induced increases in CRF messenger RNA (mRNA) in mice with a cocaine history.

Results:

Pretreatment with ICI-118,551 or antalarmin blocked swim-induced reinstatement of CPP. Reinstatement by clenbuterol was also blocked by antalarmin. ICI-118,551 pretreatment prevented swim-induced increases in CRF mRNA in the BNST. Effects in the amygdala were not observed. Conclusions These findings indicate that, during stress, norepinephrine, via beta 2-ARs, either directly or indirectly activates CRF-releasing neurons in the BNST that interface with motivational neurocircuitry to induce reinstatement of cocaine conditioned reward.

Introduction

Understanding the mechanisms through which stress promotes relapse to drug use by cocaine addicts continues to be a critical research objective. Outcome measures for current treatment approaches aimed at relapse prevention remain poor, and stress is a pervasive trigger for relapse as it is typically unavoidable in daily life. In addition, there is currently no FDA-approved medication for cocaine addiction, making it a critical unmet need. Stress-induced relapse to cocaine use can be modeled in mice using a cocaine-induced conditioned place preference (CPP) approach in which, after extinction, preference can be re-established (i.e., reinstated) using stressors such as forced swim (Kreibich et al 2009, Mantsch et al 2010).

A role for noradrenergic signaling in stress-induced reinstatement of cocaine-conditioned reward has been established. Using the CPP/reinstatement approach in mice, our laboratory has found that functional antagonism of noradrenergic neurotransmission

via administration of the α 2-adrenergic receptor (AR) agonist clonidine blocks swim-induced reinstatement of extinguished CPP (Mantsch et al 2010), while disinhibition of noradrenergic activity via administration of yohimbine or the highly selective α 2-AR antagonist BRL 44,408 is sufficient to reinstate (Mantsch et al 2010, Vranjkovic et al 2012). These findings parallel those in rats demonstrating α 2-adrenergic AR agonist blockade of shock-induced reinstatement following cocaine self-administration (SA; (Erb et al 2000)) and sufficiency of central administration of norepinephrine or systemic delivery of yohimbine for reinstatement of cocaine seeking (Brown et al 2011, Brown et al 2009, Feltenstein & See 2006). More importantly, they are consistent with reports that α 2-AR agonists attenuate drug craving in human cocaine addicts upon presentation of stress-associated imagery (Jobes et al 2011).

Stress-induced increases in noradrenergic activity appear to induce reinstatement of cocaine-conditioned reward in part via activation of beta-AR. We have found that swim-induced reinstatement of cocaine-induced CPP is blocked by the nonselective β -AR antagonist propranolol (Mantsch et al 2010) and is not observed in beta-AR-deficient mice (Vranjkovic et al 2012) while the non-selective beta-AR agonist isoproterenol induces reinstatement (Vranjkovic et al 2012). More specifically, our findings suggest that beta 2-ARs are necessary for stress-induced reinstatement of cocaine-conditioned reward, as swim-induced reinstatement of CPP is blocked by the selective beta 2-AR antagonist ICI-118,551 and administration of the selective beta 2-AR agonist clenbuterol is sufficient for reinstatement of CPP (Vranjkovic et al 2012).

The sites at which stress-induced increases in beta-AR activation promote reinstatement behavior appear to include the bed nucleus of the stria terminalis (BNST)

and central amygdala (CeA), both of which receive dense noradrenergic projections (Ricardo & Koh 1978, Woulfe et al 1988), express beta-ARs (Asanuma et al 1991, Cecchi et al 2007, Rainbow et al 1984), and are required for stress-induced reinstatement of lever pressing following SA in rats (Erb et al 2001, Leri et al 2002, McFarland et al 2004) or cocaine-induced CPP in mice (Briand & Blendy 2010). In rats, delivery of a cocktail of beta 1- and beta 2-AR antagonists directly into either the BNST or the CeA prevents shock-induced reinstatement of cocaine seeking following self-administration (Leri et al 2002). The BNST and CeA are components of the extended amygdala and serve as key interfaces between the stress and reward systems. Both regions are highly interconnected with the mesocorticolimbic system through which they likely regulate drug-seeking behavior and reinstatement of cocaine-conditioned reward, although the precise pathways and mechanisms through which this regulation occurs are not fully understood.

The link between the mesocorticolimbic circuitry that has been implicated in relapse and beta-AR actions in the BNST and amygdala likely involves the neuropeptide, corticotropin releasing factor (CRF). CRF is a key mediator of stress induced reinstatement of cocaine seeking following SA in rats (Mantsch et al 2008) (Buffalari et al 2012, Erb et al 2006, Erb et al 1998, Graf et al 2011, Shaham et al 1998) and has been reported to contribute to stress-induced reinstatement of lever pressing following SA via actions in the BNST (Erb et al 2001) and the ventral tegmental area (VTA; (Blacktop et al 2011, Wang et al 2005). In the BNST, evidence suggests that stress-induced increases in CRF arise in part from local release from intrinsic cell populations (Silberman et al 2013). Moreover, CRF release into the VTA is thought to involve projections from the BNST

and the CeA (Rodaros et al 2007). In light of the requirement for beta-AR signaling in these regions for stress-induced cocaine seeking in rats following SA (Leri et al 2002) and evidence suggesting CRF-dependence of beta-AR actions in BNST (Nobis et al 2011, Silberman et al 2013), we hypothesized that beta 2-AR activation is required for stress-induced regulation of CRF releasing neurons in one or both of these regions and for relapse to drug use during periods of stress. In support of this hypothesis, it has been reported that reinstatement in response to central norepinephrine is CRF-dependent (Brown et al 2009).

Here, we further test this hypothesis by (1) investigating the ability of the CRF-R1 receptor antagonist, antalarmin, to prevent reinstatement of cocaine-induced CPP in mice by a stressor, forced swim, and administration of the beta 2-AR agonist clenbuterol and (2) testing for the ability of the beta 2-AR selective antagonist ICI-118,551, which blocks swim-induced cocaine-conditioned reward, to also prevent swim-induced activation of CRF neurons in the BNST and amygdala, as assessed by increases in CRF mRNA measured using qPCR quantification in dissected tissue from mice with a prior history of cocaine administration.

Methods

Subjects: A total of 116 male C57BL/6 mice (8–10 weeks old; 20 mice for CPP testing and 96 for qPCR testing), purchased from Harlan Laboratories, were housed individually in a humidity- and temperature-controlled, AAALAC-accredited animal facility under a 12 h/12 h light/dark cycle (lights on at 0700 hours) with food and water

available ad libitum, except when in the experimental chambers. All procedures were carried out in compliance with the NIH guidelines and the Guide for Care and Use of Laboratory Animals and were approved by Marquette University.

Conditioned place preference apparatus: Behavioral testing was conducted as previously described (Mantsch et al. 2010; Vranjkovic et al. 2012). Six ENV-3013 mouse CPP chambers from Med Associates, Inc. were used. The stainless-steel and polyvinyl chloride chambers consisted of three distinct compartments separated by 5 cm wide×5.9 cm high manual guillotine doors. The two 46.5×12.7×12.7-cm side compartments consisted of a white compartment with a 6.35×6.35-mm stainless-steel mesh floor and a black compartment with a stainless-steel grid rod floor consisting of 3.2-mm rods spaced 7.9 mm apart. The side compartments were attached via a gray colored 7.2-cm-long center compartment with a smooth floor. The clear tops of the compartments were hinged to permit placement and removal of the mice. Ceiling lights were attached to each top. To balance unconditioned side preferences, only the light in the black compartment was illuminated during the training, testing, and extinction phases. Automated data collection was accomplished by using photobeams (six beams for the white and black test areas and two beams for the center gray area) that were evenly spaced across the length of the chamber and interfaced with a computer containing MED-PC software (MED Associates). Using this automated photobeam system, entry into a side compartment was defined as consecutive breaks of the first two-photocell beams in that compartment located adjacent to the door separating that compartment from the center compartment. Exiting of a side compartment (and entry into the center compartment) was indicated by occlusion of the beams in the center compartment.

Cocaine-induced conditioned place preference: Cocaine-induced conditioned place preference (CPP) was conducted using an unbiased approach by pairing one compartment with cocaine and the other with saline as previously described (Mantsch et al. 2010). These assignments were made randomly. On the first day of CPP, mice were placed into the center compartment of the chamber and given free access to all three compartments for 30 min in the absence of cocaine or saline to determine preconditioning preference. During the 8-day conditioning phase of the experiment, mice received cocaine (15 mg/kg, i.p.) on the odd days and saline injections on the even days. Immediately after the injection, mice were confined (guillotine door closed) to the drug-appropriate compartment for 30 min. A day after the final conditioning session, the mice were tested for the expression of cocaine-induced CPP by placing them in the center compartment and allowing them full access to the apparatus for 30 min. CPP was defined as the change in time spent (in seconds) in the cocaine-paired compartment after conditioning compared with the initial preconditioning session. Mice were determined to exhibit a CPP if they spent more time in the cocaine-paired compartment during the post-conditioning session compared to the preconditioning session.

Extinction: Daily extinction training was conducted after conditioning. During the extinction sessions, the mice had free access to the entire apparatus for 30 min following placement in the center compartment with both guillotine doors open. Mice underwent daily extinction training until the extinction criterion was met (50 % reduction in the preference for the cocaine-paired compartment). Mice were tested for reinstatement 24 h after this criterion was reached.

Reinstatement: Aside from exposure to a reinstating stimulus, the reinstatement test sessions were identical to extinction conditions: mice were provided free access to the entire apparatus for 30 min following placement in the center compartment with both guillotine doors open. Most mice were tested multiple times for reinstatement. The sequence of reinstatement test conditions was counter-balanced using a Latin square design. The mice underwent additional extinction prior to each subsequent test and were not tested again until the criterion for extinction was once again reached. Reinstatement was defined as the difference in the time spent (in seconds) in the cocaine-paired compartment between the reinstatement test day and the prior extinction day.

Drugs: Cocaine HCl (15 mg/kg) was obtained from the National Institute on Drug Abuse (NIDA) through the NIDA Drug Supply Program. The CRF-R1 antagonist antalarmin (10 mg/kg), the beta 2-adrenergic receptor (AR) agonist clenbuterol (4 mg/kg), and the beta 2-AR antagonist ICI-118,551 (1 mg/kg) were purchased from Sigma-Aldrich. Cocaine, clenbuterol, and ICI-118,551 were dissolved in saline (0.9 % bacteriostatic saline). Antalarmin was dissolved in 5 % DMSO. All drugs were administered i.p. in a volume of 0.1 ml per 25 g body weight.

Experiment 1: role of beta-2 AR and CRF-R1 receptors in stress-induced reinstatement of CPP

Forced swim-induced reinstatement Stress-induced reinstatement was induced using a forced swim (FS) protocol as previously reported (Kreibich & Blendy 2004, Mantsch et al 2010). Briefly, mice were placed into a 30 cm high × 20 cm deep cylindrical polypropylene container filled with water (20–25 °C) for 6 min. After the forced swim, mice were placed back into their home cages for 3 to 4 min before being introduced into

the center compartment of the CPP apparatus with free access to the entire apparatus for reinstatement testing, as described above.

The role of beta 2-ARs and CRF-R1 receptors in swim-induced reinstatement We have previously reported that either pharmacological blockade of the β 2-AR or genetic beta 2-AR deletion prevents stress-induced, but not cocaine-induced, reinstatement of extinguished cocaine-induced CPP in mice (Mantsch et al. 2010; Vranjkovic et al. 2012). To confirm the role of beta 2-ARs in swim-induced reinstatement and to determine the role of CRF-R1 receptors, mice ($n = 8$) underwent cocaine-induced CPP and extinction as described above before testing for swim-induced reinstatement following pretreatment with the beta 2-AR antagonist ICI-118,551 (1 mg/kg i.p.), the CRF-R1 antagonist antalarmin (10 mg/kg i.p.), or vehicle. The mice received injections 30 min prior to forced swim. The sequence of the treatments was counter-balanced using a Latin square design.

Experiment 2: effects of CRF-R1 antagonism on beta 2-AR agonist-induced reinstatement

We previously reported that the beta-2-AR agonist clenbuterol reinstates extinguished cocaine-induced CPP (Vranjkovic et al. 2012). To determine if the beta 2-AR induces cocaine- conditioned reward via a CRF-dependent mechanism, the mice ($n = 12$) were tested for reinstatement in response to administration of the beta 2-AR agonist clenbuterol (4 mg/kg, i.p.) or vehicle 30 min after pretreatment with CRF-R1 receptor antagonist antalarmin (10 mg/kg, i.p.; Kreibich et al., 2009) or vehicle. The mice were introduced into the experimental chambers 30 min after clenbuterol administration and tested for reinstatement. The sequence of the treatments was counter-balanced using a

Latin square design. Notably, we have found that reinstatement of extinguished CPP by this dose of clenbuterol is not attenuated by pretreatment with the beta-1 AR antagonist, betaxolol (10 mg/kg, i.p.), suggesting that its agonist actions are selective for the beta-2 AR and/or that beta-1 AR, if activated, do not contribute to reinstatement (Ext: 558.28 ± 72.45 ; Clen/Betax: 1075.11 ± 94.03 ; Significant reinstatement, paired t test; $p < 0.001$).

Experiment 3: role of beta 2-ARs in swim-induced effects on crf mRNA in the BNST and amygdala

In order to examine the role of beta 2-ARs on stress-induced CRF mRNA, mice received an injection of cocaine (15 mg/kg, i.p.) or saline on alternating days for 8 days. Following the cocaine treatment, mice were given an 8-day withdrawal period before exposure to the stressor. Mice were pretreated with either ICI-118,551 (1 mg/kg, i.p.) or vehicle 30 min prior to forced swim. After forced swim, the mice were placed back in their home cages for 30 min prior to sacrifice. The remaining mice did not undergo forced swim but instead were euthanized 60 min after the ICI-118,551 or vehicle injection. The groups were as follows: vehicle, vehicle + forced swim, ICI, ICI + forced swim.

Tissue extraction: Mice were killed by cervical dislocation and their brains were rapidly removed and flash frozen by submersion for 30 s in a beaker filled with 2-methylbutane sitting in dry ice and brains were then stored at -80°C . A cryostat was used to cut one 500- μm -thick coronal section from frozen brain tissue at the level of the BNST (+0.45 to -0.05 mm from Bregma; **Fig. 2.3a**), and tissue punches were taken from the BNST using a tissue punch kit (1.00 mm in diameter), with hemispheres pooled into one sample for each mouse. In the same brain, a cryostat was used to cut two 500- μm -thick coronal

sections from frozen brain tissue at the level of the amygdala (−0.82 to −1.82 mm from Bregma; **Fig. 2.3b**), and tissue punches were taken from the amygdala, with hemispheres pooled into one sample for each mouse. The tissue punches were stored at −80 °C for later quantitative real-time polymerase chain reaction (qPCR) analysis.

RNA was extracted from frozen brain punches using a phenol: chloroform method. Due to the small amount of tissue, tissue from two mice was pooled for each sample. Briefly, tissue punches were homogenized in Trizol using a 20 ga. followed by a 25 ga. needle. Homogenized samples were incubated at room temperature with chloroform and then centrifuged at 11,000×g for 15 min. The upper aqueous phase was separated into a new tube and isopropanolol was added. Following incubation at room temperature, samples were centrifuged at 12,000×g for 10 min. The isopropanolol was poured off and samples were washed twice in 70 % ethanol followed by centrifugation at 12,000×g for 5 min. The pellet was finally stored in 70 % ethanol at −20 °C.

Reverse transcription and qPCR: For complementary DNA (cDNA) production, the ethanol was poured off and the RNA pellet was allowed to air dry before being re-suspended in RNase free water. Samples were then treated with DNase (Invitrogen) to remove genomic DNA. Following DNase treatment, cDNA was created using Oligo(dT) primers, and a Superscript II reverse transcription kit (Invitrogen). cDNA was diluted 1:3 and stored at −20 °C for qPCR analysis. All qPCR was run using the StepOne real-time PCR system (Applied Biosystem). For qPCR analysis, reactions containing PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences), 20 μM of forward and reverse primers, and cDNA were loaded into the wells of a 48-well plate on ice. The cycling parameters were 95 °C for 30s followed by 40cycles of 95°C (5s), 60°C (15s), and 70°C

(10s) followed by a melting curve to ensure amplification of a single product. All reactions were performed in triplicate and the mean threshold cycle for each gene product for each sample was used for analysis. The mRNA levels of CRF were normalized to the housekeeping gene, TATA binding protein (TBP). Primer sequences were generously provided by Dr. Julie Blendy (Cleck et al 2008).

Results

Prior to testing for reinstatement, mice underwent CPP and extinction as previously described (Mantsch et al. 2010). All mice displayed cocaine-induced CPP and met criteria for extinction. Five mice were excluded from testing because they did not display CPP (two mice) or did not extinguish (three mice). The mean time spent in the cocaine-paired compartment prior to and after conditioning and during extinction for each CPP experiment (Exp. 1 and 2) is shown in Table 2.1.

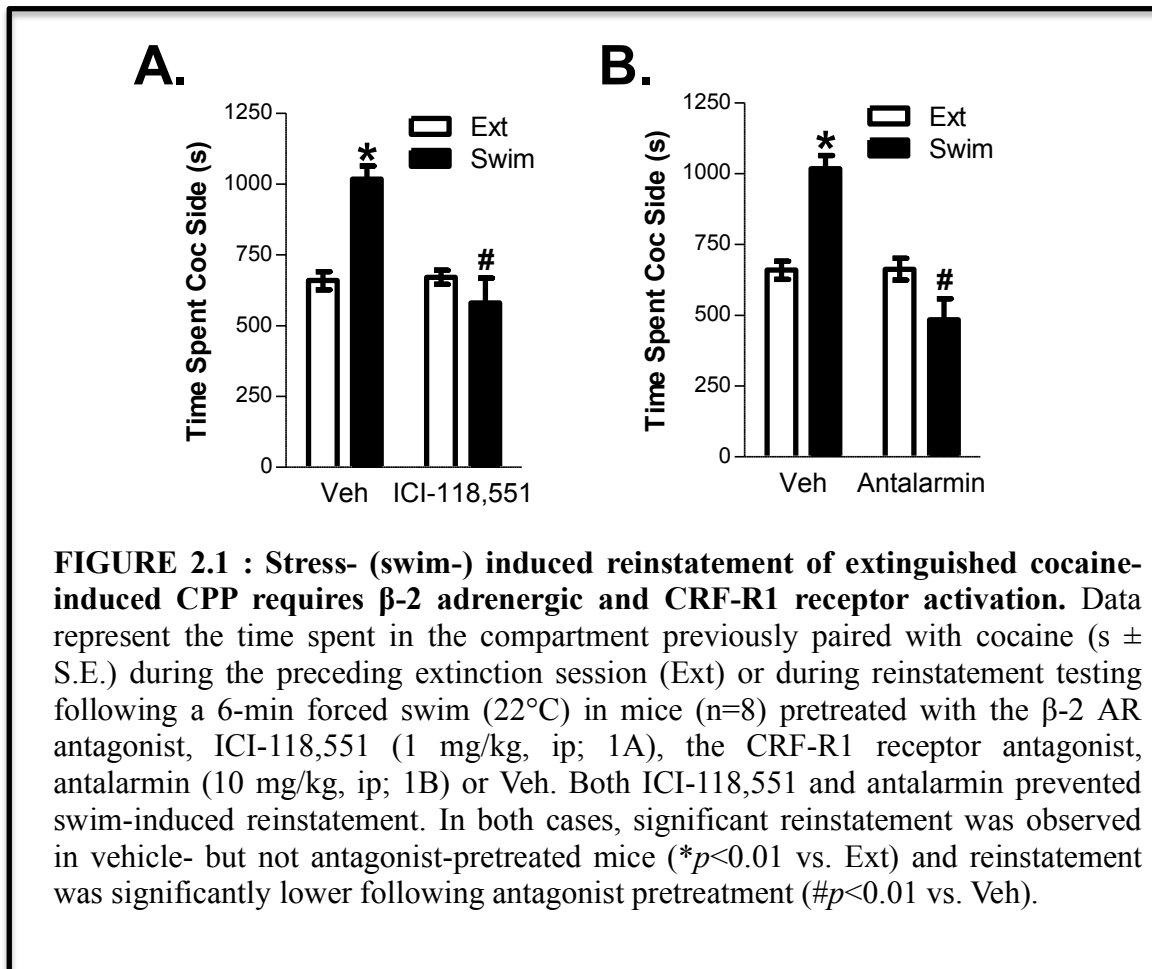
TABLE 1 Time Spent in Cocaine-Paired Compartment (sec \pm S.E)

	<i>Pre-Cond.</i>	<i>Post-Cond</i>	<i>First Ext</i>	<i>Last Ext</i>
<i>Experiment 1</i>	528.12	957.47*	927.34	639.84**
(n= 8)	(\pm 106.68)	(\pm 37.99)	(\pm 120.56)	(\pm 33.10)
<i>Experiment 2</i>	558.58	995.80*	1012.45	573.97**
(n= 12)	(\pm 72.59)	(\pm 80.67)	(\pm 167.34)	(\pm 53.85)

Table 2.1: Cocaine-induced conditioned place preference (CPP) and extinction. Mice underwent CPP using an unbiased 8-day (4 x alternating 15 mg/kg, ip cocaine and vehicle administration) approach. Data represent the mean time (sec \pm S.E.) spent in the cocaine compartment prior to conditioning (Pre-Cond.), after conditioning (Post-Cond.), and on the first and last (prior to the first reinstatement test) days of extinction (First and Last Ext) in mice used for Experiments 1 and 2. Mice used in both experiments displayed significant CPP (* p <0.01; paired t-test Post-Cond. vs. Pre-) and showed extinction (** p <0.001; paired t-test, First Ext. vs. Last).

Experiment 1: role of beta 2-AR and CRF-R1 receptors in stress-induced reinstatement of CPP

For this experiment, mice (n=8) were tested for the effects of vehicle, the CRF-R1 antagonist antalarmin, and the beta 2-AR antagonist ICI-118,551 on reinstatement of extinguished CPP following forced swim. The pretreatments in combination with forced swim were tested in counterbalanced sequence determined using a Latin square design. The effects are shown in **Fig. 2.1**.



1.1: Effects of the beta 2-AR antagonist, ICI-118,551, on stress- induced reinstatement of CPP. We previously reported that the beta 2-AR antagonist, ICI-118,551, prevented swim-induced re- instatement of CPP in mice (Vranjkovic et al. 2012). This effect of ICI-118,551 (1 mg/kg, i.p.) was also observed in the present study (**Fig. 2.1a**). Consistent with our earlier report, two- way repeated measures ANOVA showed significant overall effects of forced swim ($F(1,7)=22.423$; $p<0.01$) and ICI- 118,551 ($F(1,7) = 8.829$; $p < 0.05$) and a significant interaction between swim-induced reinstatement and ICI-118,551 pre- treatment ($F(1,7) = 14.272$; $p < 0.01$). ICI-118,551 prevented swim-induced reinstatement. Post hoc testing showed that significant reinstatement was observed in

vehicle but not ICI-118,551 pretreated mice ($p < 0.01$ vs. the prior extinction session) and was significantly reduced following ICI-118,551 pretreatment ($p < 0.01$ vs. vehicle).

1.2: Effects of the CRF-R1 receptor antagonist, antalarmin on stress-induced reinstatement of CPP The neuropeptide CRF has also been implicated in stress-induced cocaine seeking (Erb et al 1998, Shaham et al 1998). To test for the involvement of CRF in swim-induced reinstatement of CPP, mice received pretreatment with the CRF-R1 receptor antagonist, antalarmin (10 mg/kg, i.p.). Antalarmin prevented reinstatement following forced swim (**Fig. 2.1b**). Two-way repeated measures ANOVA showed significant overall effects of forced swim ($F(1,7) = 5.710$; $p < 0.05$) and antalarmin ($F(1,7) = 13.642$; $p < 0.01$) and a significant interaction between swim-induced reinstatement and antalarmin pretreatment ($F(1,7) = 67.375$; $p < 0.01$). Post hoc testing showed that significant reinstatement was observed in vehicle-pretreated but not antalarmin-pretreated mice ($p < 0.01$ vs. extinction) and was significantly reduced following antalarmin pretreatment ($p < 0.01$ vs. vehicle).

Experiment 2: effects of CRF-R1 antagonism on beta 2-AR agonist-induced reinstatement

We previously reported that the administration of beta 2-AR agonist, clenbuterol (4 mg/kg, i.p.), is sufficient to reinstate extinguished cocaine-induced CPP in mice (Vranjkovic et al. 2012). Here, we demonstrate that, like forced swim, clenbuterol-induced reinstatement is blocked by pretreatment with the CRF-R1 receptor antagonist, antalarmin. Pretreatment with antalarmin (10 mg/kg, i.p.) prevented clenbuterol-induced reinstatement (**Fig. 2.2**).

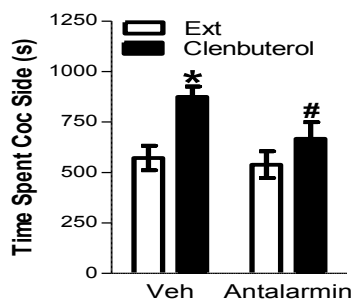


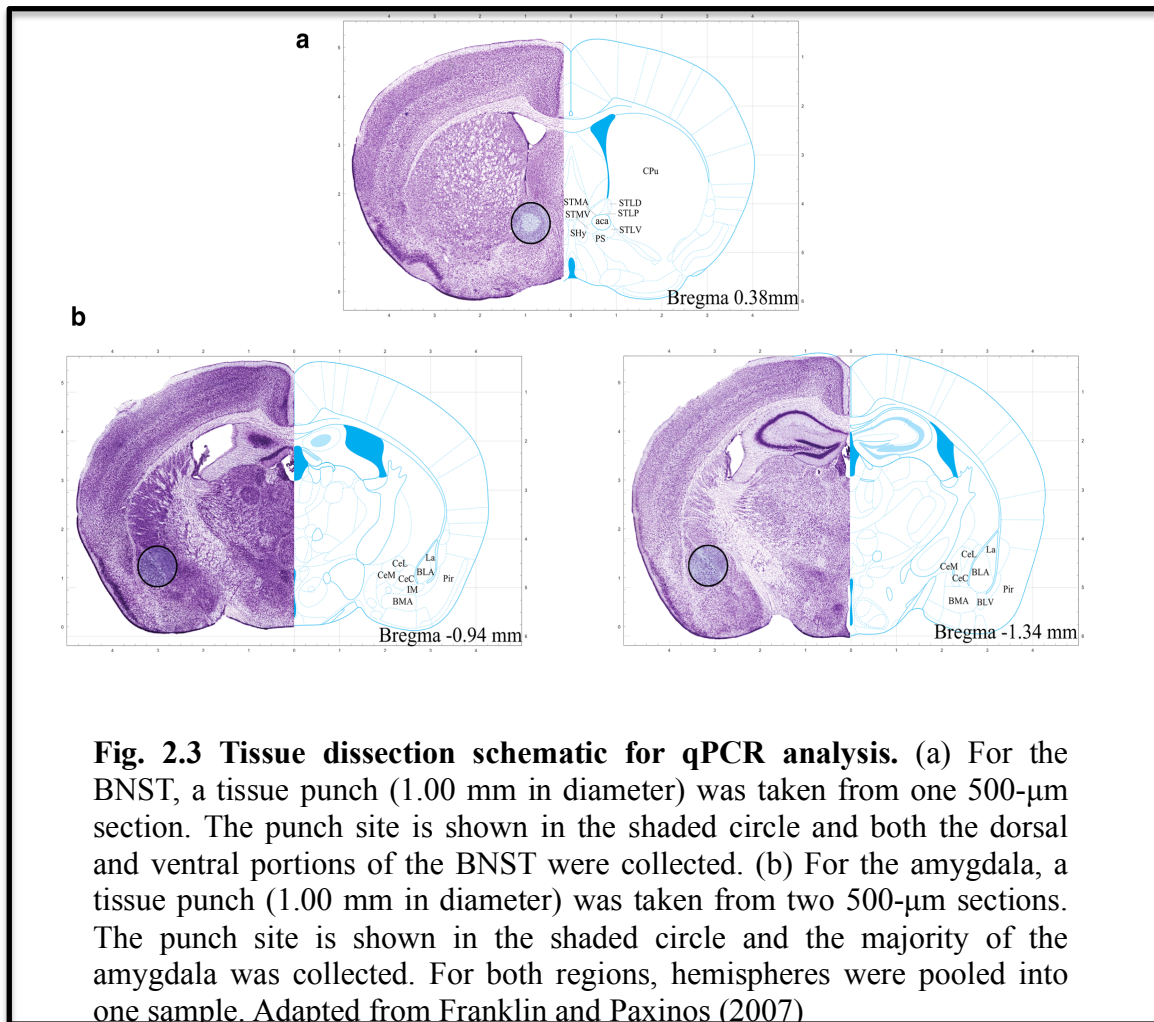
FIGURE 2.2: Clenbuterol-induced reinstatement is significantly decreased by pretreatment with the CRF-R1 receptor antagonist, antalarmin. Data represent the time spent in the compartment previously paired with cocaine ($s \pm$ S.E.) during the preceding extinction session (Ext) or during reinstatement testing following administration of clenbuterol (4 mg/kg, ip) after pretreatment with the CRF-R1 receptor antagonist, antalarmin (10 mg/kg, ip; $n=9$) or vehicle ($n=12$). Significant reinstatement was observed in vehicle- but not antalarmin-pretreated mice ($*p<0.01$ vs. Ext) and reinstatement was significantly lower following antalarmin pretreatment ($\#p<0.05$ vs. Veh).

Twelve mice were tested for reinstatement following vehicle pretreatment. Nine were tested for reinstatement following antalarmin. Since some mice were not tested for effects of both vehicle and antalarmin on clenbuterol-induced reinstatement, a mixed two-way clenbuterol reinstatement (repeated measure) \times antalarmin pretreatment condition (between subjects measure) ANOVA was used. A significant overall effect of clenbuterol administration ($F(1,19)=26.335$; $p<0.001$) and a significant interaction between antalarmin pretreatment and clenbuterol-induced reinstatement ($F(1,19) = 4.294$; $p=0.050$) were observed. Antalarmin prevented clenbuterol-induced reinstatement. Significant reinstatement was observed in vehicle-pretreated but not antalarmin-pretreated mice ($p < 0.01$ vs. extinction), and time spent in the cocaine-paired

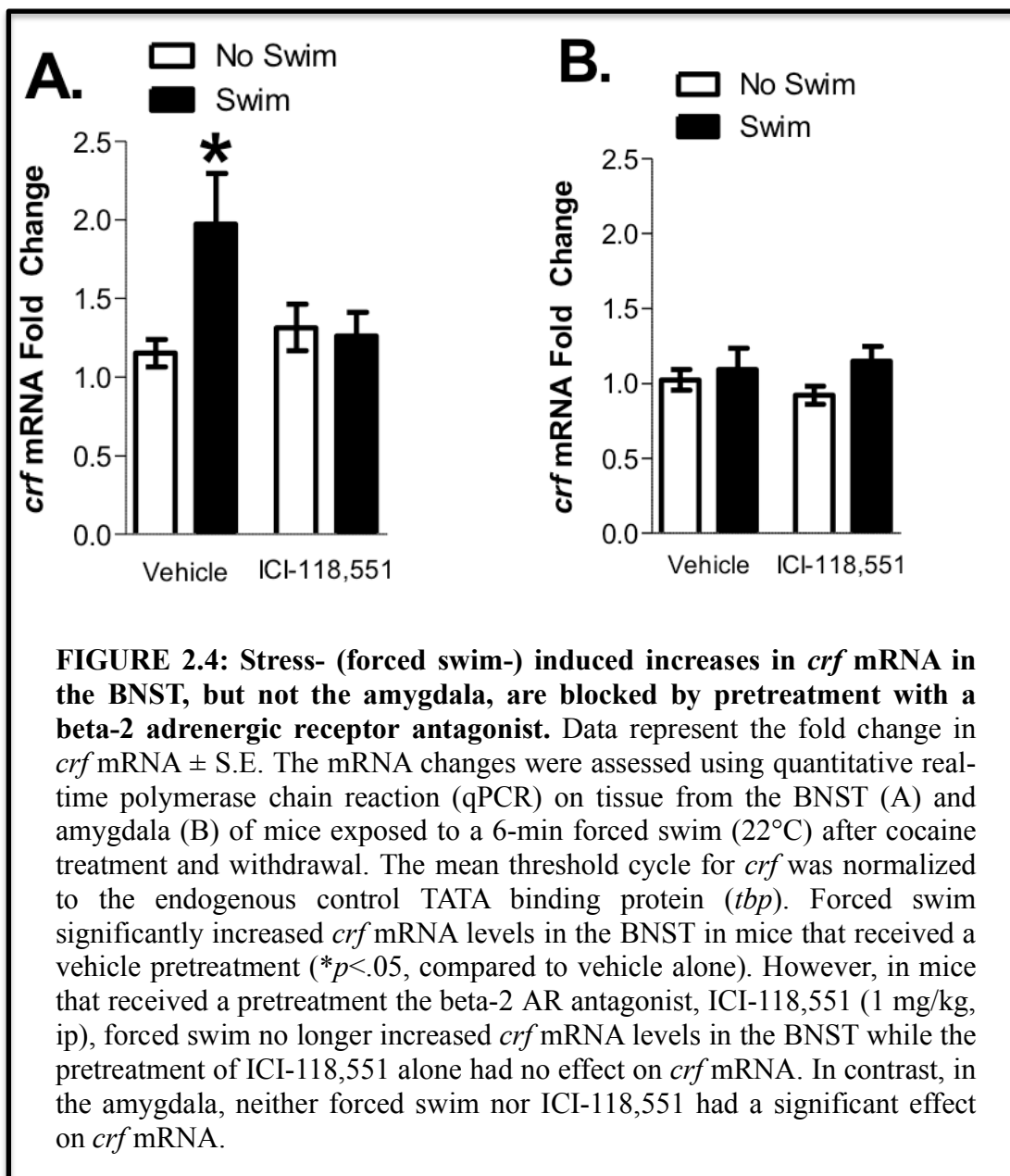
compartment was increased in mice pretreated with vehicle compared to mice pretreated with antalarmin ($p=0.05$).

Experiment 3: Role of beta 2-ARs in stress-induced effects on CRF mRNA in the BNST and amygdala

Since reinstatement of CPP following administration of the beta 2-AR agonist, ICI-118,551, was dependent on CRF receptor activation, we hypothesized that beta 2-AR receptors regulate, either directly or indirectly, CRF-producing neurons in brain regions previously implicated in CRF-dependent stress-induced cocaine seeking. To test this hypothesis, we quantified CRF mRNA in the BNST and amygdala dissected from cocaine-treated mice 30 min following the termination of a 6-min forced swim using qPCR after pretreatment with ICI-118,551 (1 mg/kg, i.p.) or vehicle. Schematics depicting the areas targeted for dissection are included in **Fig. 2.3**.



3.1: Effects on crf mRNA in the BNST: The BNST is involved in stress-induced cocaine seeking following SA in rats (Leri et al 2002, McFarland et al 2004), and both beta -AR signaling and CRF actions in the BNST are important for stress-induced reinstatement (Erb & Stewart 1999, Leri et al 2002, Wang et al 2006), suggesting that the BNST is a critical site in which beta 2-ARs can either directly or indirectly regulate CRF. Here, we examined the effect of pretreatment with the beta 2-AR antagonist ICI-118,551 (1 mg/kg, i.p.) on swim-induced levels of CRF mRNA in the BNST following cocaine treatment and withdrawal (**Fig. 2.4a**).



Two-way ANOVA did not show a significant main effect of forced swim ($F(1,36)=3.737$, $p=0.061$) or ICI-118,551 ($F(1,36)=1.919$, $p=0.174$) on CRF mRNA levels in the BNST but did show a significant interaction between forced swim and ICI-118,551 ($F(1,36)=4.851$, $p<0.05$). Mice that received vehicle pretreatment and forced swim had significantly higher CRF mRNA levels in the BNST compared to the vehicle

treatment alone ($p < 0.05$). However, when mice were pretreated with ICI-118,551, forced swim no longer increased CRF mRNA levels in the BNST ($p > 0.05$ compared to vehicle treatment), while the treatment with ICI-118,551 alone had no significant effect on CRF mRNA levels ($p > 0.05$, compared to vehicle treatment alone).

3.2: Effects on crf mRNA in the amygdala Like the BNST, the amygdala has been implicated in stress-induced cocaine seeking following SA in rats (McFarland et al 2004). Moreover, beta-AR signaling in the central nucleus of the amygdala contributes to stress-induced reinstatement (Leri et al 2002) and forced swim following protracted withdrawal after binge cocaine access has been reported to increase CRF mRNA levels in the amygdala (Cleck et al 2008). Thus, the amygdala is a potential site at which CRF is either directly or indirectly regulated by beta 2-ARs to promote cocaine use. Here, we examined the effect of the beta 2-AR antagonist ICI-118,551 (1 mg/kg, i.p.) on swim-induced levels of CRF mRNA in the amygdala following cocaine treatment and withdrawal (**Fig. 2.4b**). In contrast to the BNST, a two-way ANOVA did not show a significant main effect of forced swim ($F(1,39) = 2.033$, $p = 0.162$), ICI-118,551 ($F(1,39) = 0.046$, $p = 0.83$), or an interaction between forced swim and ICI-118,551 on CRF mRNA in the amygdala ($F(1,39) = 0.558$, $p = 0.459$).

Discussion

The main finding of this study is that an interaction between beta 2-ARs and CRF-R1 receptors is critical for stress-induced reinstatement of extinguished cocaine-induced CPP in mice. Additionally, beta 2-AR signaling is required for the stress-

induced regulation of CRF mRNA levels in the BNST. We found that not only is CRF-R1 activation necessary for swim induced reinstatement of cocaine-induced CPP but it is also necessary for reinstatement in response to the beta 2-AR agonist clenbuterol, suggesting that beta 2-AR signaling is upstream from CRF actions in the neural pathway responsible for stress-induced reinstatement of cocaine-conditioned reward. In support of this hypothesis, we also found that forced swim can significantly increase CRF mRNA levels in the BNST, a region necessary for stress-induced reinstatement, and that this effect can be prevented by pretreatment with the beta 2-AR antagonist ICI-118,551. Surprisingly, this effect does not occur in the amygdala. Taken altogether, these data raise the possibility that beta 2-AR-dependent activation of CRF-expressing neurons in the BNST mediates stress-induced reinstatement of cocaine-conditioned reward, a process that likely contributes to relapse.

The BNST is an important site for integration of stress and reward networks (Flavin & Winder 2013). The BNST receives heavy innervation from most subregions of the amygdala (Dong et al 2001a, Weller & Smith 1982), a structure known to be involved in stress, fear and anxiety, and projects to regions involved in neuroendocrine, autonomic and behavioral control (Dong & Swanson 2006) such as the PVN and the amygdala (Cullinan et al 1993, Dong et al 2001a, Dong et al 2001b). In addition to its connectivity with stress-related structures, BNST efferent projections target regions involved in motivation and reward, such as the nucleus accumbens (Dong et al 2001b) and the VTA (Georges & Aston-Jones 2001, Georges & Aston-Jones 2002). The BNST is activated during stress-induced reinstatement (Briand et al 2010, Brown et al 2011) and is necessary for stress-induced reinstatement in mice and rats (Briand & Blendy 2010,

Erb & Stewart 1999, Leri et al 2002, McFarland et al 2004). In addition, it is an important region for CRF actions that mediate stress-induced reinstatement following SA, as CRF-R1 activation in the BNST is both necessary (Erb et al. 2001; Erb and Stewart 1999) and sufficient (Erb and Stewart 1999) for reinstatement, and stress can increase CRF mRNA in the BNST in rats (Funk et al 2006). Indeed, our own findings support this hypothesis by demonstrating that CRF-R1 receptor activation is necessary for forced swim induced reinstatement of CPP in mice and that the same stressor increases CRF mRNA levels in the BNST of mice with a prior history of cocaine, albeit in the absence of CPP. These data suggest that the BNST is a major site of regulation of CRF actions during stress-induced reinstatement of cocaine-conditioned reward.

In addition to CRF, the noradrenergic system plays an important role in reward and stress (Flavin & Winder 2013, Weinshenker & Schroeder 2007). In particular, this system is heavily involved in stress-induced reinstatement behaviors. For example, reducing norepinephrine levels through alpha 2-AR agonism blocks stress-induced reinstatement of cocaine seeking following SA in rats (Erb et al. 2000) and reinstatement of cocaine-conditioned reward in mice (Mantsch et al. 2010), as well as stress-associated cocaine craving in humans (Jobes et al 2011). Norepinephrine is also sufficient for reinstatement as elevation of norepinephrine levels through central administration of norepinephrine or alpha 2-AR antagonism promotes reinstatement behaviors in both rats and mice (Brown et al. 2011; Brown et al. 2009; Feltenstein and See 2006; Mantsch et al. 2010).

We have previously demonstrated, using the mouse CPP/ reinstatement approach that, specifically, beta 2-ARs are required for stress-induced reinstatement (Mantsch et al.

2010; Vranjkovic et al. 2012). Reinstatement of CPP by either forced swim or yohimbine is blocked by the beta 2-AR-selective antagonist, ICI-118,551, but not the beta1-AR-selective antagonist, betaxolol, while administration of the beta 2-AR-selective agonist, clenbuterol, is sufficient to reinstate CPP. Our current findings confirm this role for beta 2-ARs. In rats, the BNST, which receives dense noradrenergic innervation through the ventral noradrenergic bundle (Ricardo & Koh 1978, Woulfe et al 1988) and displays increased norepinephrine levels following stress (Pacak et al 1995), appears to be a key site for beta-AR regulation of cocaine seeking during stress. Blockade of beta 1- and beta 2-ARs in the BNST using a cocktail of ICI-118,551 and betaxolol prevents footshock-induced reinstatement of cocaine seeking following SA in rats (Leri et al. 2002).

The precise mechanism through which beta 2-ARs regulate reinstatement of cocaine seeking and CPP is unclear, but we hypothesize that it involves effects on CRF neurons in the BNST. Beta-AR activation increases excitatory transmission in the BNST (Egli et al 2005), and these effects appear to be mediated in part via a CRF-R1-dependent mechanism (Nobis et al 2011). In addition, norepinephrine-induced reinstatement is prevented by CRF receptor antagonism, but CRF-induced reinstatement is not blocked by inhibition of noradrenergic neurotransmission (Brown et al 2009), suggesting that noradrenergic signaling is upstream from CRF in the chain of events that lead to reinstatement. Our own findings support this possibility, as we found that reinstatement of CPP induced by the beta 2-AR agonist clenbuterol is blocked by the CRF-R1 antagonist antalarmin and that stress-induced increases in CRF mRNA in the BNST are prevented by the beta 2-AR antagonist ICI-118,551. CRF released into the BNST may originate from CRF-releasing afferent projections to the BNST or from local cell

populations (Veinante et al 1997). The latter possibility is supported by findings from Silberman et al. (2013) suggesting that norepinephrine depolarizes CRF-containing neurons intrinsic to the BNST, thereby releasing CRF locally, which then enhances glutamatergic neurotransmission via presynaptic effects.

CRF neurons in the BNST also send efferent projections to a number of brain regions, most notably the VTA (Rodaros et al. 2007). The VTA is a heterogeneous region that is the source of dopamine neurons that comprise the mesocorticolimbic system and has a well-established role in stress-induced cocaine seeking (Mantsch et al 2014, McFarland et al 2004). CRF actions in the VTA are necessary for stress-induced reinstatement and are mediated by activation of CRF-R1 receptors (Blacktop et al 2011). Emerging evidence suggests that the BNST-VTA pathway is essential for stress-induced cocaine seeking and reinstatement of CPP (Mantsch et al. 2014). For example, reinstating stimuli such as forced swim increase c-fos expression specifically in BNST neurons that project to the VTA (Briand et al. 2010). The BNST sends both glutamatergic and GABAergic projections to the VTA (Georges & Aston-Jones 2001, Kudo et al 2012), which appear to have distinct roles in reward and aversion (Jennings et al 2013). However, the relative roles of GABAergic vs. glutamatergic projections from the BNST to the VTA in the regulation of stress-induced reinstatement behaviors are not clear. Notably, withdrawal from chronic ethanol exposure enhances basal glutamatergic regulation of VTA-projecting BNST neurons in a CRF-R1 dependent manner (Silberman et al. 2013). Understanding how projections from the BNST to the VTA promote relapse to drug use during stress and delineation of the processes in the BNST that regulate these projections represent important goals for future research.

In general, the role for CRF and NE in stress-induced reinstatement behaviors, as demonstrated in the present study, extends to other classes of abused drugs. Antagonism of CRF receptors prevents stress-induced reinstatement following alcohol (Le et al 2000), heroin (Shaham et al 1997), and nicotine (Bruijnzeel et al 2009, Zislis et al 2007). SA, as does suppression of noradrenergic neurotransmission using alpha-2 AR agonist drugs (Le et al 2005, Shaham et al 2000, Zislis et al 2007). However, subtle differences in the mechanisms underlying stress-induced reinstatement appear to exist. For example, the alpha-1 AR antagonist, prazosin, blocks stress-induced reinstatement (Le et al 2011), while the highly selective alpha-2 AR antagonist, RS-79948, fails to evoke reinstatement following alcohol SA in rats (Dzung Le et al 2009). Thus, application of the current findings to other drugs of abuse will require further investigation.

There is some evidence that suggests that targeting ARs in human cocaine addicts may be an effective treatment strategy. In humans, alpha 2-AR agonists, which functionally antagonize noradrenergic neurotransmission, reduce stress-induced (Jobes et al 2011) and cue-induced (Fox et al 2012) cocaine craving. The effectiveness of beta blockers in cocaine users classified as dependent has also been examined (Kampman et al 2001). It is noteworthy that, while the beta-blocker propranolol did not universally improve treatment outcome measures, positive responses were observed in subjects who displayed more severe cocaine withdrawal symptoms. Considering that such symptoms are largely stress-related, we view these findings as supportive of a role for beta-ARs in stress-induced relapse.

Similar to the BNST, the amygdala is a region known to be involved in both reward and stress. The amygdala is comprised of a number of subnuclei that are highly

interconnected with regions involved in stress-related responses such as the PVN and BNST, as well those involved in reward-related processes, such as the NAc, the PFC, and the VTA (McDonald 1991a, McDonald 1991b, Rodaros et al 2007, Silverman et al 1981). As is the case for the BNST, it has been reported that the amygdala is essential for stress-induced reinstatement (McFarland et al. 2004) and that the CeA sends CRF-positive projections to the VTA (Rodaros et al. 2007). Moreover, beta 2-AR signaling in the CeA (Leri et al. 2002) and a projection from the CeA that regulates CRF release in the BNST (Erb et al. 2001) have been reported to be necessary for stress-induced cocaine seeking following SA in rats, suggesting that beta 2-AR regulation of CRF neurons may occur in the amygdala as well. Surprisingly, we found that forced swim stress did not alter CRF mRNA levels in the amygdala. This is in contrast to a prior study demonstrating swim-induced increases in CRF mRNA levels in the amygdala of mice with a prior history of cocaine (Cleck et al 2008). However, in that study, mice received greater amounts of cocaine and the increase in CRF mRNA levels was only observed following prolonged withdrawal (Cleck et al. 2008). Importantly, in the present study, mice were tested for CRF mRNA responses after repeated exposure to cocaine but in the absence of the CPP context. It is not clear how conditioning to cocaine and testing for swim effects on CRF mRNA measured following exposure to the CPP chambers could have influenced our results. It should also be noted that since our dissections were gross dissections of the amygdala due to the small amount of tissue yielded from a mouse brain, we cannot exclude the possibility that a more specific dissection of the CeA may yield different results. In contrast to its regulatory role in anxiety, CRF actions in the CeA do not appear to be necessary for stress-induced reinstatement following SA or CPP (Erb and Stewart

1999; Wang et al. 2006). Our findings also suggest that, while CeA regulation of CRF release into the BNST is necessary for stress-induced reinstatement behaviors (Erb et al. 2001), the source of CRF may consist of CRF projections from regions other than the CeA or cells that intrinsic to the BNST and not CRF neurons originating in the CeA.

Our current findings support a hypothesis that places beta2- AR signaling upstream from CRF actions in the BNST in the neural pathway that mediates stress-induced reinstatement of cocaine-conditioned reward. While there are long-established roles for AR and CRF individually in stress-induced reinstatement, it has become clear that an interaction between beta2-AR signaling and CRF producing neurons, possibly in the BNST is likely critical. For this reason, further investigation of the precise mechanisms through which beta2-ARs contribute to the stress-induced regulation of CRF neurons in the BNST is needed. Finally, our findings also suggest that medications targeting beta2-ARs hold some promise for potential treatment aimed at minimizing the contribution of stress to relapse.

Chapter III

Stress-induced cocaine seeking requires a beta-2 adrenergic receptor-regulated pathway from the ventral bed nucleus of the stria terminalis that regulates CRF actions in the ventral tegmental area

Abstract

The ventral bed nucleus of the stria terminalis (vBNST) has been implicated in stress-induced cocaine use. Here we demonstrate that, in the vBNST, corticotropin releasing factor (CRF) is expressed in neurons that innervate the ventral tegmental area (VTA), a site where the CRF receptor antagonist antalarmin prevents the reinstatement of cocaine seeking by a stressor, intermittent footshock, following intravenous self-administration in rats. The vBNST receives dense noradrenergic innervation and expresses β adrenergic receptors (ARs). Footshock-induced reinstatement was prevented by bilateral intra-vBNST injection of the β -2 AR antagonist, ICI-118,551, but not the β -1 AR antagonist, betaxolol. Moreover, bilateral intra-vBNST injection of the β -2 AR agonist, clenbuterol, but not the β -1 agonist, dobutamine, reinstated cocaine seeking, suggesting that activation of vBNST β -2 AR is both necessary for stress-induced reinstatement and sufficient to induce cocaine seeking. The contribution of a β -2 AR-regulated vBNST-to-VTA pathway that releases CRF was investigated using a disconnection approach. Injection of ICI-118,551 into the vBNST in one hemisphere and antalarmin into the VTA of the contralateral hemisphere prevented footshock-induced reinstatement, whereas ipsilateral manipulations failed to attenuate stress-induced cocaine seeking, suggesting that β -2 AR regulate vBNST efferents that release CRF into the VTA, activating CRF receptors, and promoting cocaine use. Last, reinstatement by

clenbuterol delivered bilaterally into the vBNST was prevented by bilateral vBNST pretreatment with antalarmin, indicating that β -2 AR-mediated actions in the vBNST also require local CRF receptor activation. Understanding the processes through which stress induces cocaine seeking should guide the development of new treatments for addiction.

Introduction

In many cocaine addicts, relapse to drug use is triggered by the onset of craving in response to episodes of stress. Due to the uncontrollable and often unavoidable nature of stress, its contribution to relapse is particularly problematic when managing cocaine addiction.

Using rodent reinstatement models, preclinical researchers have begun to define the neurocircuitry that underlies stress-induced relapse. One prominent pathway that has been implicated consists of a dopaminergic projection from the ventral tegmental area (VTA) to the medial prefrontal cortex, where dopamine D1 receptor activation increases the activity of a glutamatergic pathway to the nucleus accumbens that is critical for cocaine seeking (Capriles et al 2003, McFarland et al 2004). During stress, the VTA is regulated by the neuropeptide corticotropin releasing factor (CRF; (Wang et al 2005), in part via CRF-R1 receptors (Blacktop et al 2011), to evoke cocaine seeking.

The VTA receives inputs from brain regions involved in integration of the stress response, including those comprising the “extended amygdala”: the nucleus accumbens shell, central amygdala, and bed nucleus of the stria terminalis (BNST;(Phillipson 1979). Among these structures, the BNST, which serves as an interface between stress and motivational/reward systems, appears to be particularly important for stress-induced

cocaine seeking, in that its inactivation prevents stress-induced reinstatement in rodents ((Briand et al 2010, McFarland et al 2004). A subgroup of neurons projecting from the BNST to the VTA express CRF (Rodaros et al 2007) and therefore may be responsible for stress-induced increases in VTA CRF and CRF-dependent cocaine seeking. However, CRF projections to the VTA from the ventral BNST (vBNST), the subregion implicated in stress-induced cocaine seeking (McFarland et al., 2004), have not been described. The BNST, particularly the vBNST, receives dense noradrenergic innervation (Phelix et al 1992) and expresses β adrenergic receptors (ARs; (Cecchi et al 2007)), which have been reported to regulate efferent projections (Nobis et al 2011), including those that innervate the VTA (Dumont & Williams 2004, Silberman et al 2013). Both stress-induced reinstatement (Erb et al 2001, Erb & Stewart 1999) and noradrenergic regulation of BNST efferents to the VTA (Silberman et al., 2013) require BNST CRF receptor activation, suggesting that norepinephrine in the BNST promotes local CRF release, thereby activating a second CRF-releasing pathway to the VTA.

Considering the important role for noradrenergic signaling in stress-induced reinstatement in rodents (Erb et al 2000, Mantsch et al 2010) and craving in human cocaine-dependent populations (Jobes et al 2011), AR regulation of a vBNST-to-VTA CRF-releasing pathway is a likely mechanism through which stress promotes cocaine seeking. We have found that β -2 AR activation is both necessary and sufficient for stress-induced reinstatement in mouse models (Vranjkovic et al 2012). Moreover, it has been demonstrated that a mixture of β -AR antagonists, administered into the vBNST, prevents stress-induced reinstatement (Leri et al 2002). In this study, we demonstrate direct CRF neuronal projections from the vBNST to the VTA and examine the relationship between

stress-induced β -2 AR activation in the vBNST and CRF-R1 receptor-dependent actions in the VTA that lead to cocaine seeking.

Materials and Methods:

Subjects

Male Sprague Dawley rats (Harlan Laboratories) ~90-d-old at the time of delivery (325 g) were used. Rats were housed singly in a temperature- and humidity-controlled, Association for Assessment and Accreditation of Laboratory Animal Care accredited facility under a 12 h light/dark cycle (lights off at 07:00 h) and had access to food and water at all times, except during the food training periods during which they were kept at 90% of their free-feeding body weight. All procedures were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Drugs

Cocaine HCl was acquired from the National Institute on Drug Abuse through its drug supply program. The selective β -1 AR antagonist betaxolol HCl, the selective β -2 AR antagonist (-)-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI-118,551) HCl, the selective β -1 AR agonist dobutamine HCl, the selective β -2 AR agonist clenbuterol, and the axonal transport inhibitor colchicine HCl were purchased from Sigma-Aldrich. Cocaine was dissolved in bacteriostatic saline. Other drugs were dissolved in sterile water. Colchicine was delivered intra cerebroventricularly in a volume of 2 μ l over a 5 min period. Intracranial drug injections occurred in a volume of 0.2 μ l/side over a 1 min period.

Catheter and cannula implantation

For intravenous self-administration (SA) and reinstatement testing, rats were implanted with catheters into the jugular vein under ketamine HCl (100 mg/kg i.p.) and xylazine (2 mg/kg, i.p.) anesthesia, as described previously (Blacktop et al 2011), along with 11 mm 26-gauge guide cannulae aimed at the vBNST (0.6 mm posterior to bregma; 3.5 mm lateral to midline; 6.9 mm ventral to the skull surface at a 15° angle), and/or the VTA (5.6 mm posterior to bregma; 2.2 mm lateral to midline; 6.9 mm ventral to the skull surface at a 12° angle) for intracranial injections. The tips of the guide cannulae were aimed 0.5 mm above the target injection sites.

Cocaine self-administration

Following a 2 week recovery from surgery, rats were trained to self-administer cocaine (1.0 mg/kg/inf, i.v.) by pressing a response lever under an FR1 schedule in a computer-interfaced operant conditioning chamber (Med Associates) during daily 2 h sessions. During these sessions, the active (front) lever was extended into the chamber and the corresponding stimulus light was illuminated. Pressing this lever resulted in a cocaine infusion (200 µl over 5 s), followed by a 25 s time-out period during which the stimulus light was extinguished but the lever remained extended. Responding on a second, inactive lever was recorded but had no programmed consequences. Once stable SA under the FR1 schedule was observed (>15 infusions), the requirements for SA were gradually increased until rats displayed stable responding under a FR4 schedule (<10% variation from the mean over 3 sessions), at which time daily access was increased to 6 h. Rats were permitted to self-administer daily under these conditions for 14 consecutive days after which they underwent extinction training.

Extinction training and reinstatement testing

Extinction sessions were identical to SA conditions except that (1) the sessions were 2 h in duration and (2) the cocaine solution was replaced with saline. Daily extinction training sessions were conducted until rats displayed <15 cocaine lever responses for consecutive sessions (range, 6–10 d) at which time rats were tested for reinstatement. Reinstatement sessions were identical to extinction sessions except that they were preceded by exposure to shock and/or intracranial drug delivery. Shocks (0.5 mA, 0.5 s duration) were delivered on average every 40 s (range, 10–70 s) through the grid floors of the chambers over a 15 min period that immediately preceded the 2 h reinstatement test session (Blacktop et al 2011, Graf et al 2011). During the shock session, the levers were retracted and the stimulus lights were off, but a houselight in the chamber was illuminated. Reinstatement was defined as an increase in cocaine lever responding relative to the prior extinction session. When rats were tested multiple times for reinstatement, test sessions were separated by additional extinction session and were required to display <15 cocaine lever responses before retesting.

Effects on food-reinforced lever pressing

In most cases where significant reductions in shock-induced reinstatement were observed, the effects of the same manipulations on lever pressing reinforced by food delivery (45 mg sucrose-sweetened food pellets; BioServ) were also examined in separate rats to confirm that reduced reinstatement was not attributable to motor impairment that interfered with the ability of the rats to press the lever. Following surgical intracranial implantation of guide cannulae and recovery, these rats were maintained at 90% of their free-feeding body weights and trained to self-administer food pellets by pressing a lever

under a FR4 schedule. Effects on food-reinforced responding were tested after stable patterns of lever pressing were observed (<10% variation from the mean over 3 sessions).

Histological confirmation of injection sites

The accuracy of cannula implantation was confirmed postmortem in each rat after cardiac perfusion with 60 ml 0.15% NaCl followed by 60 ml of 2.5% buffered neutral formalin under sodium pentobarbital anesthesia (55 mg/kg). Brains were removed and stored in 2.5% buffered formalin before vibratome sectioning (40 μ m), slide mounting, and staining with cresyl violet for examination using a light microscope. Rats with injection sites outside of the VTA or the vBNST were excluded from data analysis.

Experiment 1: effects of intra-BNST β AR antagonists on stress-induced reinstatement

We initially examined the role of β -1 and β -2 AR activation in the vBNST in stress-induced reinstatement by testing rats for shock-induced reinstatement following bilateral intra-vBNST injection of the β -1 AR selective antagonist, betaxolol (1 nmol/307 ng per side), the β -2 AR antagonist selective antagonist, ICI-118,551 (1 nmol/277 ng per side) or vehicle (sterile water). Concentrations were selected based on selectivity for human β receptor subtypes (Baker 2005). Moreover, the antagonist concentrations were previously used to selectively target brain β ARs (Cecchi et al 2007, Delfs et al 2000, LaLumiere et al 2010, Leri et al 2002). Rats received vBNST injections 15 min before shock. Nine total rats were used for this experiment. Five rats were tested for shock-induced reinstatement following pretreatment with vehicle, betaxolol, and ICI-118,551; two rats were tested following only vehicle and betaxolol; and two rats were tested for following only vehicle

and ICI-118,551. An additional group of rats ($n = 6$) was tested for the effects of ICI-118,551 and vehicle on food-reinforced lever pressing.

Experiment 2: reinstatement by intra-vBNST injection of β -AR agonists

To further examine the role of vBNST β -1 and β -2 ARs in stress-induced cocaine seeking, a separate group of rats was tested for the ability of bilateral intra-vBNST injection of the β -1 AR selective agonist dobutamine (1 nmol/ 301 ng per side) or the β -2 AR selective agonist clenbuterol (36 pmol/10 ng per side) to induce reinstatement. Concentrations were again chosen based on selectivity for human β receptor subtypes (Baker 2010). Further, the clenbuterol dose has been previously used to selectively target brain β -2 ARs (LaLumiere et al 2010, Roozendaal et al 2008). Ten total rats were used for this experiment. Seven rats were tested for reinstatement in response to dobutamine, clenbuterol, and vehicle. An additional three rats were tested only with clenbuterol and vehicle.

Experiment 3: effect of VTA CRF receptor antagonism on stress-induced reinstatement

To confirm our earlier finding that shock-induced reinstatement requires CRF receptor activation in the VTA (Blacktop et al., 2011), we also tested rats for the effects of intra-VTA pretreatment with the CRF-R1 receptor antagonist, antalarmin ((Webster et al 1996); 1.32 nmol/500 ng/side). We (Blacktop et al., 2011) and others (Lowery-Gionta et al 2012) have used this antalarmin dose to investigate CRF-R1 receptor-dependent contributions to behavior. Six rats were used for this experiment. Each of them was tested for the effects of both antalarmin and vehicle on shock-induced reinstatement.

Experiment 4: identification of CRF-positive neurons in the vBNST that project to the VTA

Retrograde tracing using CTb.

We used cholera toxin B subunit (CTb) as a retrograde tracer to identify neurons in the vBNST with direct efferent projections to the VTA. Rats were anesthetized with ketamine and xylazine and placed in a stereotaxic frame. Heat-sterilized Hamilton syringes were used to pressure inject 0.2 μ l biotinylated CTb (List Biological Laboratories) unilaterally into the VTA over a 10 min period (posterior to bregma -5.6 mm, lateral -2.6 mm, ventral -8.6 mm; Paxinos and Watson, 2006). Syringes were left in place for 20 min after injections and slowly removed from the brain over a 10 min period.

Colchicine treatment.

Because it has been reported that inhibition of axonal transport using pretreatment with the microtubule polymerization inhibitor colchicine is necessary for detection of CRF-immunoreactive cell bodies within the vBNST (Sakanaka et al 1986) anesthetized rats received injections of colchicine (1.25 μ mol/500 μ g in 2 μ l) into a lateral ventricle (posterior to bregma -1.0 mm, lateral 1.4 mm, ventral -5.3 mm) 10 d after injection with CTb, thus avoiding interference with retrograde transport of CTb.

Tissue collection and preparation.

Twenty-four hours after the colchicine injections, rats were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and perfused transcardially with 30 ml of 0.9% NaCl, followed by 60 ml of cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Brains were quickly removed and immersed in fixative for 24 h at 4°C . Brains were washed twice in 0.1 M sodium phosphate buffer for 12 h each, after which they were

cryoprotected in 30% sucrose in 0.1 M-phosphate buffer for 3 d at 4°C. Brains were rapidly frozen and serial coronal sections (30 µm) were cut on a cryostat. Sections were stored at -20°C until immunofluorescence procedures were conducted.

Fluorescence immunocytochemistry.

Immunofluorescence for CTb and CRF was conducted on free-floating 30 µm sections. Sections were rinsed three times for 10 min in 0.05 M KPBS and once for 10 min in 0.1 M glycine in 0.05 KPBS, followed by blocking in 3% normal donkey serum in 0.4% Triton KPBS for 20 min. Sections were then incubated for 48 h at 4°C with rabbit anti-CRF (supplied by Dr Wylie Vale, The Salk Institute) diluted 1:8000 in 0.1% Triton KPBS with 3% normal donkey serum. Sections were then rinsed 3× 10 min in 0.05 M KPBS, followed by incubation with an AlexaFluor 488-conjugated donkey anti-rabbit secondary antibody (Catalog #A11008; 1:500; Life Technologies) in 0.05 M KPBS for 2 h at room temperature, and rinsed three times for 10 min in 0.05 M KPBS. For detection of biotinylated CTb, sections were incubated for 24 h at 4°C in goat anti-biotin polyclonal antibody (Catalog # SP 3000; Vector Laboratories) diluted 1:30,000 in 0.1% Triton KPBS and 3% normal donkey serum. After rinsing, sections were incubated 2 h with AlexaFluor 598-conjugated donkey anti-goat IgG (Catalog #A-110581; 1:500; Life Technologies) and washed three times for 10 min in 0.05 M KPBS. Sections were briefly rinsed in distilled water and mounted onto gel-coated SuperFrost Plus slides and coverslipped using Vectashield mounting medium with DAPI (Catalog #H-1200; Vector Laboratories). Photomicrographs were acquired using a Retiga 2000R digital camera (QImaging) on a Nikon 80i microscope using NIS Elements software (Nikon Instruments).

Experiment 5: effects of vBNST and VTA disconnection on stress-induced cocaine seeking

A disconnection approach was used to determine whether a β 2-AR activated, CRF-dependent pathway between the vBNST and the VTA is required for stress-induced cocaine seeking. At the time of catheterization, separate groups of rats were implanted with two cannulae aimed unilaterally at the vBNST and VTA in either the contralateral or the ipsilateral hemisphere(s) of the brain. The effect of pathway disconnection was examined in six rats by injection of the β -2 AR antagonist ICI 118,551 (1 nmol/277 ng) into the vBNST in one hemisphere and the CRF-R1 receptor antagonist antalarmin (1.32 nmol/500 ng) into the VTA of the other before testing for shock-induced reinstatement. For comparison, the same rats were tested for shock-induced reinstatement following injection of vehicle into each region. As a control to ensure that reductions in reinstatement were attributable to disconnection, a second groups of rats ($n = 7$) received a unilateral injection of ICI 118,551 into the vBNST in one hemisphere and a unilateral injection of antalarmin into the VTA in the same hemisphere before testing for shock-induced reinstatement. These rats were also tested for the effects of ipsilateral vehicle injections. For this experiment, the hemispheres into which cannulae were implanted were randomized across rats in each group such that in the contralateral treatment group, half of the rats received ICI 118,551 injections into the left vBNST and antalarmin injections into the right VTA, whereas the other half received ICI 118,551 injections into the right vBNST and antalarmin injections into the left VTA. Likewise, half of the rats in the ipsilateral treatment group received drug injections into sites in the right hemisphere, whereas the remaining rats received injections into sites in the left hemisphere. To

examine the potential contribution of nonspecific behavioral suppression to the effects on reinstatement, an additional group of rats ($n = 6$) was tested for the effects of contralateral drug and vehicle injections on food-reinforced lever pressing.

Experiment 6: role of vBNST CRF-R1 receptors in cocaine seeking induced by vBNST β -2 AR activation

It has been reported that CRF-R1 antagonist injections directly into the vBNST can also block stress-induced cocaine seeking (Erb et al 2001, Erb & Stewart 1999). To determine whether β -2 ARs in the vBNST regulate efferent pathways to the VTA to produce cocaine seeking via a mechanism that also involves local CRF actions, we tested for the ability of intra-BNST pretreatment with antalarmin to block reinstatement in response to local delivery of the β 2 AR agonist clenbuterol. Reinstatement in response to bilateral intra-BNST delivery of clenbuterol (36 pmol/10 ng per side) was examined following bilateral intra-BNST pretreatment with antalarmin (1.32 nmol/500 ng/side) or vehicle (10 min pretreatment) in counterbalanced sequence in six rats. To test the hypothesis that CRF-R1 receptor activation in the vBNST is downstream from β -2 AR activation in the sequence of events that mediate stress-induced cocaine seeking, a separate group of six rats was tested for reinstatement in response to bilateral intra-vBNST CRF (63 pmol/300 ng/side; Erb and Stewart, 1999) following bilateral intra-vBNST pretreatment with the selective β -2 AR antagonist, ICI-118,551 (1 nmol/277 ng per side) or vehicle (10 min pretreatment).

Statistical analyses

Statistical analyses were conducted using Predictive Analytics Software statistics software (SPSS). Statistical significance was determined using ANOVA followed, when

appropriate, by further analyses of main effects using ANOVA and/or *post hoc* testing using Bonferroni-corrected *t* tests.

Results

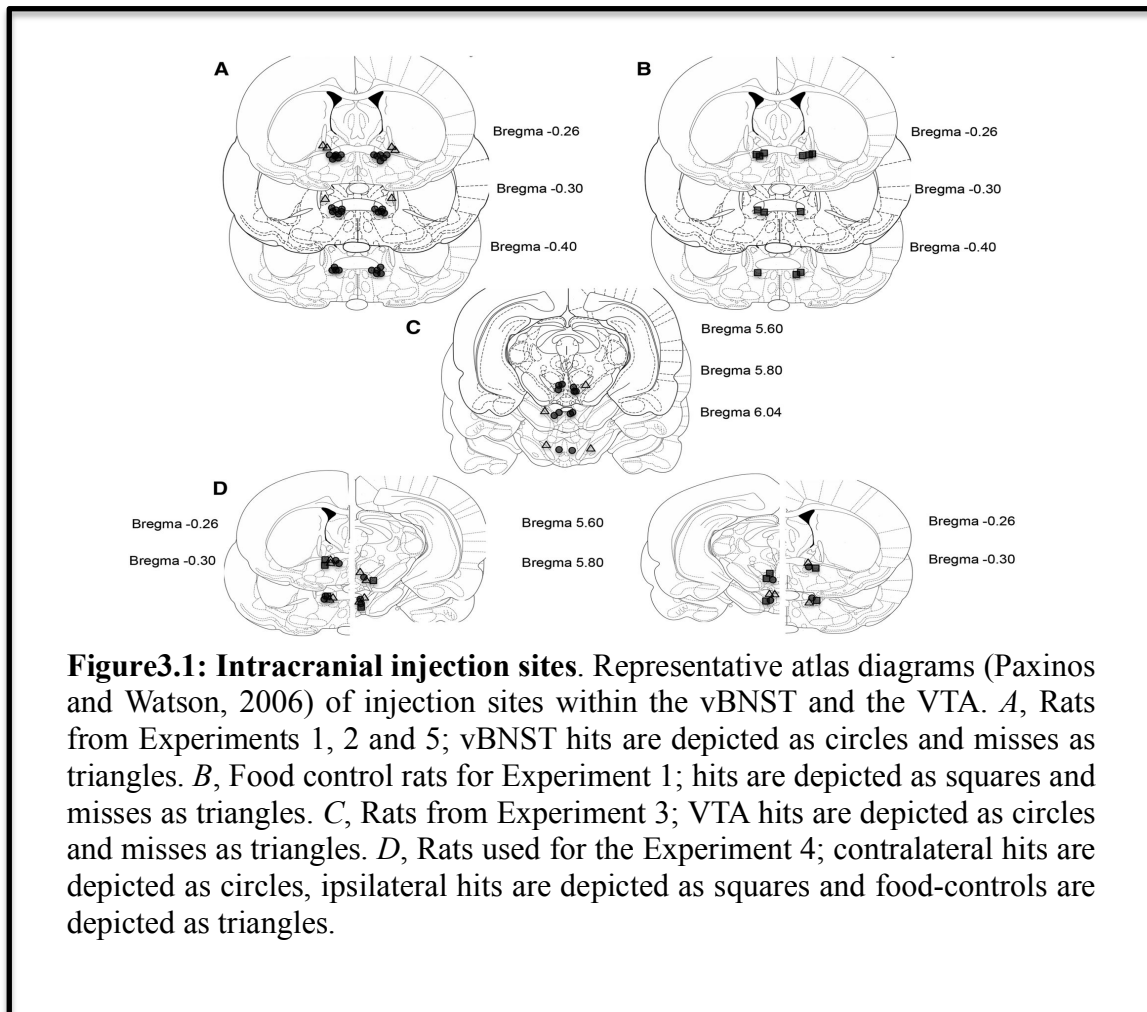
Cocaine SA and extinction in rats used for the various reinstatement experiments are shown in Table 3.1 and did not differ across experiments.

<i>Experiment</i>	<i>Cocaine SA (infusions/6-h session ± S.E.)</i>		<i>Extinction (responses/2-h session ± S.E.)</i>	
	<i>SA Day 1</i>	<i>SA Day 14</i>	<i>First Ext</i>	<i>Last Ext</i>
<i>Exp. 1 (n=9)</i>	77.22 ± 7.71	92.78 ± 8.19	66.33 ± 11.50	9.89 ± 2.35
<i>Exp. 2 (n=10)</i>	68.57 ± 8.36	89.38 ± 7.07	104.20 ± 24.21	9.70 ± 1.14
<i>Exp. 3 (n=6)</i>	75.10 ± 5.86	90.21 ± 7.26	70.20 ± 29.14	11.60 ± 1.47
<i>Exp. 5 (n=13)</i>	71.82 ± 4.73	85.74 ± 4.01	68.09 ± 12.83	12.00 ± 2.39
<i>Exp. 6 (n=6)</i>	74.78 ± 5.05	88.21 ± 5.63	131.00 ± 29.14	7.00 ± 1.98

Table 1: *Cocaine SA and extinction.* Data represent cocaine SA (infusions/6-h session ± S.E) on days 1 and 14 of SA testing and cocaine lever responding (responses/2-h session ± S.E.) on the first day of extinction training (First Ext) and the last day of extinction training prior to reinstatement testing (Last Ext) in rats from each of the reinstatement experiments

In general, each group of rats displayed escalation, as previously demonstrated under these conditions, and showed reduced cocaine seeking across extinction testing, which consisted of an average of 8.24 (±0.28) sessions before extinction criteria were met. The injection sites for rats used for each experiment are depicted in Figure 3.1. A

total of 11 rats were excluded from analyses due to cannula misplacement. In all cases, drug effects were not observed with inaccurate injection.

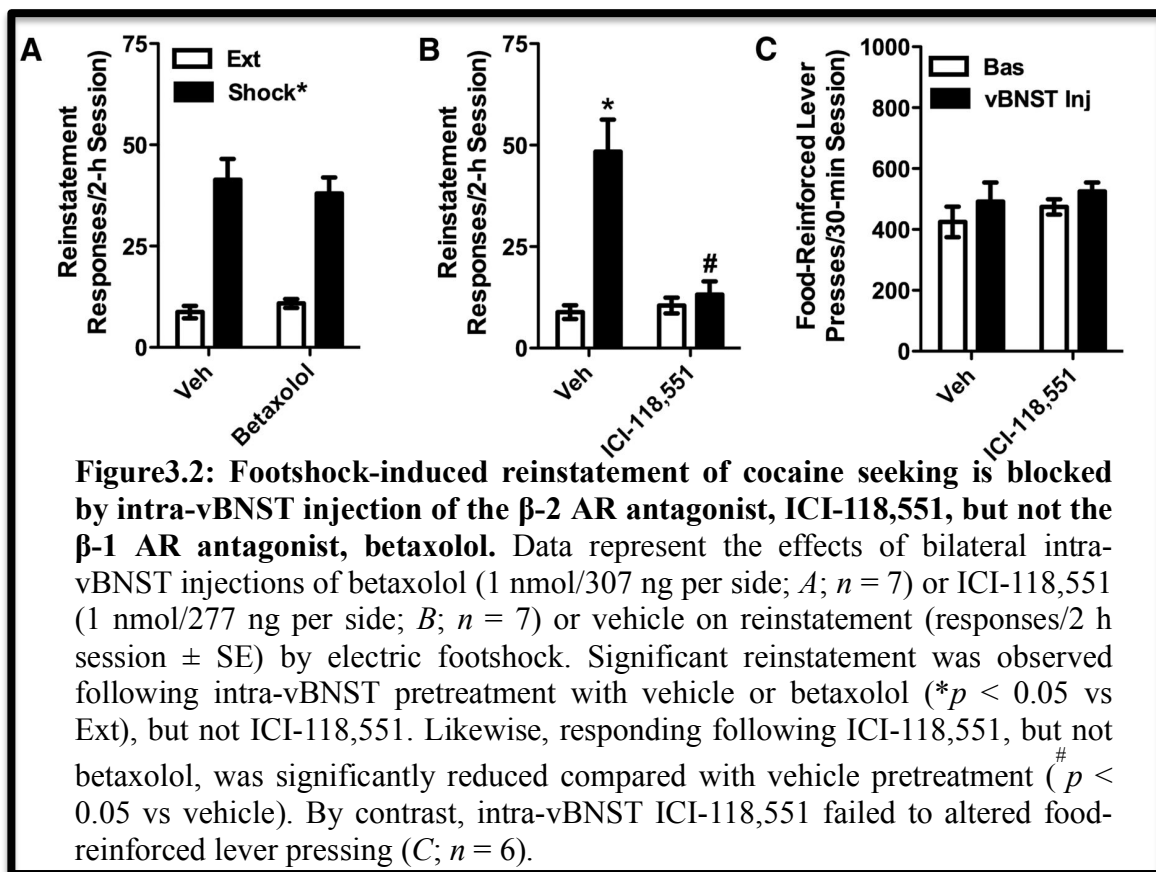


Experiment 1: effects of intra-BNST β AR antagonists on stress-induced reinstatement

The effect of bilateral intra-vBNST injection of the β -1 AR antagonist betaxolol (1 nmol/307 ng per side) on shock-induced reinstatement was examined in seven rats and is shown in **Figure 3.24**. Two-way repeated measures (reinstatement \times betaxolol)

ANOVA showed a significant overall reinstatement effect ($F_{(1,6)} = 65.437; p < 0.001$) but no main effect of betaxolol pretreatment or interaction between betaxolol treatment and reinstatement. Shock produced robust reinstatement regardless of whether rats were pretreated with betaxolol or vehicle. The effects of bilateral intra-vBNST injection of the β -2 AR antagonist ICI-118,551 (1 nmol/277 ng per side) pretreatment on shock-induced reinstatement were also examined in seven rats and are shown in **Figure 3.2B**. Two-way repeated-measures ANOVA showed a significant reinstatement effect ($F_{(1,6)} = 52.475; p < 0.001$) and a significant main effect of ICI-118,551 pretreatment ($F_{(1,6)} = 10.224; p < 0.05$), as well as a significant reinstatement \times ICI-118,551 pretreatment interaction ($F_{(1,6)} = 20.213; p < 0.01$). Shock reinstated cocaine seeking in vehicle but not ICI-118,551 pretreated rats ($p < 0.01$ vs extinction; Ext) and reinstatement was reduced in ICI-118,551 pretreated versus vehicle pretreated rats ($p < 0.01$). To assess potential motor impairment resulting from intra-vBNST ICI-118,551 delivery that may have nonspecifically interfered with reinstatement responding, a separate group of six rats was tested for effects of intra-vBNST ICI-118,551 on sucrose pellet-reinforced responding. ICI-118,551 pretreatment failed to alter lever pressing under these conditions (**Fig. 3.2C**). In three of the rats that were tested, guide cannulae were misplaced such that they received injections into areas that comprise the dorsal BNST. Notably, in these rats, shock-induced reinstatement was observed in both vehicle-pretreated (Ext: 8.67 ± 1.76 responses vs shock: 32.33 ± 2.03) and ICI-118,551-pretreated (Ext: 12.67 ± 1.76 responses vs shock: 28.33 ± 4.81) rats. Effects of shock were specific to the previously active lever as shock failed to increase responding on a previously inactive lever in vehicle-pretreated rats (Ext: 1.37 ± 0.51 responses vs shock: 1.02 ± 0.44). Neither

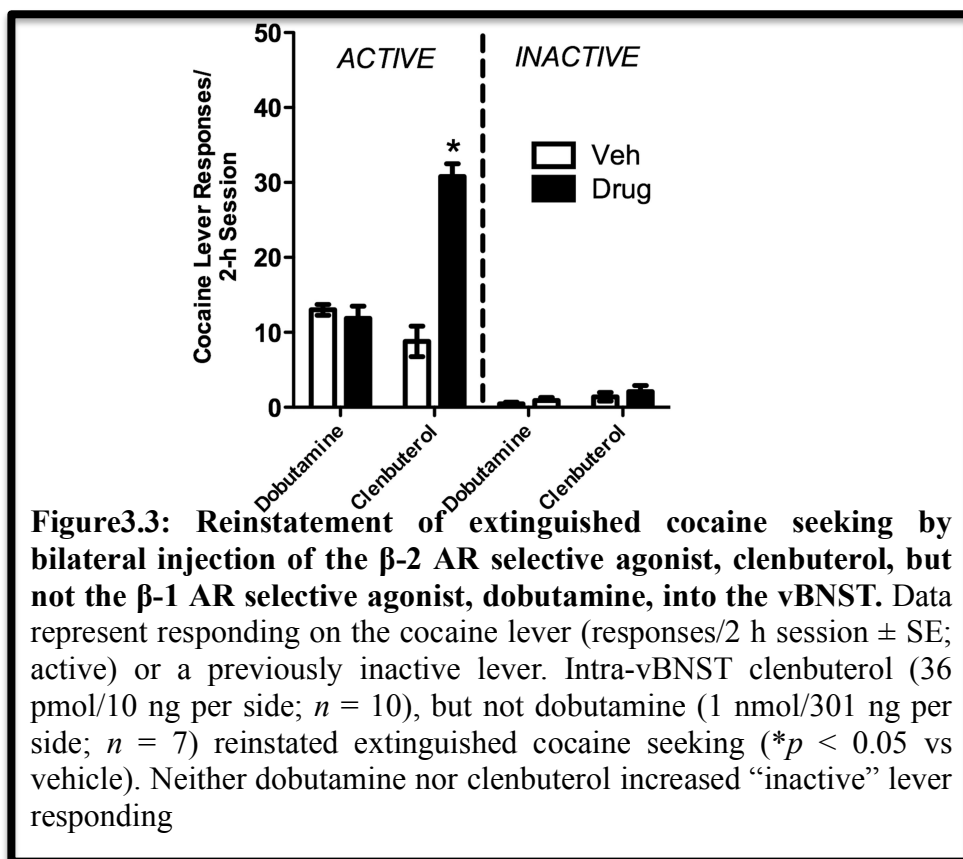
betaxolol nor ICI-118,551 affected inactive lever responding during reinstatement testing (vehicle: 1.02 ± 0.44 responses; betaxolol: 1.00 ± 0.33 ; ICI-118,551: 0.75 ± 0.31).



Experiment 2: reinstatement by intra-vBNST injection of β -AR agonists

Following SA and extinction, bilateral intra-vBNST delivery of clenbuterol (36 pmol/10 ng per side) but not dobutamine (1 nmol/ 301 ng per side), reinstated cocaine seeking (**Fig. 3.3**). A paired *t* test showed that, compared with vehicle, pretreatment with clenbuterol significantly increased previously active lever pressing ($t_{(9)} = 11.156$; $p < 0.001$). Neither dobutamine (0.88 ± 0.22 responses) nor clenbuterol (0.83 ± 0.55 responses) increased responding on a previously inactive lever relative to vehicle

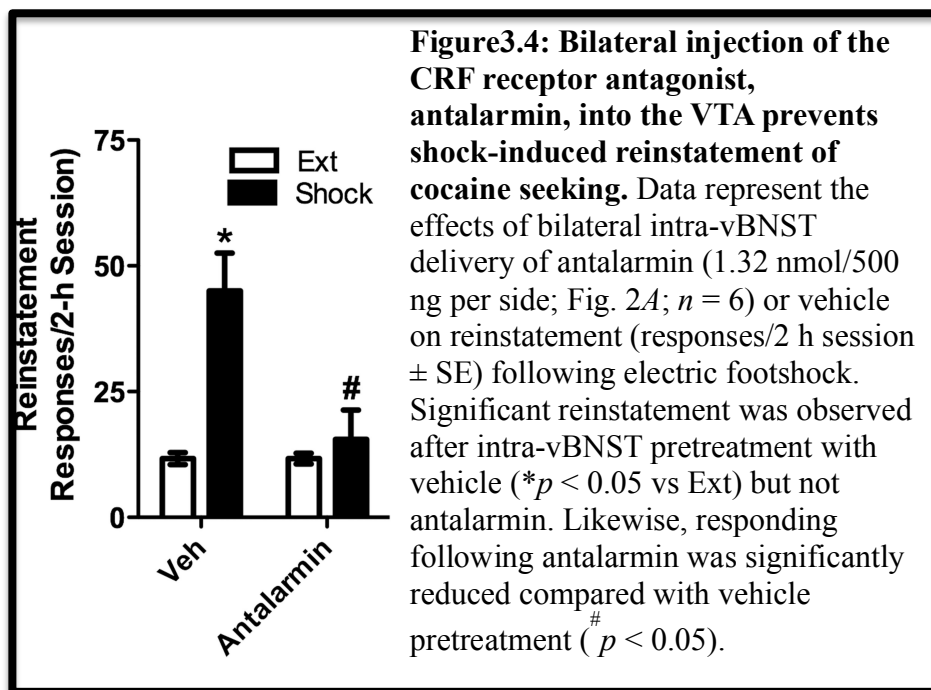
pretreatment (0.63 ± 0.18) responses. Guide cannulae were misplaced in three rats such that they received injections into sites within the dorsal BNST. In contrast to rats that received intra-vBNST injections, neither clenbuterol (Ext: 8.67 ± 1.99 vs clenbuterol: 5.33 ± 5.95) nor dobutamine (Ext: 10.00 ± 2.07 vs dobutamine: 9.33 ± 7.60) produced reinstatement in these rats.



Experiment 3: effect of VTA CRF receptor antagonism on stress-induced reinstatement

As previously reported (Blacktop et al., 2011), bilateral injection of the CRF-R1 receptor selective antagonist, antalarmin (1.32 nmol/500 ng per side) into the VTA prevented reinstatement. The effect of bilateral intra-VTA antalarmin pretreatment on shock-induced reinstatement was examined in six rats as shown in **Figure 3.4**. Two-way

repeated-measures ANOVA showed a significant reinstatement effect ($F_{(1,5)} = 12.971; p < 0.05$) and a significant main effect of antalarmin pretreatment ($F_{(1,5)} = 9.175; p < 0.05$), as well as a significant reinstatement \times antalarmin pretreatment interaction ($F_{(1,5)} = 8.461; p < 0.05$). As previously reported, shock reinstated cocaine seeking in vehicle but not antalarmin pretreated rats ($p < 0.05$ vs Ext) and reinstatement was reduced in antalarmin pretreated versus vehicle-pretreated rats ($p < 0.05$). As we have already demonstrated that intra-VTA antalarmin delivery does not alter food-reinforced lever pressing (Blacktop et al., 2011), we did not test for these effects in this study. In two of the rats that were tested, guide cannulae were misplaced such that they received injections outside of the VTA. Notably, in these rats, shock-induced reinstatement was observed in both vehicle-pretreated (Ext: 13 and 12 responses vs shock: 35 and 29 responses) and antalarmin-pretreated (Ext: 5 and 12 responses vs shock: 39 and 41 responses) rats.



Effects of shock were specific to the previously active lever as shock failed to increase responding on a previously inactive lever in vehicle-pretreated rats (Ext: 1.12 ± 0.29 responses vs shock: 0.89 ± 0.65). Antalarmin did not affect inactive lever responding during reinstatement testing (vehicle: 0.89 ± 0.65 responses; antalarmin: 0.81 ± 0.52).

Experiment 4: identification of CRF-positive neurons in the vBNST that project to the VTA

To determine whether CRF-positive neurons within the vBNST project to the VTA, we injected CTb unilaterally into the VTA and looked for the presence of CTb/CRF double-labeled perikarya in the vBNST in four rats. Representative photomicrographs from one rat are shown in **Figure 5**. CTb-immunoreactive (CTb-ir) perikarya were observed in the vBNST ipsilateral, to the VTA injection site (average of 45 CTb-ir perikarya/ipsilateral vBNST). By contrast, and consistent with a previous report (Dong & Swanson 2006) CTb immunoreactivity in the vBNST contralateral to the VTA injection site was minimal (average of four immunoreactive cells/contralateral vBNST). CRF-like-immunoreactive (CRF-ir) perikarya and fibers were observed in the vBNST (**Fig. 3.5**). Under higher-magnification, three cell types were observed in the vBNST: CTb-ir/CRF-immunonegative, CTB-ir/CRF-ir, and CTb-immunonegative/CRF-ir cells. Notably, most CRF-positive cells in the ipsilateral vBNST were also CTb-immunoreactive (~80%). By contrast, dual-labeled CRF- and CTb-positive cells were not observed in the contralateral vBNST.

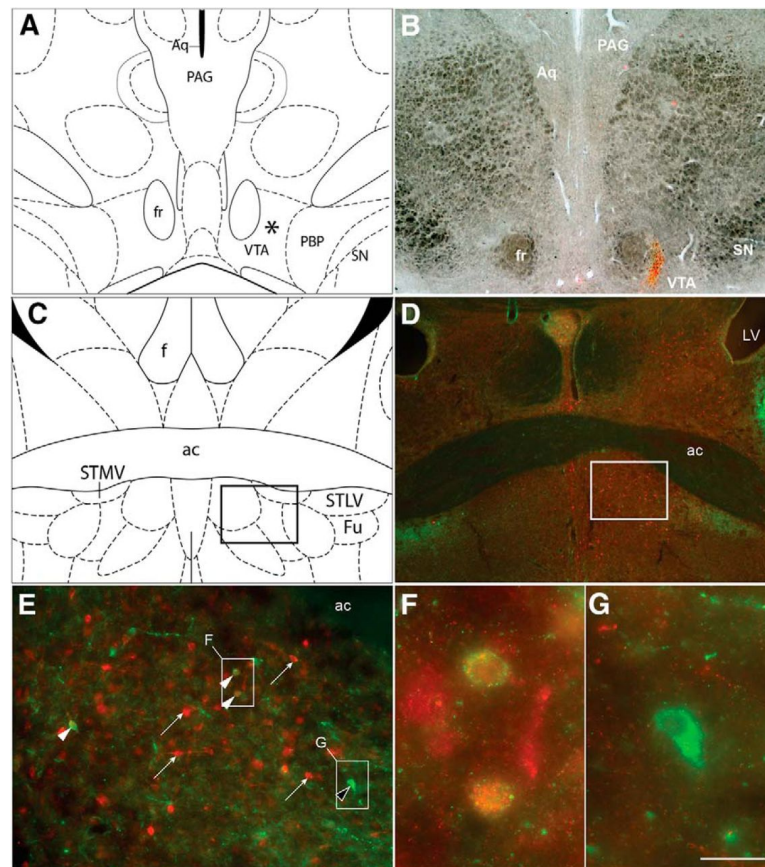
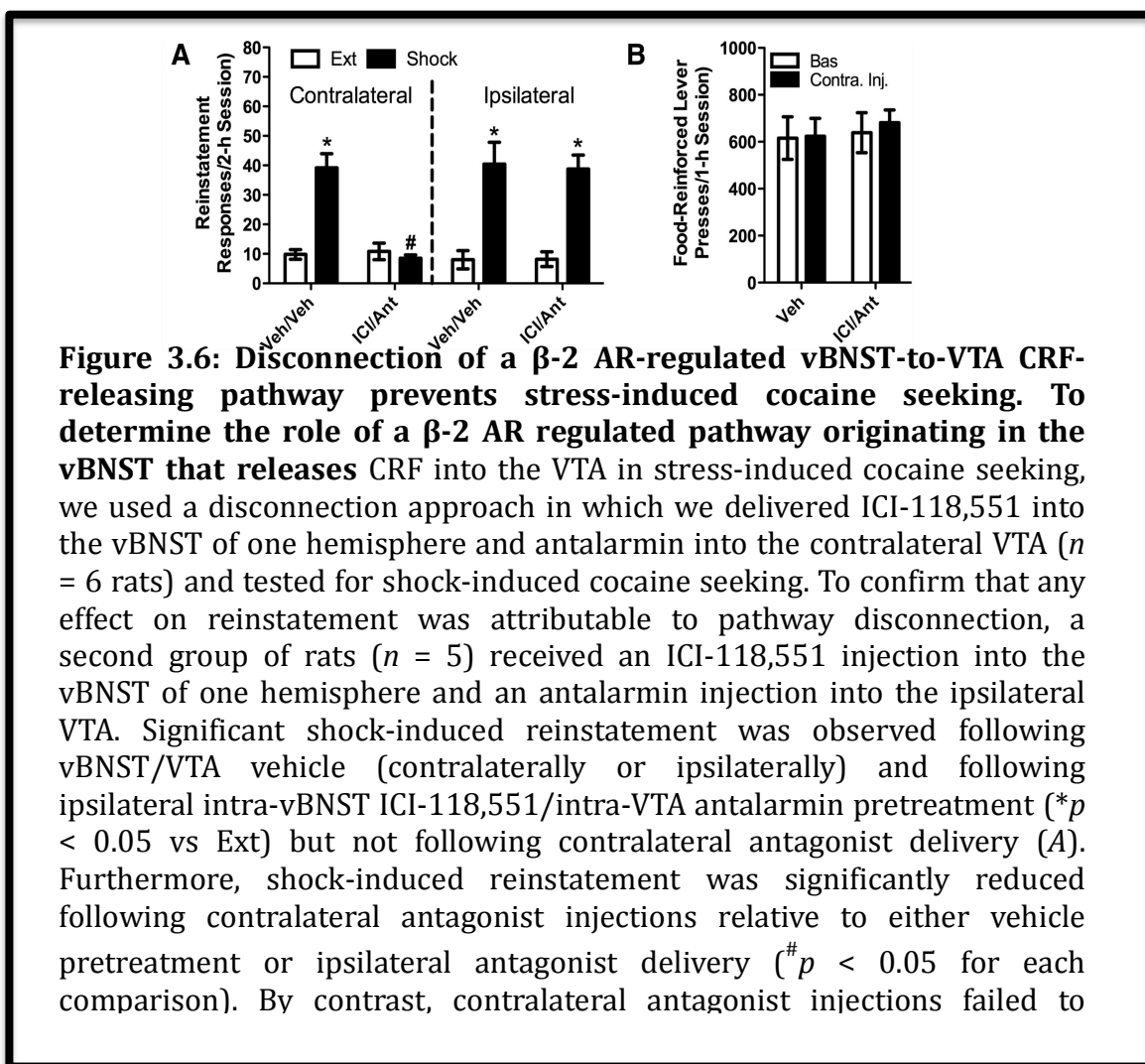


Figure 3.5: CRF neurons within the vBNST project to the VTA. *A*, Line drawing (adapted from Paxinos and Watson, 2006) of a coronal section containing the area of the VTA targeted for CTb microinjections and depicted in *B*. *B*, Representative photomicrograph depicting the VTA in brightfield, and CTb immunofluorescence in red. *C*, Line drawing of a coronal section containing the vBNST used for immunostaining of CRF and CTb. *D–G*, Representative photomicrographs depicting immunostaining for CRF (green) and CTb (red) in the BNST ipsilateral to the VTA injection site. Rectangle in *D* indicates area shown at higher-magnification in *E*. *E*, White arrowheads indicate CRF-ir/CTb-ir (VTA-projecting) neurons; white arrows indicate CTb-ir/CRF-immunonegative neurons; black arrowhead indicates a CRF-ir/CTb-immunonegative cell. Rectangles in *E* represent areas shown at higher-magnification in indicated panels. *F*, CRF-ir/CTb-ir neurons in vBNST. *G*, CRF-positive/CTb-immunonegative cell in BnST. Scale bars: *B*, *D*, 500 μm ; *E*, 100 μm ; *F*, *G*, 33 μm . ac, Anterior commissure; Aq, cerebral aqueduct; f, fornix; fr, fasciculus retroflexus; Fu, bed nucleus of the stria terminalis, fusiform part; PAG, periaqueductal gray; PBP, parabrachial pigmented nucleus of the VTA; SN, substantia nigra; STL, bed nucleus of the stria terminalis, lateral division, ventral part; STMV, bed nucleus of the stria terminalis, medial division, ventral part.

Experiment 5: effects of vBNST and VTA disconnection on stress-induced cocaine seeking

To determine the role of a β -2 AR regulated pathway originating in the vBNST that releases CRF into the VTA in stress-induced cocaine seeking, we used a disconnection approach in which we delivered ICI-118,551 (1 nmol/277 ng) into the vBNST of one hemisphere and antalarmin (1.32 nmol/500 ng) into the contralateral VTA ($n = 6$ rats). To confirm that any effect on reinstatement was attributable to pathway disconnection, a second group of rats ($n = 5$) received ICI-118,551 into the vBNST of one hemisphere and antalarmin into the ipsilateral VTA. Contralateral but not ipsilateral antagonist delivery prevented shock-induced reinstatement (**Fig. 3.6A**), suggesting that, during stress, β -2 AR in the vBNST regulate one or more pathways that release CRF into the VTA to induce cocaine seeking. A three-way ANOVA showed a significant interaction between reinstatement test condition (shock vs extinction; repeated measure), antagonist pretreatment (ICI-118,551/antalarmin vs vehicle/vehicle treatment; repeated measure), and injection site (contralateral vs ipsilateral; between-subjects; $F_{(1,9)} = 7.169$; $p < 0.05$). Further analysis revealed a significant interaction between antagonist treatment and shock-induced reinstatement only in rats that received contralateral antagonist injections ($F_{(1,5)} = 25.851$; $p < 0.01$). *Post hoc* testing showed that significant shock-induced reinstatement was observed following vBNST/VTA vehicle (contralaterally or ipsilaterally) and following ipsilateral intra-vBNST ICI-118,551 and intra-VTA antalarmin pretreatments ($p < 0.05$ for each comparison) but not following contralateral antagonist delivery. Furthermore, shock-induced reinstatement was significantly reduced following contralateral antagonist injections relative to either

vehicle pretreatment or ipsilateral antagonist delivery ($p < 0.05$ for each comparison). To assess potential motor impairment resulting from the disconnection approach that may have nonspecifically interfered with reinstatement responding, a separate group of six rats were tested for effects on sucrose pellet-reinforced responding. Contralateral intra-vBNST ICI-118,551 and intra-VTA antalarmin delivery failed to alter lever pressing under these conditions (**Fig. 3.6B**).

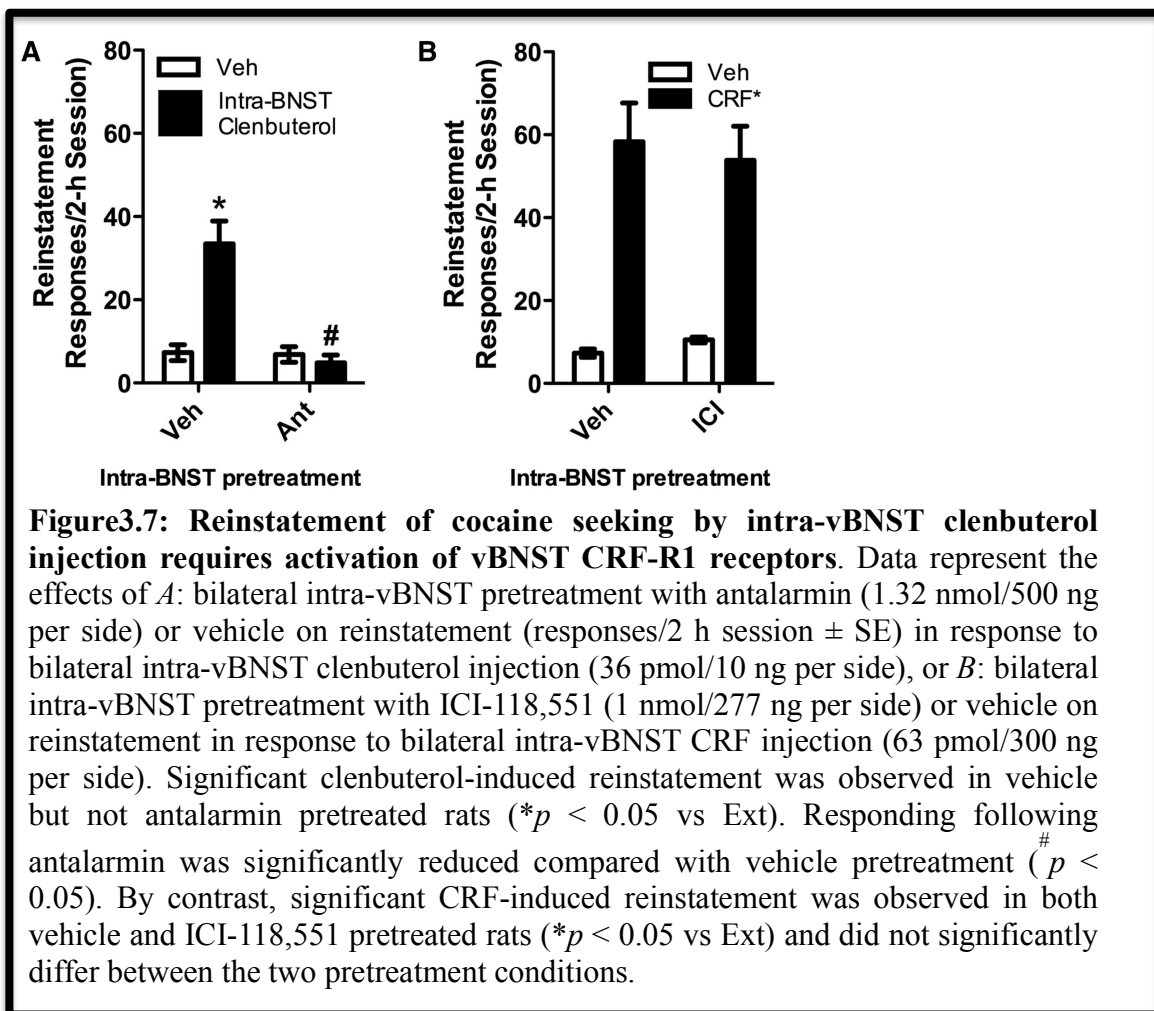


Effects of shock were specific to the previously active lever as shock failed to increase responding on a previously inactive lever in vehicle/vehicle-pretreated rats (Ext: 0.88 ± 0.35 responses vs shock: 1.11 ± 0.27). Neither ipsilateral nor contralateral manipulations affected inactive lever responding during reinstatement testing (vehicle/vehicle: 1.11 ± 0.27 responses; ipsilateral: 0.97 ± 0.66 ; contralateral: 0.93 ± 0.66).

Experiment 6: role of vBNST CRF-R1 receptors in cocaine seeking induced by vBNST β -2 AR activation

Because it has been reported that CRF receptors in the BNST are also required for stress-induced cocaine seeking (Erb and Stewart, 1999; Erb et al., 2001), and because BNST β ARs have been proposed to modulate efferent projections to the VTA via regulation of local CRF actions (Silberman et al., 2013), we hypothesized that reinstatement induced by vBNST β -2 AR activation requires local CRF receptor activation. To test this hypothesis we delivered antalarmin (1.32 nmol/500 ng per side) bilaterally into the vBNST before bilateral vBNST injection of clenbuterol (36 pmol/10 ng per side). As shown in **Figure 3.7**, intra-vBNST antalarmin delivery prevented intra-vBNST clenbuterol-induced cocaine seeking ($n = 7$). Two-way repeated-measures ANOVA showed a significant reinstatement effect ($F_{(1,6)} = 25.910$; $p < 0.01$) and a significant main effect of antalarmin pretreatment ($F_{(1,6)} = 29.883$; $p < 0.01$), as well as a significant reinstatement \times antalarmin pretreatment interaction ($F_{(1,6)} = 35.968$; $p < 0.01$). Clenbuterol reinstated cocaine seeking in vehicle but not antalarmin-pretreated rats ($p < 0.01$ vs Ext) and reinstatement was reduced in antalarmin- versus vehicle-pretreated rats

($p < 0.01$). By contrast, bilateral intra-vBNST pretreatment with the β -2 AR antagonist, ICI-118,551 (1 nmol/277 ng per side) failed to attenuate reinstatement in response to intra-vBNST CRF delivery (63 pmol/300 ng per side).



Two-way repeated-measures ANOVA showed an overall effect of CRF ($F_{(1,5)} = 46.925$) but no overall effect of ICI-118,551 pretreatment or no CRF \times ICI-118,551 interaction. Altogether, these findings indicate that CRF receptor activation is likely downstream from β -2 AR activation in the sequence of events responsible for stress-induced reinstatement. Effects of intra-BNST clenbuterol (2.14 ± 0.67 responses vs 1.38 ± 0.73

responses during extinction) and CRF (0.66 ± 0.42 vs 0.00 ± 0.00 responses during extinction) on previously inactive lever responding were not observed. Likewise, neither intra-BNST antalarmin (antalarmin/clenbuterol: 1.16 ± 0.16 responses) nor intra-BNST ICI-118,551 (ICI/CRF: 2.5 ± 1.93 responses) significantly altered inactive lever pressing during reinstatement.

Discussion

Our results extend earlier findings that the reinstatement of cocaine seeking by a stressor, electric footshock, requires CRF actions in the VTA (Wang et al., 2005; Blacktop et al., 2011) and vBNST β AR activation (Leri et al., 2002) by showing that vBNST β -2 AR activation is both necessary and sufficient for stress-induced reinstatement. Moreover, using a disconnection approach in which we block β -2 ARs in the vBNST of one hemisphere and VTA CRF-R1 receptors in the contralateral hemisphere, we demonstrate that vBNST β -2 ARs regulate a CRF-releasing pathway to the VTA that is necessary for stress-induced cocaine seeking. As VTA CTb delivery labels CRF-positive cells in the ipsilateral vBNST, it is likely that this pathway consists (at least partly) of vBNST CRF neurons that project directly to the VTA. Consistent with reports that CRF actions in the BNST are required for stressor-induced cocaine seeking (Erb and Stewart, 1999; Erb et al., 2001), we demonstrate that vBNST CRF-R1 activation is necessary for reinstatement induced by local delivery of the β -2 AR agonist, clenbuterol, suggesting that β -2 AR-mediated activation of this BNST-to-VTA pathway also requires local CRF receptor activation.

Findings from laboratory studies in human cocaine addicts (Jobes et al., 2011) and preclinical experiments in rodents (Erb et al., 2000; Mantsch et al., 2010) point to a role

for noradrenergic signaling in the stress-induced relapse to cocaine use. Specifically, our studies in mice suggest that β -2 ARs are critical for stress-induced cocaine seeking (Vranjkovic et al., 2012). As a target for ascending noradrenergic projections (Ricardo & Koh 1978, Weller & Smith 1982, Woulfe et al 1990) and a key site for integration of stress and reward networks (Flavin & Winder 2013, Stamatakis et al 2014), the BNST is a likely location at which stress-induced increases in norepinephrine regulate drug use. Although shock-induced increases in norepinephrine have not been reported, a variety of distinct stressors, including immobilization (Pacak et al 1995), visceral pain (Deyama et al 2009), and exposure to a shock-conditioned stimulus (Onaka & Yagi 1998), fox odor (Fendt et al 2005), or an aversive tastant (Park et al 2012), have been demonstrated to increase noradrenergic neurotransmission in the BNST. In particular, the vBNST receives very dense noradrenergic innervation (Egli et al 2005, Georges & Aston-Jones 2001, Woulfe et al 1990). Moreover, delivery of a mixture of β -1 and β -2 AR antagonists into the vBNST has been reported to prevent shock-induced reinstatement in rats (Leri et al., 2002). Our data extend these findings by demonstrating that β -2 but not β -1 AR activation in the vBNST is necessary for shock-induced cocaine seeking and that intra-vBNST injection of a β -2 AR- but not a β -1 AR-selective agonist is sufficient to reinstate.

Beta-2 ARs are expressed throughout the BNST (Cecchi et al., 2007). Whereas in the dorsal BNST β AR activation can have either excitatory or inhibitor effects on synaptic transmission, β AR effects in the vBNST are predominantly inhibitory (Egli et al., 2005) and include inhibition of neurons that project directly to the VTA (Dumont and Williams, 2004). In the dorsal BNST, β -AR regulation of neuronal activity requires CRF-R1 activation (Nobis et al., 2011). Although the involvement of CRF in norepinephrine-

dependent effects in the vBNST has not been established, our finding that local antalarmin pretreatment blocks reinstatement by intra-vBNST clenbuterol delivery supports a role for local CRF actions in the regulation of cocaine seeking by norepinephrine and β -2 AR activation. Consistent with this possibility, previous studies demonstrated that (1) noradrenergic terminals synapse on the dendrites of CRF-positive neurons in the vBNST(Phelix et al 1994) (2) β -2 AR-mediated reinstatement requires CRF-R1 activation(McReynolds et al 2014), and (3) the β -2 AR antagonist, ICI-118,551, blocks stress-induced increases of BNST CRF mRNA (McReynolds et al., 2014). Overall, the findings are consistent with reports that cocaine seeking induced by central norepinephrine delivery is blocked by CRF receptor antagonism, whereas suppression of noradrenergic function using the α -2 AR agonist, clonidine, does not affect reinstatement in response to central CRF delivery(Brown et al 2009).

Notably, the magnitude of cocaine seeking in response to intra-vBNST clenbuterol was lower than that observed following either shock or CRF. This could be attributed to (1) secondary effects of BNST β -2 AR activation that are not engaged by shock or CRF and offset responding, (2) unique attributes of clenbuterol itself (e.g., pharmacokinetics, ligand-specific receptor desensitization), or (3) the requirement for additional stress-responsive mediators to fully engage the processes that lead to cocaine seeking.

Our findings suggest that the vBNST influences cocaine seeking via regulation of the VTA. Consistent with this possibility, stress-induced reinstatement in mice is associated with activation of VTA-projecting BNST neurons (Briand et al., 2010) and a role for a vBNST-VTA pathway in the expression of cocaine seeking has been reported

(Sartor & Aston-Jones 2012). In light of these findings, it is surprising that vBNST β -ARs have been found to exert inhibitory effects on neurons that project to the VTA, likely via stimulation of local GABA release (Dumont and Williams, 2004). Both GABAergic and glutamatergic projections from the BNST to the VTA have been identified (Georges & Aston-Jones 2001, Georges & Aston-Jones 2002, Jennings et al 2013, Kudo et al 2012, Sartor & Aston-Jones 2012). Moreover, CRF-positive terminals within the VTA coexpress either the glutamatergic neuronal marker, vesicular glutamate transporter 2, or the GABAergic neuronal marker, glutamic acid decarboxylase, and make contacts that have morphological characteristics of either excitatory or inhibitory synapses, suggesting that CRF can be coreleased into the VTA along with either GABA or glutamate (Tagliaferro & Morales 2008). Further investigation is needed to (1) confirm the neurochemical phenotype of VTA-projecting vBNST CRF-positive neurons, (2) clarify the mechanism through which β -2 ARs regulate these neurons and determine how this regulation might change following cocaine exposure, and (3) examine the likelihood that β -2 AR in the vBNST may also regulate CRF and/or CRF actions in the VTA via parallel multisynaptic pathways. Consistent with the last possibility, BNST efferents can influence the VTA through multisynaptic pathways that include regions, such as the lateral habenula and rostromedial tegmental area (Dong & Swanson 2006, Kaufling et al 2009, Lammel et al 2012).

It has been found that (1) shock elevates VTA CRF levels (Wang et al., 2005), (2) intra-VTA CRF delivery is sufficient to induce cocaine seeking (Wang et al., 2005; Blacktop et al., 2011), and (3) VTA CRF receptor activation is required for stress-induced reinstatement (Wang et al., 2005; Blacktop et al., 2011) in rats. Our

demonstration that intra-VTA antalarmin delivery prevents shock-induced reinstatement is consistent with these reports. As the antalarmin dose used may also block CRF-R2 receptors, our findings are not definitive regarding which CRF receptor subtype is involved. However, we have reported that, in rats tested under identical conditions, intra-VTA delivery of a CRF-R2-selective antagonist does not prevent shock-induced reinstatement, whereas intra-VTA injection of a CRF-R1 but not a CRF-R2-selective agonist is sufficient to induce cocaine seeking (Blacktop et al., 2011).

CRF actions in the VTA are complex and involve both excitatory and inhibitory effects that vary depending on the receptor and site (Beckstead et al 2009, Hahn et al 2009, Riegel & Williams 2008, Ungless et al 2003, Wanat et al 2013, Wanat et al 2008). Likely VTA targets for CRF include mesocortical DA cells, which are activated during stress (Deutch et al 1991). Elevated medial prefrontal cortex DA, via activation of DA receptors, is necessary for stress-induced reinstatement (Capriles et al., 2003; McFarland et al., 2004). We demonstrate that the mechanisms through which vBNST β -2 ARs regulate this CRF projection to the VTA requires local CRF actions, likely at CRF-R1. This finding is consistent with reports that (1) depolarization of BNST neurons by the nonselective β -AR agonist, isoproterenol, is CRF-dependent (Nobis et al., 2011), (2) CRF in the BNST depolarizes neurons that project to the VTA (Silberman et al., 2013), and (3) stress-induced increases in CRF mRNA in the BNST in mice are β -2 AR-dependent (McReynolds et al., 2014). Moreover it is consistent with reports that intra-BNST antalarmin delivery prevents stress-induced cocaine seeking (Erb and Stewart, 1999; Erb et al., 2001). Although the requirement for vBNST CRF is evident, the source of this CRF has not been identified and could include intrinsic cell populations within the BNST

and/or CRF-releasing projections originating in other regions (e.g., the central amygdala). A clearer mechanistic understanding awaits determination of BNST β -2 AR receptor localization as it relates to that of CRF and CRF-R1.

Although it is likely that the mechanisms through which stress promotes relapse are not identical across drug classes, both CRF-R1 receptor activation (Bruijnzeel et al 2009, Deutch et al 1991, Le et al 2000, Shaham et al 1997) and central noradrenergic signaling (Erb et al 2000, Le et al 2005, Shaham et al 2000, Yamada & Bruijnzeel 2011) are necessary for stress-induced reinstatement of heroin-, nicotine-, and alcohol-seeking in rats. Moreover, in the case of heroin, stress-induced drug seeking requires CRF (Wang et al 2006) and noradrenergic (Wang et al 2001) signaling within the BNST. Notably, similar processes in the BNST may be engaged during withdrawal from ethanol (Francesconi et al 2009, Huang et al 2010, Silberman et al 2013) and opioids (Delfs et al 2000, Fuentealba et al 2000), suggesting that this pathway to the VTA, or parallel pathways originating in other BNST subregions may also contribute to withdrawal-related drug seeking.

To summarize, we report that, during stress, norepinephrine released into the vBNST activates β -2 ARs, which via a CRF-dependent process, activate neurons that release CRF into the VTA, thereby leading to cocaine seeking. Identification of the processes responsible for stress-induced cocaine seeking should guide the development of new treatment approaches aimed at managing relapse, particularly in individuals whose use is stress-related.

Chapter IV

Enhanced CRFR1 receptor-dependent regulation of a ventral tegmental area to prelimbic cortex projection establishes susceptibility to stress-induced cocaine seeking

ABSTRACT

The ability of stress to trigger cocaine seeking in humans and rodents is variable and is determined, in part, by the amount and pattern of prior drug use. This study examined the role of a corticotropin releasing factor- (CRF-) regulated dopaminergic projection from the ventral tegmental area (VTA) to the prelimbic cortex in shock-induced cocaine seeking and its recruitment under self-administration conditions that establish relapse vulnerability. Rats with a history of daily long-access (LgA; 14 x 6 hrs/day) but not short-access (ShA; 14 x 2 hrs/day) self-administration showed robust shock-induced cocaine seeking. This was associated with a heightened shock-induced prelimbic cortex Fos response and activation of VTA neurons that project to the prelimbic cortex, as defined by Fos co-labeling with the retrograde tracer, cholera toxin B. Both shock-induced reinstatement and the prelimbic cortex Fos response were prevented by bilateral intra-VTA injections of the CRFR1 receptor antagonist, antalarmin. Pharmacological disconnection of the CRF-regulated dopaminergic projection to the prelimbic cortex by injection of antalarmin into the VTA in one hemisphere and the D1R antagonist SCH23390 into the prelimbic cortex of the contralateral hemisphere prevented shock-induced cocaine seeking, while antagonist administration within the same hemisphere or disconnection of the VTA projection to infralimbic cortex was without effect. LgA, but not ShA, cocaine self-administration resulted in increased CRFR1 mRNA levels in the VTA as measured using in situ

hybridization. Altogether, these findings suggest that excessive cocaine use establishes susceptibility to stress-induced relapse by recruiting CRF regulation of a key stressor-responsive mesocortical dopaminergic pathway.

INTRODUCTION

Relapse to drug use remains a barrier to the effective treatment of cocaine addiction. While a number of stimuli can elicit relapse, stress is a particularly problematic relapse trigger due to its unpredictable and often unavoidable nature. Stress-induced relapse is studied in rats using the self-administration (SA)/reinstatement approach in which the ability of a stressor, most commonly electric footshock, to re-establish extinguished cocaine-directed lever pressing is assessed (Mantsch et al., 2015). Studies using this approach have begun to define the neurocircuitry and mechanisms that contribute to stress-related relapse and have demonstrated involvement of dopamine D1 receptor activation in the prelimbic cortex (Capriles et al 2003; McFarland et al 2004) and corticotropin releasing factor (CRF) activation of CRFR1 receptors in the ventral tegmental area (VTA; Blacktop et al 2011). Based on these findings, a role for a CRF-regulated dopamine projection from the VTA to the prelimbic cortex in stress-induced cocaine seeking has been proposed but has not been directly demonstrated.

The mesocortical dopamine system has long been known to be highly responsive to stress (Thierry et al., 1976; Reinhard Jr et al 1982; Deutch et al 1985; Speciale et al 1986). Moreover, VTA dopamine cells that project to the prelimbic cortex are activated by stressors, as measured *ex vivo* using Fos immunoreactivity or slice electrophysiology in neurons labeled by retrograde tracers injected into the prelimbic cortex (Deutch et al 1991; Lammel et al 2011). Although studies examining the regulation of mesocortical

dopamine by VTA CRF have produced mixed results (see e.g., Kalivas et al., 1987), reports that icv CRF increases dopaminergic neurotransmission in the prefrontal cortex (Dunn and Berridge, 1987; Lavicky and Dunn, 1993) and that CRF promotes excitation of VTA dopamine neurons via both pre- and post-synaptic mechanisms (Ungless et al 2003; Korotkova et al 2006; Riegel and Williams 2008; Wanat et al 2008, Beckstead et al 2009; Hahn et al 2009, Williams et al 2014) suggest that stress-induced CRF release into the VTA may underlie increases in mesocorticolimbic dopamine release. Consistent with this possibility, Refojo et al (2011) reported that selective deletion of CRFR1 receptors in VTA dopamine neurons significantly reduced stress-induced increases in dopamine in the prefrontal cortex in mice.

Evidence that CRF regulation of mesocortical dopamine is responsible for stress-induced cocaine seeking is indirect. Stress-induced reinstatement is associated with elevated extracellular CRF in the VTA (Wang et al., 2005) and extracellular dopamine in the prelimbic cortex (McFarland et al 2004) and VTA (likely reflecting somatodendritic release; Wang et al, 2005). Moreover, CRF receptor antagonism in the VTA prevents both stress-induced cocaine seeking (Wang et al, 2005; Blacktop et al 2011) and increases in VTA dopamine (Wang et al, 2005), while stress-induced cocaine seeking is prevented by D1 receptor antagonism in the prelimbic cortex (Capriles et al 2003; McFarland et al 2004). In this study we more directly test the hypothesis that stress-induced reinstatement requires CRF regulation of a dopaminergic pathway from the VTA to the prelimbic cortex.

We have found that stress-induced cocaine seeking depends on the prior history of cocaine use. Under our experimental protocol, footshock induces reinstatement in rats

with a history of long-access (LgA; 14 x 6 hrs/day) but not short-access (ShA; 14 x 2 hrs/day) cocaine SA (Mantsch et al 2008). Moreover, cocaine seeking in response to intra-VTA CRF delivery is also only observed following SA under LgA conditions (Blacktop et al., 2011), suggesting that cocaine use promotes stress-induced cocaine seeking by recruiting CRF regulation of the mesocortical pathway. Although a role for VTA CRF-R2 receptors in stress-induced cocaine seeking has been reported (Wang et al 2005), we and others have demonstrated a role for CRFR1 receptors (Blacktop et al 2011; Chen et al 2014). Therefore, in the present study we also test the hypothesis that stress regulation of the mesocortical dopamine pathway is recruited following LgA SA as a result of increased VTA CRFR1 receptor expression.

MATERIALS AND METHODS

Subjects

Adult male Sprague Dawley rats (Harlan Laboratories) ~ 90-d old at the time of delivery (325g) were housed individually in a temperature- and humidity-controlled, Association for Assessment and Accreditation of Laboratory Animal Care accredited facility under a 12 h/12h dark/light cycle (lights off at 700h) and had access to food and water at all times, except during food training periods during which they were maintained at 90% of their free-feeding body weight. All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals.

Drugs

Cocaine HCl was acquired from the National Institute on Drug Abuse through its Drug Supply Program. The selective dopamine D1 receptor antagonist SCH-23390 and the CRFR1 receptor antagonist antalarmin were purchased from Sigma-Aldrich. All drugs were dissolved in bacteriostatic saline. Intracranial drug doses were selected based

on previously published results (Blacktop et al 2011; Capriles et al 2003; Vranjkovic et al 2014).

Self-Administration and Extinction

All rats in the study underwent cocaine or saline self-administration followed by extinction training. Rats self-administered cocaine/saline through indwelling jugular catheters implanted under ketamine HCl (100mg/kg i.p.) and xylazine (2mg/kg, i.p.) anesthesia, as described previously (Blacktop et al., 2011; Vranjkovic et al., 2014). Following a 5-day post-surgery recovery period, rats were trained to self-administer cocaine (0.5 mg/kg/inf, i.v.) by pressing a lever under a FR1 schedule of reinforcement in computer-interfaced operant conditioning chambers (Med Associates) during daily 2-h sessions. During these sessions, the active lever was extended into the chamber and the corresponding light was illuminated. Pressing this lever resulted in activation of a pump, which infused cocaine (200 μ l over 5 sec) followed by a 5-sec time-out phase during which the stimulus light was extinguished, but the lever remained extended. Responding during the time-out phase did not activate the pump. Responding on a second (inactive) lever was also recorded but had no consequences. Once stable self-administration under the FR1 schedule was observed (>15 infusions for three consecutive days), the criterion for self-administration was gradually increased until rats displayed stable responding under a FR4 schedule (within 10% of the 3-session mean) at which time they entered into self-administration testing. Depending on the experiment and experimental group, the conditions under which self-administered cocaine during the 14-day testing phase varied. Some rats continued to have access to cocaine (0.5 mg/kg/inf) during 2-h daily sessions (short-access; ShA rats). Some rats were provided daily access to cocaine (0.5 mg/kg/inf) during 6-h sessions (long-access; LgA rats). Saline control rats were provided

daily access to saline during 2-h sessions (Sal rats). Following completion of the 14-day self-administration phase, rats underwent extinction training. During the 2-h extinction training sessions, the cocaine solution was replaced with saline (saline control rats continued to have access to saline). The experimental conditions were otherwise identical to those during self-administration. Depending on the experiment, extinction training either continued for ten days (Experiments 1, 2, 3, and 5) or until a response based criterion was reached (<15 active lever responses two consecutive sessions; Experiment 4). Following extinction training, rats were tested for shock-induced reinstatement of cocaine seeking and/or were killed and their brains processed for immunohistochemistry or in situ hybridization.

Shock-Induced Reinstatement

With the exception of rats in Experiment #5, all rats were tested for shock-induced reinstatement of extinguished lever pressing. The 2-hr reinstatement sessions were identical to the extinction sessions except that they were preceded by the delivery of intermittent mild non-noxious footshock, in some cases following intracranial drug injections. Shocks (0.5mA, 0.5sec duration) were delivered on average every 40 seconds (range 10- 70 sec) throughout the grid floors of the self-administration chambers over a 15 min period that immediately preceded the 2hr reinstatement test session (Blacktop et al., 2011; Vranjkovic et al., 2014). During the shock period, the levers were retracted and the stimulus lights were not illuminated; the levers were extended into the chamber and the lights turned on immediately following the shock period. Reinstatement was defined as an increase in active lever responding relative to the prior extinction session.

Statistical Analyses

Statistical analyses were conducted using Predictive Analytics SoftWare statistics software (SPSS, Inc.). Statistical significance was determined using ANOVA followed, when appropriate, by further analyses of main effects using ANOVA and/or post-hoc testing using Bonferroni-corrected t-tests.

Experiment #1: Relationship between prefrontal cortical Fos immunoreactivity and shock-induced reinstatement of cocaine seeking.

We have previously shown that robust shock-induced reinstatement of extinguished cocaine seeking is observed following daily long-access (LgA; 6 hrs) but not short-access (ShA; 2 hrs) cocaine self-administration (Mantsch et al., 2008). To examine the relationship between shock-induced cocaine seeking and activation of the prelimbic and infralimbic regions of the prefrontal cortex, separate groups of rats underwent 14 days of self-administration testing under ShA (14 x 0.5 mg/kg/inf cocaine; 2 hrs/day) or LgA (14 x 0.5 mg/kg/inf cocaine; 6 hrs/day) conditions or were provided access to saline for two hours daily (14 x saline; 2 hrs/day; Sal). Following self-administration, rats received extinction training (2 hrs daily, as described above). As it has been reported that many cocaine-induced neurobiological alterations that contribute to drug seeking depend on the time period that has elapsed following self-administration (Grimm et al., 2001; Conrad et al., 2009; Ma et al., 2014), for these experiments we chose not to use extinction criteria for reinstatement testing/Fos determination. Rather, all rats underwent extinction training for ten consecutive days. Following extinction, all rats were tested for reinstatement in response to shock (ShA rats, n=8; LgA rats, n=7; Sal rats, n=10) or under shock-free conditions (ShA rats, n=10; LgA rats, n=10; Sal rats, n=10) and, immediately after the 2-h reinstatement session, were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and perfused transcardially with 0.9% NaCl,

followed by 60 ml of cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Brains were removed and post-fixed in paraformaldehyde over-night at 4°C. Brains were then washed twice in 0.1M sodium phosphate buffers for 12 hrs. Afterwards, brains were cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 3 days at 4°C. Brains were frozen and serial coronal sections (25 µm) containing the prelimbic and infralimbic cortices (+3.24 to 2.54) were cut at 200 µm intervals on a freezing microtome and placed in cryoprotectant. Sections were stored at -20°C until immunohistochemistry procedures were conducted. Immunohistochemical analysis of Fos expression was performed on free-floating sections by using an avidin-biotin peroxidase protocol (Hoffman et al., 2008). Fos immunolocalization was visualized using the primary polyclonal rabbit anti-Fos antibody (sc-52, Santa Cruz Biotechnology; 1:10,000). Free-floating sections were washed 3 times for 5 min in 0.5M KPBS and then incubated in 2% hydrogen peroxide in 0.5MKPBS containing 0.4% triton for 15 min to block endogenous peroxidase activity. Sections were washed three more times and then incubated in 3% natural donkey serum for one hour. Afterwards the sections were incubated in the primary antibody containing 0.4% triton X-100 for 24 hours in 4°C. Next, sections were washed again, and then incubated for one hour in biotinylated horse anti-rabbit IgG secondary antibody (PI-1000; Vector Laboratories; 1:600). Sections were washed once again and then incubated for one hour in ABC solution (PK-4000; Vectastain Elite ABC kit; Vector Laboratories). After another series of washes, sections were incubated 3 times for 5min each in 0.175 M sodium acetate (7.0 pH). Sections were treated with a nickel-enhanced diaminobenzidine method (black-nuclear reaction) (SK-4100 DAB; Vector Laboratories) and the sections were again incubated in 0.175 M sodium acetate and then washed in KPBS. Tissue was

then mounted on super-frost slides and dehydrated the next day. Fos immunoreactivity was quantified using Image J software. The total numbers of Fos-positive cells within each cortical region (i.e., the sum across regions all sections) were determined and compared across groups.

Experiment #2: Role of VTA CRFR1 receptors in shock-induced cocaine seeking and cortical subregion activation

Rats were implanted with jugular catheters bilateral cannula (11-mm, 22-gauge) aimed at the caudal VTA (5.6 mm posterior to bregma; 2.2 mm lateral to midline; 6.9 ventral to skull surface at a 12° angle; Paxinos and Watson, 2006) and underwent cocaine self-administration under LgA conditions and extinction prior to testing for reinstatement in response to shock or under shock-free conditions. Prior to the 2-hour reinstatement session, rats received bilateral intra-VTA injections with the CRFR1 receptor antagonist, antalarmin (1.32 nmol/500 ng/side; n=9) or vehicle (n=8). We (Blacktop et al., 2011; Vranjkovic et al., 2014) and others (e.g., Lowery-Gionta et al., 2012) have used this antalarmin dose to investigate CRF-R1 receptor-dependent contributions to behavior. Microinfusions were delivered in a volume of 0.25 µl/side over a 1-min period followed by an additional 1-min period to allow for drug diffusion, ten minutes prior to the 15-min shock or shock-free period. Immediately after the 2-hr session, rats were processed (as described for Exp #1) for immunohistochemical analysis of Fos expression in the prelimbic and infralimbic cortices. The accuracy of cannula implantation into the VTA was confirmed post-mortem following staining of sections containing VTA with cresyl violet for examination using a light microscope. Rats with injection sites outside of the targeted regions were excluded from data analysis.

Experiment #3: Relationship between Fos immunoreactivity in VTA neurons that project to the prelimbic cortex and shock-induced reinstatement of cocaine seeking.

To determine if activation of VTA neurons that project to the prelimbic cortex is associated with shock-induced reinstatement of extinguished cocaine seeking, rats underwent LgA self-administration for 14 days and received bilateral injections with the retrograde tracer cholera toxin b subunit (CTb) into the prelimbic cortex. Rats were anesthetized with ketamine and xylazine and placed in a stereotaxic frame. Heat-sterilized Hamilton syringes were used to pressure inject 0.2 μ l biotinylated CTb (10%; List Biological Laboratories; #104; Campbell, CA) unilaterally into the prelimbic cortex over a 10-min period (at a 6 degree angle anterior to Bregma 3.2 mm; lateral 0.4 mm; ventral 3.2 mm; Paxinos and Watson, 2006). Syringes were left in place for 20 min after injections and slowly removed from the brain over a 10-min period. Following injections, rats were allowed to recover for seven days prior to undergoing daily extinction training until the extinction criterion was reached (<15 responses for 3 consecutive sessions) at which point they were tested for cocaine seeking in response to shock (n=6) or under shock-free conditions (n=5). Immediately following the 2-h reinstatement session, rats were processed (as described above) for CTb/Fos labeling of VTA neurons using dual immunohistochemistry. Fos was visualized using a nickel-enhanced DAB reaction as described above. CTb was visualized by similar methods except for the addition of an avidin-biotin blocking step (SP-2100) prior to incubation with the CTb primary antibody (List Biology #703; 1:8000). After the 24-hour incubation period, sections were washed and incubated in horse anti-goat secondary antibody (PI-9500; Vector Laboratories; 1:1,000). Sections were washed again and incubated for one hour in ABC solution. Sections were then washed and treated with sodium acetate and were treated using the DAB method (brown cytoplasmic staining) for

visualization of CTb. Sections were washed and then mounted on super-frost slides and dehydrated the next day. Photomicrographs were acquired using a Retiga 2000R digital camera (QImaging) on a Nikon 80i microscope using NIS Elements software (Nikon Instruments). Fos-, CTb- and dual Fos/CTb- immunoreactive cells were identified and counted using Image J software. Using a similar approach, it has been previously reported that restraint stress selectively increases Fos expression in VTA neurons that project to the prelimbic cortex in rats (Deutch et al., 1991).

Experiment #4: Effect of pharmacological disconnection of the CRF-regulated dopaminergic pathway from the VTA to the prelimbic cortex on shock-induced cocaine seeking.

A disconnection approach was used to determine whether a CRFR1 receptor-activated dopaminergic projection from the VTA to the prelimbic cortex is required for stress-induced cocaine seeking. At the time of catheterization, separate groups of rats were implanted with two cannulae each aimed unilaterally at the VTA (at a 12 degree angle posterior to Bregma -5.6 mm; lateral 2.2 mm; ventral -6.9 mm; Paxinos and Watson, 2006) or the prelimbic cortex (at a 6 degree angle anterior to Bregma 3.2 mm; lateral 0.4 mm; ventral 3.2 mm; Paxinos and Watson, 2006) in either the contralateral or ipsilateral hemisphere(s) of the brain. The effect of pathway disconnection was examined by injection of the CRF-R1 receptor antagonist antalarmin (1.32 nmol/500 ng) into the VTA in one hemisphere and the dopamine D1 receptor antagonist SCH23390 (1.38mM/200 ng) into the prelimbic cortex of the other prior to testing for shock-induced reinstatement (n=5). For comparison, the same rats were tested for shock-induced reinstatement following injection of vehicle into each region. As a control to ensure that reductions in reinstatement were attributable to disconnection, a second groups of rats (n=4) received a unilateral injection of antalarmin into the VTA in one hemisphere and a unilateral

injection of SCH23390 into the prelimbic cortex in the same hemisphere prior to testing for shock-induced reinstatement. These rats were also tested for the effects of ipsilateral vehicle injections. For this experiment, the hemispheres into which cannulae were implanted were randomized across rats in each group such that in the contralateral treatment group, half of the rats received antalarmin injections into the left VTA and SCH23390 injections into the right prelimbic cortex, while the other half received antalarmin injections into the right VTA and SCH23390 injections into the left prelimbic cortex. Likewise, half of the rats in the ipsilateral treatment group received drug injections into sites in the right hemisphere, while the remaining rats received injections into sites in the left hemisphere. A third group of rats (n=4) were implanted with cannulae for disconnection of CRFR1-regulated VTA dopaminergic inputs into the infralimbic cortex. As with the prelimbic cortex disconnection experiment, these rats received an antalarmin injection into the VTA of one hemisphere and a SCH23390 injection into the infralimbic cortex of the other. The accuracy of cannula implantation was confirmed post-mortem in each rat after cardiac perfusion with 60-ml 0.15% NaCl followed by 60-ml 2.5% buffered neutral formalin under sodium pentobarbital anesthesia (55 mg/kg). Brains were removed and stored in 2.5% buffered formalin prior to vibratome sectioning (40 μ m), slide mounting, and staining with cresyl violet for examination using a light microscope. Rats with injection sites outside of the targeted regions were excluded from data analysis.

Experiment #5: Effects of cocaine self-administration under conditions that promote shock-induced cocaine seeking on CRFR1 mRNA in the VTA

We previously reported that intra-VTA CRF injections reinstate cocaine seeking in rats with a history of LgA, but not ShA self-administration (Blacktop et al., 2011). To

determine if LgA self-administration recruits CRFR1-dependent regulation of VTA dopamine neurons that project to the prelimbic cortex to increase susceptibility to shock-induced cocaine seeking, we used in situ hybridization to examine differences in the expression of CRFR1 mRNA within the VTA resulting from self-administration under ShA (n=8), LgA (n=8) and saline control (n=8) conditions. All rats were tested under their respective conditions and underwent ten days of extinction. 24 hrs after the final extinction session brains were extracted following rapid decapitation in the absence of anesthesia and were placed in methyl-2-butane for one minute at a temperature between -25 and -35°C, and placed in a -80°C freezer until sectioning. The tissue was sectioned on a cryostat to 14 µm thickness and placed onto superfrost plus slides and stored in a -80 °C freezer until in situ hybridization analysis was conducted. A 1138 bp fragment of the rat CRF-R1 cDNA (NCBI NM_030999, nucleotides 99-1236) was isolated by PCR from rat cortex cDNA and subcloned into pCRII-TOPO (Invitrogen) to create pCRII-TOPO-rCRHR1-1. For antisense riboprobe synthesis, the pCRII-TOPO-rCRHR1-1 plasmid was linearized with BamHI and transcribed with T7 RNA polymerase (Promega). The riboprobe was double labeled with ³⁵S-CTP and ³⁵S-UTP (Perkin-Elmer; 1250 Ci/mmol) and free nucleotides were removed using a Micro Bio-Spin column (Bio-Rad). In situ hybridization was performed as previously described with minimal modifications (Speert et al, 2002, Westphal et al 2010). Briefly, slides were post-fixed for 1 h in 4% phosphate-buffered paraformaldehyde and washed three times in 2X SSC. Sections were incubated in 0.1M triethanolamine containing 0.25% acetic anhydride for 10 minutes with stirring, rinsed in dH₂O, dehydrated and air-dried. Slides were then hybridized overnight at 55°C with the ³⁵S-labeled CRHR1 riboprobe (2x10⁶ cpm/slide) in 50% formamide

hybridization cocktail (Amersco) containing 10 mM DTT. After hybridization, excess unhybridized probe was removed by 2X SSC washes and by incubating slides in RNase A (37 C, 1 h). Slides were then washed in decreasing salt solutions (2x, 1x, and 0.5x SSC) before a final high-stringency wash in 0.1x SSC (65 C, 1 h). Slides were then dehydrated in ethanol, air-dried, and apposed to Kodak BioMax MR autoradiography film (Carestream) for 7 days. Four to five slides (1 slide/series with 4 sections/slide) from each rat were included and were processed simultaneously to allow direct comparisons in the same regions and minimize variation. Autoradiography films were scanned and analyzed using densitometry in ImageJ (Version 1.48g5). Signal pixels within the area of interest were defined as having a gray value of 3.5 standard deviations above the mean gray value of the background region. Both the number of pixels (area) and average gray values (mean optic density/OD) above background were determined and multiplied to generate an integrated densitometric measurement (integrated OD). CRFR1 signal from multiple sections (spanning bregma -5.2 to -6.4) was analyzed and averaged to generate one single integrated OD per region per rat/per rat. Based on prior functional/anatomical distinctions (Oades et al., 1987), the VTA was subdivided into rostral (-5.2 to -5.6 mm bregma) and caudal regions (-5.6 to -6.2 bregma). The caudal VTA did not include the VTA tail (rostromedial tegmental area; -6.6 to -6.9 mm bregma (Jhoun et al., 2009; Matsui and Williams, 2011; Barrot et al., 2012).

RESULTS

Cocaine self-administration and extinction

Self-administration (infusions on days 1 and 14 of self-administration testing and total mg/kg cocaine intake across all sessions) and extinction data (responses during the final 2-h session prior) for each experiment are shown in Table 1.

Table 1

<i>Exp.</i>	<i>Group</i>	<i>SA (infusions/session ± S.E.)</i>		<i>Total Intake (mg/kg)</i>	<i>Last Ext (resp/session ± S.E.)</i>
		<i>SA Day 1</i>	<i>SA Day 14</i>		
<i>Exp. 1</i>	Saline/No Shock	14.2 ± 3.5	4.3 ± 0.9	---	6. ± 1.58
	Saline/Shock	9.9 ± 1.2	3.8 ± 6.7	---	5.3 ± 1.2
	ShA Coc/No Shock	27.9 ± 1.5	18.4 ± 4.7	354.9 ± 19.8	9.4 ± 2.8
	ShA Coc/Shock	27.7 ± 1.3	22.7 ± 4.7	363.8 ± 31.4	5.1 ± 1.4
	LgA Coc/No Shock	79.3 ± 3.9	82.8 ± 9.2	1062.3 ± 83.1	11.7 ± 2.3
	LgA Coc/Shock	75.1 ± 3.5	83.7 ± 7.7	1027.0 ± 77.2	11.5 ± 3.0
<i>Exp. 2</i>	Vehicle/No Shock	83.6 ± 4.3	91.6 ± 6.3	1007.0 ± 48.0	8.0 ± 1.8
	Vehicle/Shock	76.0 ± 9.1	94.0 ± 4.9	1092.3 ± 107.5	8.0 ± 1.8
	Antalarmin/No Shock	78.0 ± 9.0	105.0 ± 11.2	1076.3 ± 109.4	6.8 ± 2.4
	Antalarmin/Shock	90.0 ± 5.0	94.0 ± 4.9	1126.6 ± 115.1	8.0 ± 2.8
<i>Exp. 3</i>	Shock	58.4 ± 8.2	80.4 ± 9.4	887.2 ± 98.7	2.8 ± 1.8
	No Shock	71.8 ± 9.0	88.0 ± 10.6	1086.6 ± 60.0	9.3 ± 2.4
<i>Exp. 4</i>	Prelimbic/contralateral	62.6 ± 4.6	74.8 ± 5.3	907.6 ± 77.8	5.8 ± 2.2
	Prelimbic/ipsilateral	68.0 ± 5.9	92.3 ± 3.5	1063.75 ± 94.2	8.3 ± 1.9
	Infralimbic/contralateral	68.5 ± 3.1	77.8 ± 3.7	959.8 ± 67.5	7.3 ± 3.3
<i>Exp. 5</i>	Saline	3.6 ± 1.8	3.3 ± 0.8	---	3.2 ± 0.9
	ShA Coc	28.7 ± 2.6	21.2 ± 4.0	348.2 ± 52.5	26.7 ± 7.7
	LgA Coc	79.3 ± 5.9	77.6 ± 10.8	994.1 ± 69.5	20.2 ± 6.6

Experiment #1: Relationship between prefrontal cortical Fos immunoreactivity and shock-induced reinstatement of cocaine seeking

Rats underwent daily cocaine self-administration for 14 days under ShA (2 hrs/day; total intake ~359.4 mg/kg) or LgA (6 hrs/day; total intake ~1045 mg/kg) or were provided access to saline for self-administration, prior to undergoing ten days of extinction after which they were tested for cocaine seeking under shock-free conditions or following shock exposure and their brains were processed for cortical Fos analysis. Shock-induced cocaine seeking is shown in Figure 1A. Similar to previous reports (Mantsch et al., 2008), robust shock-induced reinstatement was observed in rats

following LgA cocaine self-administration but not following ShA self-administration and shock did not increase lever-pressing in control rats provided access to saline. A 3-way ANOVA reinstatement testing (extinction vs. reinstatement test; repeated measure) x self-administration (ShA, LgA, vs. Sal) x shock condition (shock vs. no shock) ANOVA showed a significant interaction among all three variables ($F_{2,38}= 12.67$; $p<0.001$). Post-hoc testing showed that shock-induced cocaine seeking, as defined by increased lever pressing relative to the prior extinction session and compared to non-shock controls, was only observed following long-access/high-intake self-administration ($p<0.01$ for each comparison). Moreover, cocaine seeking following shock was significantly higher following LgA self-administration than it was after ShA self-administration or in saline controls ($p<0.01$ for each comparison).

The numbers of Fos-positive cells in the prelimbic and infralimbic cortex following behavioral testing under shock and shock-free conditions were recorded in each group and are shown in Figures 1C (prelimbic cortex) and 1D (infralimbic cortex). Figure 1E represents a schematic identifying the regions in which Fos immunoreactivity was analyzed (Paxinos and Watson, 2013). Representative immuno-labeled sections including the cortical regions of interest from each condition/group are shown in Figures 1E-1L. A 2-way shock x SA condition ANOVA showed a significant overall effect of shock ($F_{1,45}=51.08$; $p<0.001$) and self-administration condition on the number of Fos-positive cells in the prelimbic cortex ($F_{2,45}=8.310$; $P=0.001$) and a significant shock x self-administration condition interaction ($F_{2,46}=12.828$; $p<0.001$). Post-hoc testing showed that shock increased the number of Fos-positive cells in rats tested under each condition ($p<0.05$ vs. No Shock). This increase was significantly greater in rats tested

under longer-access/high-intake conditions ($p < 0.001$ vs. Sal and ShA). Shock also modestly but significantly increased the number of Fos immunoreactive cells in the infralimbic cortex (2-way ANOVA; overall effect of shock; $F_{1,45} = 28.08$; $p < 0.001$). However, no overall effect of SA condition or SA condition x shock interaction was observed.

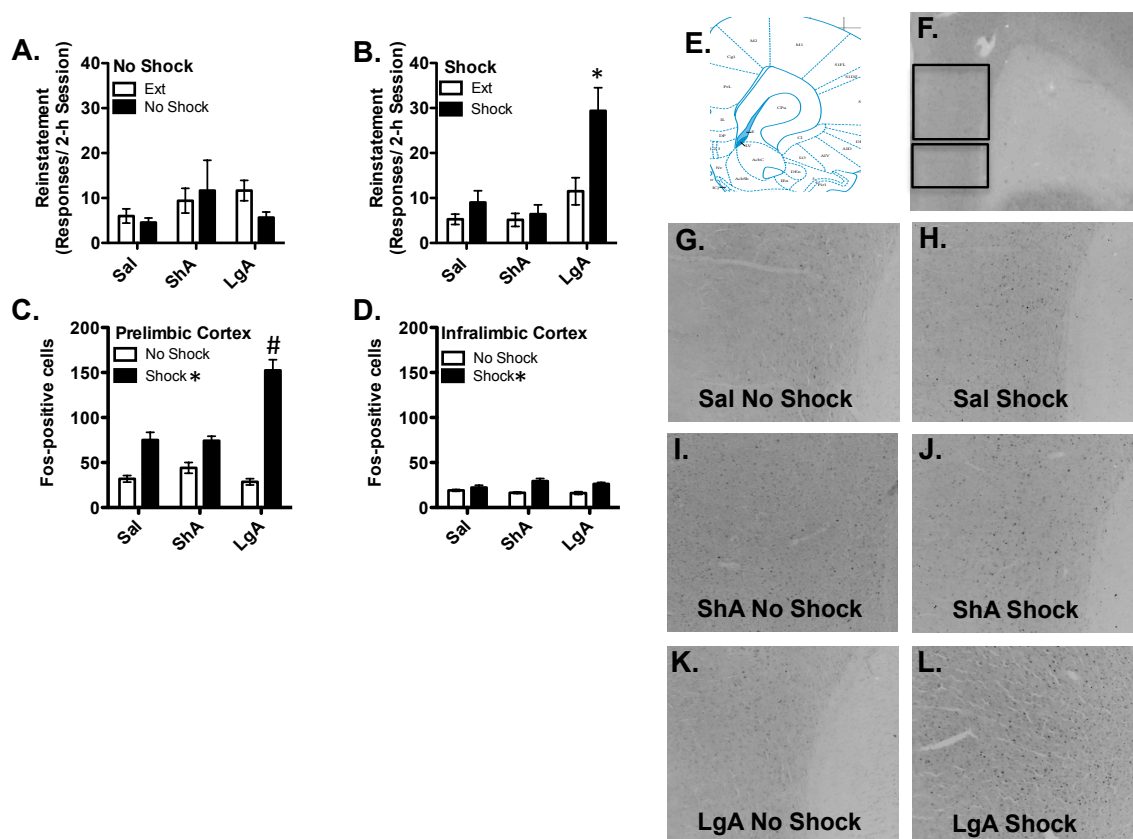


Figure 1: Stress-induced cocaine seeking is associated with a heightened Fos response in the prelimbic cortex. Data in Figures 1A and 1B represent lever pressing during extinction (Ext) and reinstatement testing either following either shock delivery or in the absence of shock in rats with a history of self-administration under short-access (ShA; 0.5 mg/kg/infusion cocaine; 14 x 2 hrs/daily), long-access (LgA; 0.5 mg/kg/infusion cocaine; 14 x 6 hrs/daily), or saline control (Sal; 14 x 2 hrs/daily) conditions. Shock only increased lever pressing during reinstatement testing following LgA SA (* $p < 0.01$ vs. prior Ext, responding in No Shock controls, and reinstatement in Sal/ShA rats). Data in Figures 1C and 1D represent the corresponding Fos responses in

the prelimbic cortex and infralimbic cortex. Shock produced overall increases in the number of Fos-positive cells in each region ($^{\#}p<0.05$; overall effect of Shock) and, in the prelimbic cortex, this response was heightened following LgA self-administration ($*p<0.01$ vs. No Shock controls and Sal/ShA rats). A schematic and image illustrating the analyzed regions (1E and 1F) and representative images depicting Fos immunoreactivity in the prelimbic cortex in each group comprise Figures 1G-L.

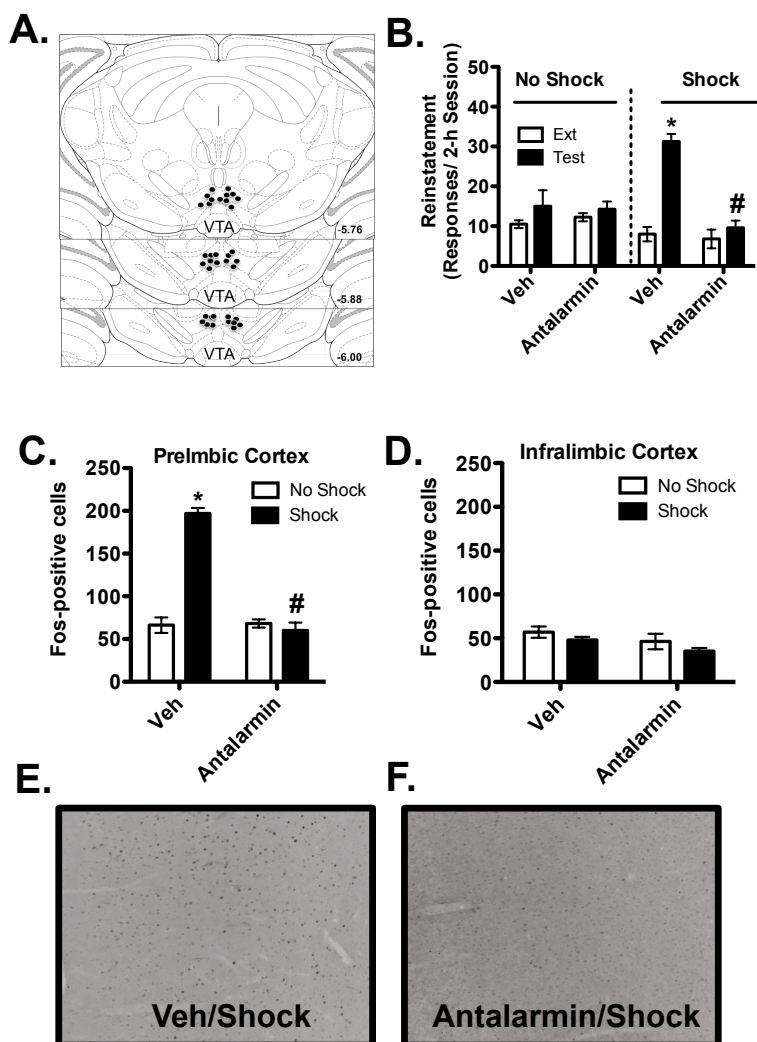


Figure 2: CRFR1 receptor antagonism in the VTA prevents shock-induced cocaine seeking and the corresponding Fos response in the prelimbic cortex. Bilateral intra-VTA injection sites for the CRFR1 receptor antagonist, antalarmin, are shown in 2A. Shock reinstated cocaine-seeking in vehicle control rats ($*p<0.01$ vs. prior Ext and No Shock controls) and intra-VTA antalarmin prevented shock induced cocaine seeking following LgA self-administration (2B; $^{\#}p<0.01$ vs. intra-VTA Veh controls) and the corresponding Fos response in the prelimbic cortex (2C; $*p<0.01$, Fos increase vs. No Shock controls in Veh rats; $^{\#}p<0.01$, Fos reduction intra-VTA antalarmin vs. veh

pretreatment) without affecting Fos in the infralimbic cortex (2D). Representative images showing the Fos responses in the prelimbic cortex following intra-VTA vehicle or antalarmin injections are shown in Figures 2E and 2F.

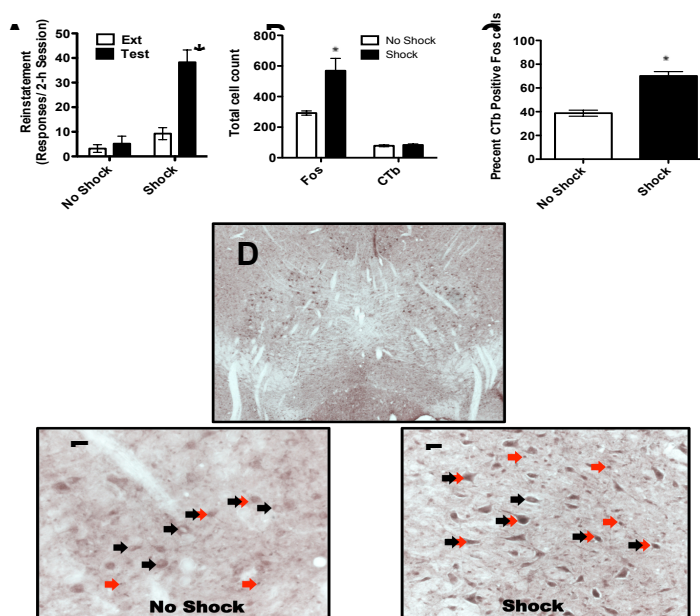
Experiment #2: Role of VTA CRFR1 receptors in shock-induced cocaine seeking and cortical subregion activation

Bilateral intra-VTA injections of the CRFR1 receptor antagonist, antalarmin, prevented shock-induced cocaine seeking rats following LgA cocaine self-administration and extinction and eliminated the corresponding increase in the number Fos immunoreactive neurons in the prelimbic cortex. Antalarmin/vehicle injection sites in the posterior VTA are shown in Figure 2A. Figure 2B shows the effects of intra-VTA antalarmin on shock-induced cocaine seeking. A 3-way reinstatement (extinction vs. reinstatement test; repeated measure) x shock condition (shock vs. no shock) x antalarmin pretreatment (VTA antalarmin vs. veh) ANOVA showed a significant interaction among all three variables ($F_{1,12}=10.79$; $p<0.01$). Post-hoc testing revealed that shock-induced cocaine seeking was observed following intra-VTA vehicle injections ($p>0.01$ vs. the prior extinction session or shock-free controls) but not after intra-VTA antalarmin delivery. Moreover, cocaine seeking in rats that received intra-VTA antalarmin injections prior to shock delivery was significantly lower than in intra-VTA vehicle controls ($p<0.01$).

Figure 2C shows the effects of intra-VTA antalarmin on the Fos response to shock in the prelimbic cortex. A 2-way antalarmin x shock-induced reinstatement ANOVA showed significant overall effects of shock ($F_{1,17}=77.86$; $p<0.001$) and intra-VTA antalarmin ($F_{1,17}=61.36$; $p<0.001$) on the number of Fos-positive cells in the prelimbic cortex and a significant shock x antalarmin interaction ($F_{1,17}=73.47$; $p<0.001$). Shock increased the number of Fos-immunoreactive cells in the prelimbic cortex in

vehicle- but not antalarmin-pretreated rats ($p < 0.01$ vs. no shock controls) and the number of Fos-positive cells in prelimbic cortex following antalarmin was significantly lower than in rats pretreated with vehicle ($p < 0.01$). By contrast, neither shock nor intra-VTA antalarmin altered Fos-positive cell numbers in the infralimbic cortex (Figure 2D). Immuno-labeled sections including the prelimbic cortex from representative rats that received VTA vehicle or antalarmin are shown in Figures 2E and 2F.

Figure 3: Shock-induced cocaine seeking induces Fos expression in VTA neurons that project to the prelimbic cortex. To determine if shock-induced cocaine seeking is associated with activation of VTA neurons that project to the prelimbic cortex, cholera toxin b subunit (CTb) was injected bilaterally into the prelimbic cortex and Fos expression in CTb-labeled cells in the VTA was quantified. Cocaine seeking was increased when rats received shock ($*p < 0.01$ vs. prior Ext and No Shock controls; Fig 3A). There was no difference in CTb labeling between rats that underwent shock-induced reinstatement and non-shock controls, but the total number of cells expressing Fos was increased by shock (Fig 3B; $*p < 0.05$ vs. No Shock Controls). Moreover, the percentage of CTb-labeled cells expressing Fos was increased in rats tested for shock-induced reinstatement relative to controls (Fig 3C; $*p < 0.05$ vs. No Shock Controls). The CTb injection site and CTb, Fos and CTb/Fos-labeled cells in the VTA in brain representative sections from each group are shown in Figure 3D-F.



Experiment #3: Relationship between Fos immunoreactivity in VTA neurons that project to the prelimbic cortex and shock-induced reinstatement of cocaine seeking

To determine if shock-induced cocaine seeking was associated with activation of VTA neurons that project to the prelimbic cortex, rats were injected bilaterally with the retrograde tracer, CTb, into the prelimbic cortex and Fos expression in CTb-labeled cells in the VTA was assessed following testing for shock-induced reinstatement or under shock-free conditions. As expected, footshock reinstated extinguished cocaine seeking (Fig 3A). A 2-way shock condition (shock vs. no shock) x reinstatement condition (extinction vs. test session) ANOVA revealed a significant shock condition x reinstatement testing interaction ($F_{1,11}=20.24$; $p=0.001$). Post-hoc testing showed that cocaine seeking was increased after exposure to shock compared to the preceding extinction session and non-shocked controls ($p<0.01$).

Shock-induced reinstatement was associated with an increase in the total number of Fos-positive cells in the VTA (2-tailed $t_{11}=2.63$; $p<0.05$), while the number of CTb-labeled VTA neurons did not differ between shocked and non-shocked rats (Fig 4B). Moreover, the percentage of CTb-positive neurons in the VTA that were co-labeled for Fos was significantly increased in rats tested for shock-induced reinstatement compared to rats tested in the absence of shock (2-tailed $t_{11}=2.95$; $p<0.05$), suggesting that shock-induced cocaine is associated with activation of VTA neurons that project to the prelimbic cortex (Fig. 4C). The CTb injection site and CTb, Fos and CTb/Fos-labeled cells in the VTA in brain representative sections from each group are shown in Figure 4D-F.

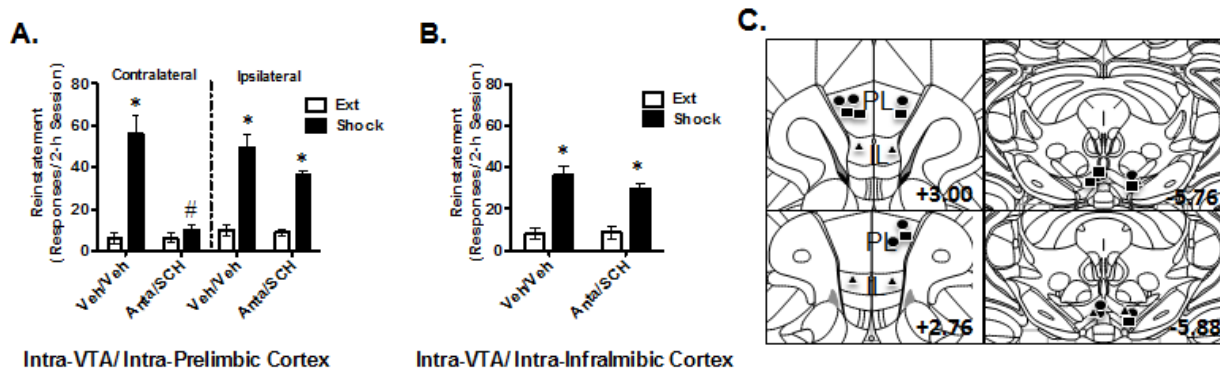


Figure 4: A CRFR1 receptor-regulated dopaminergic projection to the prelimbic cortex is required for stress-induced cocaine seeking.

To determine the role of a CRFR1 receptor-regulated dopaminergic projection from the VTA to the prelimbic cortex in stress-induced cocaine seeking, we used a disconnection approach in which we delivered antalarmin into the VTA of one hemisphere and SCH23390 into the contralateral prelimbic cortex (n=5 rats) and tested for shock-induced cocaine seeking. To confirm that any effect on reinstatement was attributable to pathway disconnection, a second group of rats (n=4) received an antalarmin injection into the VTA of one hemisphere and a SCH23390 injection into the ipsilateral prelimbic cortex. Significant shock-induced reinstatement was observed following VTA/prelimbic cortex vehicle (contralaterally or ipsilaterally) and following ipsilateral intra-VTA antalarmin/prelimbic SCH23390 pretreatment (*p<0.05 vs. Ext) but not following contralateral antagonist delivery (Fig 4A). Furthermore, shock-induced reinstatement was significantly reduced following contralateral antagonist injections relative to either vehicle pretreatment or ipsilateral antagonist delivery (#p<0.05 for each comparison). By contrast, disconnection of the VTA pathway to the infralimbic cortex

failed to alter cocaine seeking (4B; n=4). Intra-VTA and cortical injection sites for rats used for analysis are shown in Figure 4C.

Experiment #4: Effect of pharmacological disconnection of the CRF-regulated dopaminergic pathway from the VTA to the prelimbic cortex on shock-induced cocaine seeking.

To determine the role of a CRFR1 regulated pathway originating in the VTA that releases dopamine into the prelimbic cortex in stress-induced cocaine seeking, we used a disconnection approach in which we delivered antalarmin (250 ng) into the VTA of one hemisphere and the dopamine D1 receptor antagonist, SCH 23390 (200 ng) into the contralateral prelimbic (Figure 4A; n=5 rats). A 3-way repeated measures reinstatement condition (extinction vs. reinstatement test) x shock condition (shock vs. no shock) x disconnection (antalarmin/SCH 23390 vs. veh/veh) ANOVA revealed a significant interaction among all three variables ($F_{1,4}=29.02$; $p<0.01$). Post-hoc testing showed that significant shock-induced cocaine seeking as defined as an increase relative to the prior extinction session and responding by shock-free controls, was observed in vehicle control rats but not in rats that underwent pharmacological disconnection ($p<0.01$ /comparison). Moreover, cocaine seeking following shock was significantly lower in rats that received contralateral VTA antalarmin/prelimbic SCH 23390 relative to vehicle/vehicle controls ($p<0.01$).

To confirm that the effects of contralateral drug delivery on cocaine seeking were attributable to pathway disconnection, a second group of rats (Figure 4A; n=4) received antalarmin into the VTA of one hemisphere and SCH 23390 into the ipsilateral prelimbic cortex. In contrast to rats that received antagonists into the contralateral regions,

administration of antalarmin and SCH23390 to the VTA/prelimbic cortex within the ipsilateral hemisphere did not prevent shock-induced cocaine seeking. A 3-way repeated measures reinstatement condition (extinction vs. reinstatement test) x shock condition (shock vs. no shock) x ipsilateral drug treatment (antalarmin/SCH 23390 vs. veh/veh) ANOVA revealed a significant shock x reinstatement condition interaction ($F_{1,3}=56.72$; $p<0.01$). However, no main effect of or interactions involving antagonist administration were observed. Overall shock produced cocaine seeking as defined by significant shock-induced increases in all groups relative to extinction and non-shock controls ($p<0.05$ for each comparison)

In contrast to the prelimbic cortex, disconnection of the pathway from the VTA to the infralimbic cortex through antalarmin delivery into the VTA in one hemisphere and SCH 23390 delivery into the infralimbic cortex of the other hemisphere failed to alter shock-induced cocaine seeking (Figure 4B; $n=4$). A 3-way repeated measures reinstatement condition (extinction vs. reinstatement test) x shock condition (shock vs. no shock) x disconnection (antalarmin/SCH 23390 vs. veh/veh) ANOVA revealed a significant shock x reinstatement condition interaction ($F_{1,3}=82.78$; $p<0.01$). However, no main effect of or interactions involving antagonist administration were observed. Overall shock produced cocaine seeking as defined by significant shock-induced increases in all groups relative to extinction and non-shock controls ($p<0.05$ for each comparison).

VTA antalarmin/veh and cortical SCH 23390/veh injection sites are shown in Figure 4C.

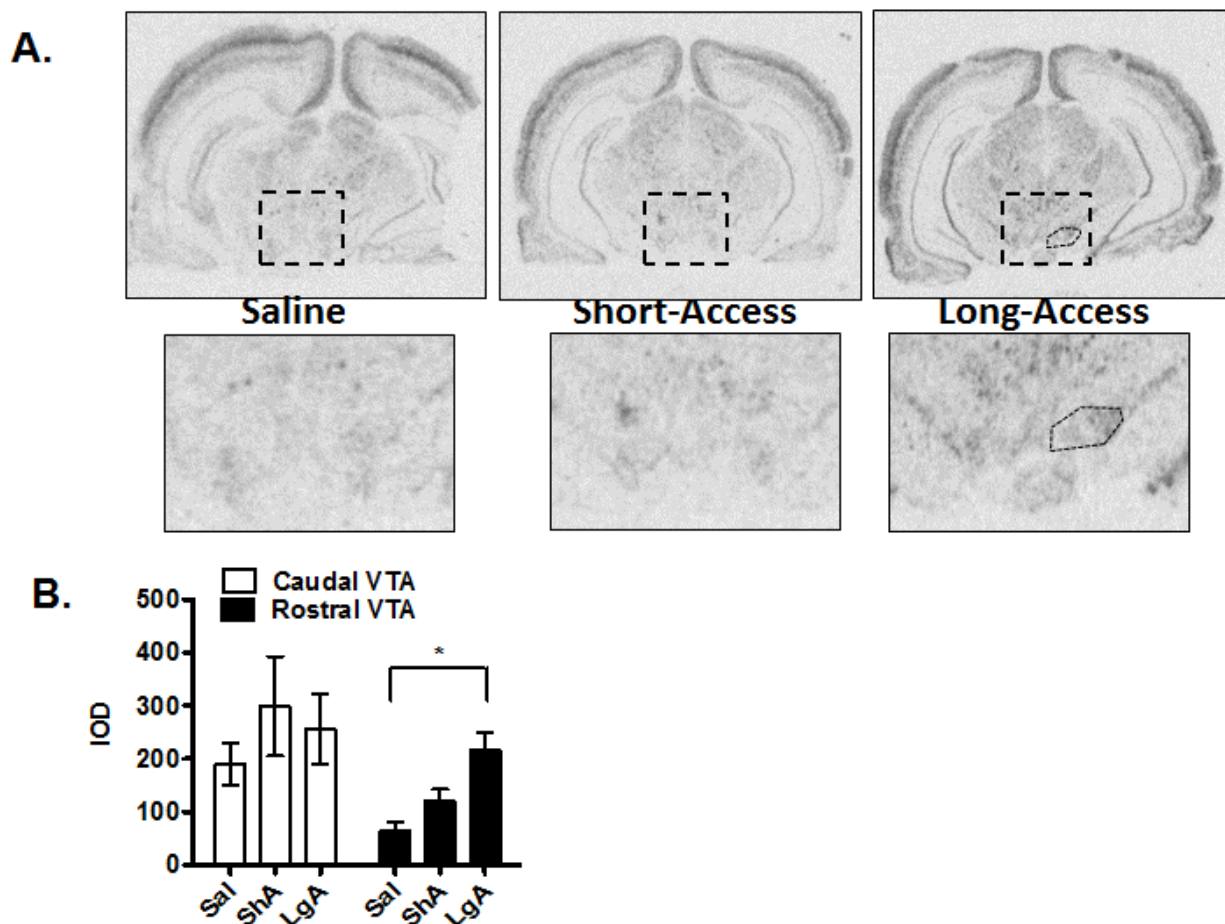


Figure 5: Cocaine self-administration under conditions that establish CRF-dependent shock-induced cocaine seeking and corresponding activation of the prelimbic cortex increases CRFR1 mRNA in the caudal VTA. CRFR1 receptor mRNA levels in the VTA were quantified by in situ hybridization using a ^{35}S -labeled CRFR1 riboprobe. Representative images of film exposed to hybridized sections containing VTA from rats following self-administration under ShA, LgA, or saline control conditions are shown in Figure 5A. Rostral (-5.2 to -5.6 mm bregma) and caudal (-5.6 to -6.2 bregma) VTA were analyzed separately and mean integrated optical densities (IODs) in each region are shown in Figure 5B. No alterations in the rostral VTA were observed. However, CRFR1 mRNA levels were significantly increased in the caudal VTA following LgA self-administration ($*p < 0.05$ vs. saline controls).

Experiment #5: Effects of cocaine self-administration under conditions that promote shock-induced cocaine seeking on CRFR1 mRNA in the VTA

To determine if the recruitment of the CRF-regulated pathway from the VTA to the prelimbic cortex was the result of increased CRFR1 receptor expression, in situ

hybridization was used to quantify VTA CRFR1 mRNA levels following self-administration and extinction in rats provided access to cocaine under ShA and LgA conditions and saline controls (Fig 5). The VTA is a complex heterogeneous structure with anatomically defined subregions that have distinct functionality (Kaufling et al; Lammel et al). The tail of the VTA notwithstanding, a key anatomical demarcation is the division of the VTA into rostral (anterior to -5.6 mm bregma) and caudal (posterior to -5.6 bregma) regions (ref). Specifically, our work examining shock-induced cocaine seeking has focused on CRF effects in the caudal VTA (ref). Thus, in this experiment, CRFR1 receptor mRNA levels were analyzed separately in the rostral (-5.2 mm to -5.6 mm bregma) and caudal (-5.6 mm to -6.2 mm bregma). Representative images of films exposed to CRFR1 riboprobe hybridized brain sections from each group containing the caudal VTA are shown in Figure 5A. Mean integrated optical rostral and caudal film densities (IOD) are shown in Figures 5B and 5C. Separate one-way ANOVAs were used to compare CRFR1 mRNA in rostral and caudal VTA across self-administration conditions. A significant overall effect of access condition was found in the caudal, but not rostral, VTA ($F_{2,23}=5.53$; $p<0.05$). Post-hoc testing showed that CRFR1 mRNA was increased relative to saline controls following LgA, but not ShA self-administration and extinction ($p<0.05$).

DISCUSSION

Although stress has been identified as a key contributor to relapse, the relationship between stress and drug-seeking behavior is complex and it has been reported that the onset of stress does not always serve as a reliable trigger for cocaine use

(Preston and Epstein, 2011; Furnari et al., 2015). A key determinant of stress-induced cocaine seeking appears to be the extent/pattern of prior use. High-frequency cocaine abusers display increased drug craving, anxiety and associated cardiovascular and hypothalamic-pituitary-adrenal responses upon exposure to stress imagery when compared to lower-frequency abusers (Fox et al., 2005). Consistent with this observation, we have found that reliable stress-induced reinstatement of cocaine seeking is observed in rats with a history of cocaine self-administration under conditions of long access (LgA)/high cocaine intake but not under conditions of short access (ShA) drug availability where intake is lower (Mantsch et al., 2008).

The glutamatergic projection from the prelimbic cortex to the nucleus accumbens core has been shown to be critical for cocaine seeking. Pharmacological inhibition of this pathway using baclofen/muscimol (McFarland et al., 2004) or TTX (Capriles et al., 2003) delivery into the prelimbic cortex prevents stress-induced cocaine seeking and associated increases in nucleus accumbens core glutamate levels (McFarland et al., 2004). Here we demonstrate that stress-induced reinstatement is associated with a heightened Fos response in the prelimbic and the infralimbic cortex.

The mesocortical dopamine projection, via dopamine D1 receptor activation in the prelimbic cortex, is a key regulator of this cortico-accumbens pathway. Injections of the D1 receptor antagonist SCH23390 (Capriles et al 2003) or the non-selective dopamine receptor antagonist fluphenazine (McFarland et al., 2004) but not the D2-like receptor antagonist raclopride (Capriles et al., 2003) injected into the prelimbic but not infralimbic cortex prevent stress-induced cocaine seeking. D1 receptors in the prelimbic cortex are predominantly expressed post-synaptically on pyramidal neurons where they

promote excitability (Seamans et al., 2001; Gonzalez-Islas and Hablitz, 2003; Tseng and O'Donnell, 2004; Sun et al., 2006). Not only do our present findings confirm that D1 receptors in the prelimbic cortex are required for shock-induced reinstatement, but they suggest that stress-induced cocaine seeking is associated with activation of VTA dopamine neurons that project to the prelimbic cortex. These findings are consistent with an earlier report by Deutch et al (1991) demonstrating a similar stress-induced response in cocaine-naïve rats. Notably, in that study, shock selectively activated VTA neurons that project to the prelimbic cortex without activating VTA neurons that project to the nucleus accumbens, consistent with reports that the mesocortical dopamine pathway is selectively activated by aversive stimuli (Lammel et al 2012).

Much evidence points to CRF in the VTA as a key regulator of the mesocortical dopamine pathway and stress-induced drug seeking. Shock-induced reinstatement of cocaine seeking is associated with increased CRF levels in the VTA (Wang et al., 2005) and intra-VTA CRF injections are sufficient to reinstate (Wang et al., 2005; Blacktop et al., 2011). Moreover, pharmacological antagonism of (Blacktop et al., 2011; Vranjkovic et al 2014) or shRNA-mediated knockdown of (Chen et al., 2014) of the CRFR1 receptor in the VTA prevents stress-induced cocaine seeking. Although studies examining the regulation of mesocortical dopamine by VTA CRF have produced mixed results (see e.g., Kalivas et al., 1987), reports that icv CRF increases dopaminergic neurotransmission in the prefrontal cortex (Dunn and Berridge, 1987; Lavicky and Dunn, 1993) and that CRF promotes excitation of VTA dopamine neurons via both pre- and post-synaptic mechanisms (Ungless et al 2003; Korotkova et al 2006; Riegel and Williams 2008; Wanat et al 2008, Beckstead et al 2009; Hahn et al 2009; Williams et al 2014) suggest

that stress-induced release of CRF into the VTA may underlie increases in mesocorticolimbic dopamine release. Consistent with this possibility, Refojo et al (2011) reported that selective deletion of CRFR1 receptors in VTA dopamine neurons significantly reduced stress-induced increases in dopamine in the prefrontal cortex in mice while Wang et al (2005) have demonstrated that increases in VTA dopamine levels associated with shock-induced reinstatement are CRF-dependent.

Here we report that the increase in Fos in the prelimbic cortex associated with shock-induced reinstatement of cocaine is prevented by intra-VTA delivery of the CRFR1 receptor antagonist, antalarmin. Further, we directly tested the role of CRFR1 regulation the mesolimbic dopamine pathway in stress-induced cocaine seeking using a pharmacological disconnection approach. Bilateral disconnection of the proposed pathway by injection of antalarmin into the VTA of one hemisphere and the D1R antagonist SCH23390 into the prelimbic cortex of the contralateral hemisphere prevented shock-induced reinstatement. By contrast administration of VTA antalarmin and PL SCH23390 in the ipsilateral hemisphere or disconnection of the CRF regulated pathway to the infralimbic cortex failed to block shock-induced reinstatement. These data suggest that, during stress, CRF released into the VTA, possibly from neurons that originate in the BNST (Rodaros et al 2007; Silberman et al 2013; Vranjkovic et al 2014), activates CRFR1 receptors on mesocortical neurons thereby promoting prelimbic dopamine release and D1 receptor activation to induce drug seeking behavior.

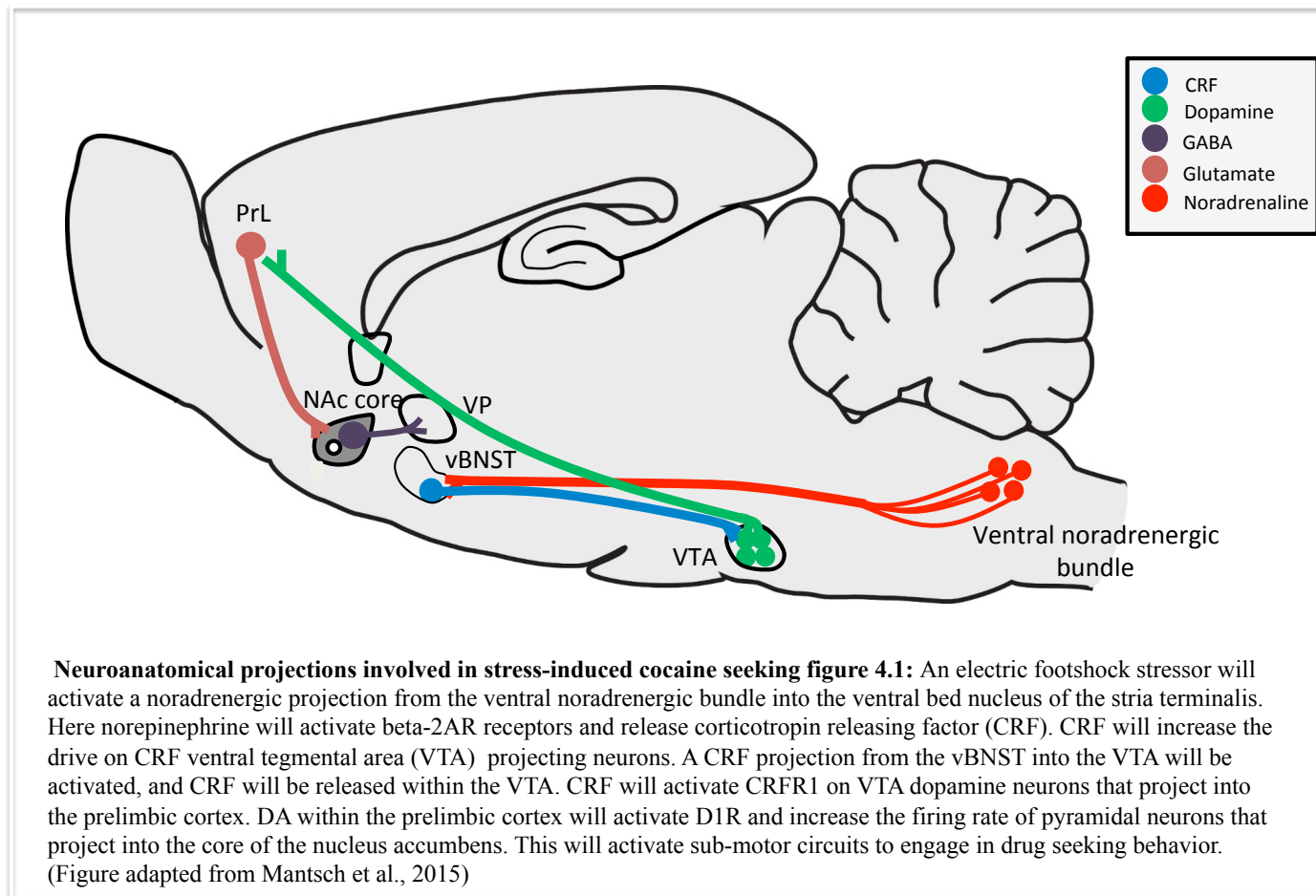
Consistent with other reports that CRF actions in the VTA depend on a prior history of cocaine exposure (Wang et al, 2005; Beckstead et al 2009; Hahn et al 2009; Williams et al 2014), we have found that intra-VTA CRF- induce reinstatements is only

observed in rats with a prior history of cocaine SA under daily extended-access/high-intake conditions. Thus, the observed heightened sensitivity to stress-induced cocaine seeking (Mantsch et al., 2008 and current findings) and the corresponding enhancement of stress regulation of the mesocortical pathway to stress can likely be attributed to increased CRFR1 receptor responsiveness at the level of the VTA. In support of this possibility, we found that CRFR1 mRNA in the posterior VTA (the site at which CRF regulates cocaine seeking) is increased following long-access SA. Notably, it has previously been reported that repeated cocaine administration increases CRF binding in the VTA (Goeders et al., 1990) and establishes CRFR1 regulation of excitatory transmission (Hahn et al., 2009). Although we assume that alterations in CRFR1 mRNA are localized to dopaminergic neurons, confirmation of this requires further investigation.

To summarize, the present findings demonstrate that 1) stress-induced cocaine seeking requires the activation of a CRFR1-regulated dopaminergic pathway from the VTA to the prelimbic cortex and 2) repeated cocaine use under patterns/intake levels observed in many cocaine abusers can establish stress-reactivity of this pathway via upregulation of CRFR1. Further understanding the regulation of this pathway and how it is recruited with excessive cocaine use (e.g., elevated glucocorticoid levels; Graf et al., 2011) should provide important insight into the addiction process.

General Discussion

Addiction is a tremendous health and financial burden on our society. Our current understanding of the brain circuitries involved in addiction is far from complete. Drug use is a stress-driven behavior partially because of the unpredictable and often uncontrollable nature of stress. The work in this dissertation will hopefully aid in the development of new and more effective treatment strategies aimed at either preventing stress-induced relapse or minimizing the role of stress in the addiction cycle. Research in our lab has focused on the idea that chronic cocaine use, as displayed by the long-access self-administration paradigm (Ahmed and Koob, 1998), increased the vulnerability to relapse during periods of stress (Mantsch et al., 2008) in a manner dependent on an escalating pattern of cocaine use, which may be dependent on the interactions of glucocorticoids and epinephrine (Mantsch et al., 2008). Findings within this dissertation will hopefully aid in the development of better treatment options. To summarize, we have found that stress-dependent relapse occurs through a corticotropin releasing factor (CRF) pathway from the bed nucleus of the stria terminalis (BNST) to the ventral tegmental area (VTA) that activates dopamine (DA) neurons that project into the prefrontal cortex to precipitate cocaine use (figure 4.1). In addition, we have found that CRF expression is increased within the BNST following a stressor that is dependent on beta-2 adrenergic receptor (AR) activation. Furthermore, long-access cocaine use significantly increased the expression of CRFR1 within the caudal VTA. This increase in CRFR1 expression primes the activity of VTA neurons that specifically project into the prefrontal cortex, and therefore causes an increase of prefrontal pyramidal activity through a dopamine receptor 1 (D1R) mechanism.



Beta-noradrenergic receptors within the BNST regulate relapse of cocaine seeking during periods of stress

Clinical and preclinical research has determined that inhibition of the noradrenergic system, through the actions of alpha-2 AR agonists such as clonidine and guanfacine, can block stress-induced craving of opiates (Sinha et al., 2007), cocaine (Jobes et al., 2011, Mantsch et al., 2010), and nicotine (Fox et al. 2012, McKee at., 2014). These studies are based on the assumption that alpha-2 ARs are presynaptic autoreceptor, although post-synaptic alpha-2 AR effects that could interfere with cocaine seeking in regions such as the prefrontal cortex have been reported. Based on these studies, we have focused our research on determining the contribution of the noradrenergic system to

stress-related relapse to cocaine use. Earlier studies have indicated that a cocktail antagonism of both beta-1 and beta-2 ARs within the bed nucleus of the stria terminalis (BNST), and the central nucleus of the amygdala (CeA) blocks footshock-induced reinstatement of cocaine seeking (Leri et al 2002). Based on these results, our lab has tested the sufficiency and necessity of activation of the individual beta-AR receptors for stress-induced relapse in both the self-administration and conditioned place preference models.

Our results indicate that the beta-2 AR is both necessary and sufficient in mediating forced swim-induced drug seeking in extinguished conditioned place preference mice (Mantsch et al 2010, McReynolds et al 2014, Vranjkovic et al 2012). Specifically, we showed that the beta-2 AR antagonist ICI 118,551 blocked forced swim-induced reinstatement (Chapter 2; Mantsch et al 2010, McReynolds et al 2014) while the non-selective beta-AR agonist isoproterenol was sufficient to induce drug seeking (Vranjkovic et al 2012), and the selective beta-2 AR agonist clenbuterol was also sufficient to induce drug-seeking behavior (McReynolds et al 2014, Vranjkovic et al 2012). In addition, using footshock-induced reinstatement in our rat self-administration model, we have shown that stress-induced reinstatement is specific to beta-2 ARs in the ventral BNST (vBNST) as intra-vBNST administration of the beta-2 AR antagonist ICI 118,551 blocked stress-induced drug seeking and intra-vBNST administration of the beta-2 AR agonist clenbuterol was sufficient to evoke stress-induced reinstatement (Chapter 3; Vranjkovic et al 2014).

Interestingly, we were able to block isoproterenol-induced reinstatement with both the selective beta-1 AR antagonist betaxolol, and the selective beta-2 AR antagonist

ICI 118,551 (Vranjkovic et al 2012). It is also important the signal redundancy in that both receptors signal through G_s . This indicates that beta-AR receptors may exert their effects on different cell types. This was interesting because antagonism of the beta-1 AR failed to block forced swim-induced reinstatement, in the CPP model, and footshock-induced reinstatement, in the self-administration model, when administered into the vBNST. This indicates that either the drugs bind to other receptors, or that there is an unknown role for beta-1 ARs in stress-induced reinstatement. Studies have shown that stress can increase the expression of both beta-1 and beta-2 ARs within the BNST (Cecchi et al 2007), and that chronic cocaine administration can decrease the beta-1 AR-mediated excitatory post-synaptic potential within the BNST (Nobis et al 2011). Our results indicate that beta-2AR receptors are important in contributing to stress-induced relapse; however, their localizations within the central nervous system is not clear. Unfortunately, beta-AR antibodies have been extremely unreliable in determining the cellular localization; however studies employing electrophysiology have determined that beta-ARs are located on glutamatergic terminals within the BNST to enhance the excitatory drive of these neurons through a CRF-dependent mechanism (Nobis et al 2011; Silberman et al 2013). In addition, experiments using HA-tags mutated onto the C-terminus of the alpha-2 AR have been used to localize the alpha-2 AR (Flavin et al 2014). This technique can be used to identify the localization of the beta-1 and beta-2-ARs within the synapse.

It is interesting that both beta-1 ARs and beta-2ARs work through the same second messenger system. Both beta-1 and beta-2 ARs are G_s -coupled receptors suggesting that downstream activation of cAMP, PKA, or CREB are involved in the

reinstatement process during stress. Interestingly, studies have shown that cAMP and CREB activation are required for forced swim- but not cocaine-induced reinstatement of conditioned place preference (Kreibich and Blendy, 2004). Therefore, it is necessary to understand the localization of these receptors within the synapse, and how their trafficking, expression, or down-stream mechanisms may change due to chronic drug use or during periods of stress.

Moreover, beta-1 ARs have a higher affinity for norepinephrine than beta-2 ARs, but here we show that the effect of stress on reinstatement is dependent upon the beta-2 AR specifically. This may be attributed to elevated norepinephrine levels during periods of stress (Pacak et al 1995), during which it is possible that the high affinity beta-1 AR is saturated and therefore is either internalized, or non-functional. This would allow for the low affinity beta-2 AR to be activated. In support of this, a similar type of affinity is seen with glucocorticoid receptors within the stress circuit as high affinity mineralocorticoid receptors are saturated under basal cortisol levels whereas low affinity glucocorticoid receptors are activated by cortisol during periods of elevated cortisol levels that are typically observed during stress. In addition, beta-2 ARs seem to have both genomic and non-genomic effects within the BNST. Electrophysiology studies have indicated that beta-ARs enhance excitatory transmission within the BNST (Nobis et al 2011), possibly through a CRF-releasing event (Silberman et al., 2013). However, we have shown that a forced swim stressor increases *crf* mRNA within the BNST, and that this increase can be blocked with antagonism of the beta-2 AR (McReynolds et al., 2014). This suggests that beta-2 ARs serve as a mechanism to reload CRF pools either within local CRF-positive neurons in the BNST or CRF-projecting neurons that originate within the BNST, and

beta-2AR receptors may also stimulate the release of CRF within the BNST. This is important because it shows that beta-ARs and CRF have a crucial interaction within the BNST.

Beta-2 ARs act upstream from CRFR1 to mediate stress-induced drug seeking

Data from our lab and others has suggested that norepinephrine drives reinstatement through a beta-AR dependent engagement of the CRF system within the BNST (Brown et al 2009, Erb et al 2000, McReynolds et al 2014, Vranjkovic et al 2014). In this dissertation we have shown this in mice wherein administration of a beta-2 AR antagonist blocked stress-dependent increases of *crf* mRNA within the BNST, and the CRFR1 antagonist antalarmin blocked clenbuterol-induced reinstatement. In addition, using the rat self-administration model, we have shown that intra-BNST clenbuterol-induced reinstatement is blocked by pretreatment with the CRFR1 antagonist antalarmin (Chapter 3; Vranjkovic et al 2014). Furthermore, we have shown that intra-BNST CRF administration induces drug-seeking behavior, and this effect is not blocked by administration of the beta-2 AR antagonist ICI 118,551 (Vranjkovic et al 2014).

This strongly suggests that beta-2 AR activation releases CRF within the BNST. This data is consistent with other studies that have shown that beta-AR activation within the BNST produces an enhancement of excitatory synaptic mechanisms (Egli et al., 2005; Nobis et al., 2011) that was dependent upon CRFR1 activation (Silberman et al., 2013). It is important to note that even though CRF signaling in the BNST is necessary for relapse (Chapter 3; Erb et al., 2001; Vranjkovic et al 2014), the source of this CRF is not known as it could be released from local CRF neurons (Veinante & Freund-Mercier 1997) or from

CRF-ergic afferents from other regions (Rodaros et al., 2007). In addition, our own data suggest that beta-2 ARs regulate CRF release within the BNST though a more direct examination of this effect is still needed.

Several different methods can be employed to study whether beta-2 AR activation is necessary for direct CRF signaling in stress-induced reinstatement in the different reinstatement models. Studies have started employing the human diphtheria toxin (toxic to humans, but not mice) receptor to selectively destroy cell bodies in a cre-dependent manner (Silberman et al., 2015). For this example, CRF-cre mice would be injected with a cre-dependent virus expressing the diphtheria toxin receptor and this virus could be specifically injected into either the BNST or CeA to produce the receptor on CRF-positive cells. Subsequently, mice would be chronically injected with the diphtheria toxin to destroy any cells expressing the diphtheria toxin receptor (Silberman et al., 2015). Using this, studies could examine whether depletion of CRF by diphtheria toxin could 1) induce forced-swim dependent reinstatement of CPP in mice; this study could examine whether CRF in cells in either the CeA or the BNST is necessary for stress-induced reinstatement. 2) This study could be used to confirm a relationship between beta-2 AR and CRFR1 activation in stress-induced reinstatement; injections of the beta-2 AR agonist clenbuterol should not be sufficient in reinstating drug-seeking behavior. 3) Electrophysiology studies could be conducted to determine whether beta-AR activation within the BNST still causes enhancement of glutamatergic transmission within the BNST that is dependent on CRFR1 activation. Beta-AR and CRFR1 could be working separately to enhance glutamatergic signaling independent from each other. In addition, injections of an AAV virus containing shRNA for CRF (Grieder et al 2014) could be

used to test whether local or CeA CRF input into the BNST regulates reinstatement. In addition, microdialysis studies may be employed to directly measure CRF release within the BNST during either a footshock stressor, or through reverse dialyzing the beta-2 AR agonist clenbuterol. These studies would be crucial in understanding the direct interaction of beta-2AR receptors and CRFR1; and their relationship in stress-induced drug seeking.

Excitatory input onto CRF neurons within the BNST

A major challenge in the field of neuroscience research on affective disorders is identifying the signaling pathways and neural circuits involved in the complex mechanisms that underlie stress-induced reinstatement. Very little studies have been done to examine the excitatory afferents that project onto CRF neurons within the BNST. Optogenetic and genetically encodable reporter strategies may be used to map excitatory afferents that directly synapse onto CRF neurons within the BNST. Anatomical studies have suggested that the BNST receives a major glutamatergic input from the infralimbic cortex, however it is important to understand the upstream structures that activate CRF neurons within the BNST, or more importantly CRF-positive BNST neurons that project to downstream structures such the VTA. Currently, the anterior insular cortex, medial prefrontal cortex, and the parabrachial nucleus have been suggested to send glutamatergic signaling onto CRF-positive cells within BNST (Swanson et al., 1982).

Activation of beta-2 AR within the BNST activates a CRF projection into the VTA.

Many studies have indicated that a CRF projection into the VTA mediates stress-induced reinstatement, however, no studies have shown a neuronal mechanism or a

functional location. A major finding in our lab is that beta-2 ARs within the vBNST recruit a CRF-projecting pathway from the vBNST to the VTA, and this pathway, in part, mediates stress-induced drug seeking (Chapter 3; Vranjkovic et al., 2014). In addition, animals that received an intra-VTA cholera toxin subunit b injection (CTb; retrograde tracer), and underwent dual immunofluorescence for CTb and CRF within the vBNST, showed that CRF-positive cells within the vBNST project to the VTA (Chapter 3; Vranjkovic et al., 2014). This directly suggests that activation of the vBNST result in release of CRF within the VTA, which will bind to CRFR1 to promote drug use during times of stress. However, data in this dissertation suggests that CRF release within the VTA is mediated by a beta-2AR, and CRFR1 interaction within the vBNST.

CRF cells that project into the VTA are either glutamatergic or GABAergic

Our studies suggest that CRF-positive neurons originate from the BNST and project into the VTA, though we did not confirm a direct CRF-releasing pathway projection. Studies stimulating the BNST through a beta-2 AR mechanism and measuring CRF release within the VTA in animals that have a knockdown of CRF, and proper controls, within the vBNST need to be conducted to confirm our theory. Furthermore, CRF-positive neurons could regulate glutamatergic or GABAergic cells within the BNST; therefore, one possibility is that CRF-positive projecting neurons could make a relay projection to another area, from which CRF is coreleased with GABA or glutamate into the VTA. Furthermore, it is not well known whether the BNST sends a predominately glutamatergic or GABAergic input into the VTA during periods of stress. Studies have shown that the majority of BNST neurons that project into the VTA are

GABAergic (Kudo et al 2012) and it is predicted that they disinhibit VTA dopaminergic neurons by synapsing onto VTA GABA neurons as studies have shown that BNST to VTA projections formed symmetric terminals that co-expressed vesicular inhibitory amino acid transporter (Kudo et al 2012). However, other studies have found that BNST glutamatergic and GABAergic projections preferentially innervate non-dopaminergic VTA neurons, and that BNST glutamatergic projection activation resulted in aversive and anxiogenic behavioral phenotypes, while BNST GABAergic projections produced rewarding and anxiolytic behavior (Jennings et al 2013). More studies need to be conducted to understand the regulation of the VTA by BNST glutamatergic/GABAergic neurons and CRF-projecting neurons, and its involvement in stress-induced reinstatement.

Studies have shown that CRF-positive terminals within the VTA synapse onto DA cells that have asymmetric projections, indicating that they are glutamatergic (Tagliaferro & Morales 2008). However, this study failed to discuss the CRF terminals that do not synapse onto DA cells. It is my strong opinion that a subset of CRF-positive terminals originating from the BNST synapse onto either GABAergic interneurons, or other GABAergic neurons where they act to disinhibit DA neurons. In addition, it is important to note that studies have shown that both presynaptic and postsynaptic CRFR1 activation can reduce the activity of VTA DA neurons (Wanat et al., 2013; Beckstead et al., 2008). This indicates that CRF within the VTA can either enhance or inhibit VTA DA firing. Therefore, it is important to understand under what conditions CRF is released within the VTA, because CRF undergoes volume transmission, and therefore a single CRF release event within the VTA may act to activate and inhibit VTA DA neurons. All

in all, others and we have suggested a beta-2 AR-driven CRF pathway within the BNST that activate a CRF projection in to the VTA. This is depicted in figure 4.2. This pathway will act to engage VTA DA cells projecting to the prelimbic cortex.

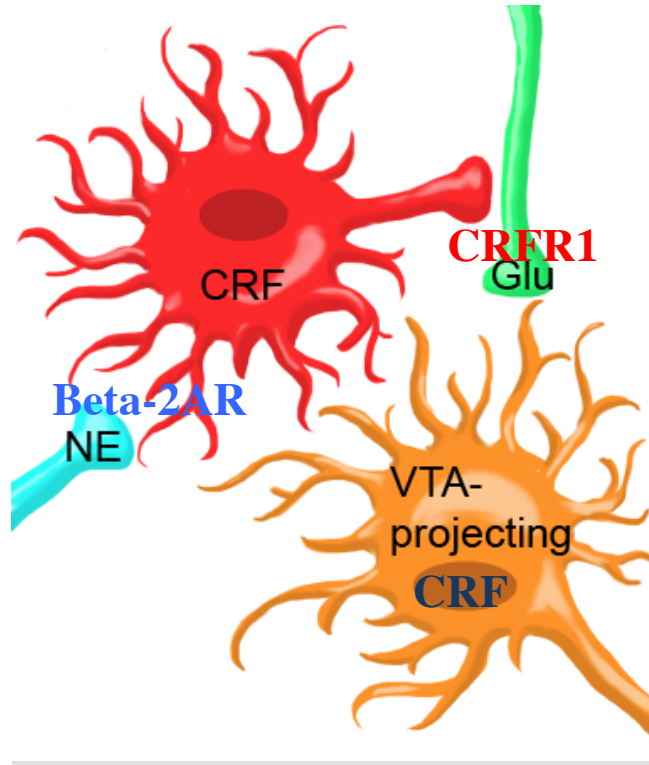


Figure 4.2: Model of NE recruitment of BNST VTA projection neurons We propose that NE activation of beta2-ARs depolarizes CRF neurons in the BNST to promote CRF release, which then activates presynaptic CRFR1 on glutamate terminals on VTA CRF-projecting BNST neurons

Susceptibility to stress-induced drug seeking is dependent on CRFR1 expression levels within the caudal VTA

Stress-induced reinstatement to a footshock stressor requires a history of extended access to cocaine. Different neurobiological mechanisms may contribute to the susceptibility to relapse during periods of stress. We have shown that CRFR1 activation is both necessary and sufficient for stress-induced drug seeking (Blacktop et al., 2011). In this dissertation we have shown that within the caudal VTA, CRFR1 expression is increased. In addition, a footshock stressor increases Fos immunoreactivity within the VTA compared to no shock controls. Furthermore, shock increased the activity of neurons that project to the prelimbic cortex. Moreover the prelimbic Fos response to shock is dependent on CRFR1 activation in the VTA (Chapter 4). These data suggests that a footshock stressor recruits a VTA projection, likely dopaminergic, to the prelimbic cortex via CRFR1 activation within the VTA as we have shown that a disconnection between VTA CRFR1 and prelimbic D1R prevents footshock-induced reinstatement. This is interesting because inhibiting dopamine signaling, with either a D1R specific antagonist, or a non-specific dopamine antagonist, within the prelimbic cortex prevents footshock-induced drug seeking (Capriles et al 2003, McFarland et al 2004). While we found that CRFR1 expression is increased within the VTA as a result of long-access cocaine self-administration, other mechanisms could be recruited to prime the activity of mesocortical neurons.

Cocaine intake-dependent neuroplastic changes within the VTA can increase the susceptibility to relapse of cocaine use during a stressor. While stress has been shown to promote CRF release within the VTA that is not dependent on a history of cocaine intake

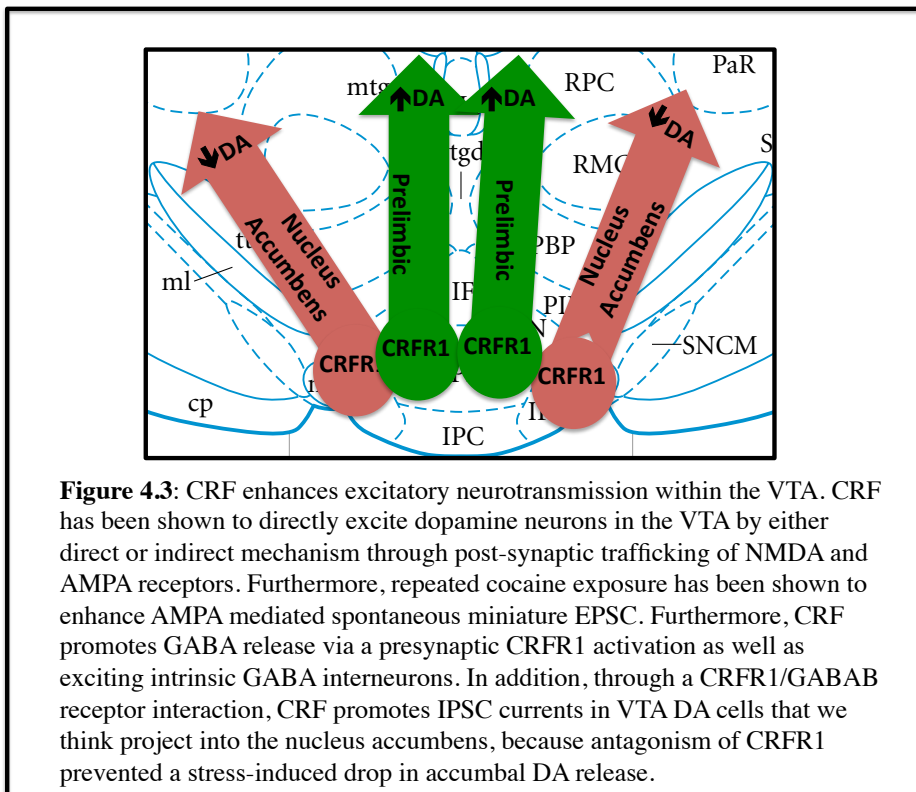
(Wang et al., 2005), no studies have examined whether CRF release is increased within the VTA in animals with a history of extended drug use. Our data from chapter 2 suggests that CRF production is increased within BNST, and that the BNST sends a direct projection of CRF to the VTA. Therefore, CRF release within the VTA may be augmented as a result of long-access cocaine use. However, the increase in *crf* mRNA observed in chapter 2 may be a reloading effect. Specifically, the beta-2 AR-mediated increase in CRF production may restore the pools of CRF in VTA terminals that have been released due to prior stressors. In addition, our lab has shown that bilateral delivery of CRF directly into the VTA fails to induce drug seeking in rats with a history of short-access self-administration (2hr/day) but produced robust drug seeking under long-access conditions suggesting that some neuroadaptation are needed. We have shown that this occurs as an increase of CRFR1 expression, but we cannot rule out other processes downstream (such as changes in the cortico-accumbens pathway), or upstream (such as actions within the BNST) from the site of CRF actions within the VTA.

Studies have shown that repeated cocaine administration has been shown to increase CRF receptor binding in the VTA (Goeders et al 1990) and also alter CRF responsiveness. Repeated cocaine administration has been shown to attenuate CRFR1-induced enhancement of GIRK channel-mediated inhibitory post-synaptic currents (Beckstead et al 2009) and establishes CRFR1-mediated regulation of glutamatergic synaptic transmission within the VTA (Hahn et al 2009). In addition, a history of cocaine use is required for CRF regulation of DA and glutamate release within the VTA (Wang et al., 2005).

We observed a gross increase in CRFR1 expression throughout the caudal VTA. However, the VTA consists of not only dopaminergic cells, but also GABAergic and glutamatergic cells. It would be interesting to see whether the increase in CRFR1 expression occurs in any specific cell type. Furthermore, it is important to determine whether the increase in CRFR1 expression is observed in any specific cell type that projects to either the prelimbic cortex, or the nucleus accumbens. While we did not explore specific projecting patterns, we did observe an increase that was specific to the caudal VTA. This is important because recent research has suggested that the caudal medial VTA contains DA neurons that specifically project into the prelimbic cortex (Lammel et al 2014, Lammel et al 2012). Recent research has shown that CRF expression is increased in the caudal, but not rostral, VTA of nicotine-dependent mice (Grieder et al 2014). In addition, the RMTg, or the tail of the VTA, has a stress-specific GABAergic input onto caudal VTA DA neurons that project into the ventral striatum (Barrot et al 2012). However, very little research has been done examining the rostral-caudal axis as it pertains to cocaine seeking during periods of stress. Studies have suggested that the rostral VTA is necessary for cocaine-taking behavior (Lee et al 2007). However, recent breakthroughs in rat and mouse genetics will allow us to specifically target VTA DA neurons that project to the prelimbic cortex, and therefore determine whether CRFR1 activation enhances the dopaminergic projection during periods of stress. Finally, other factors may mediate the heightened stress-induced cocaine seeking observed following LgA self-administration on CRFR1 function, such as downstream signaling pathways.

A VTA CRF-mediated DA projection to the prelimbic cortex is required for stress-induced drug seeking

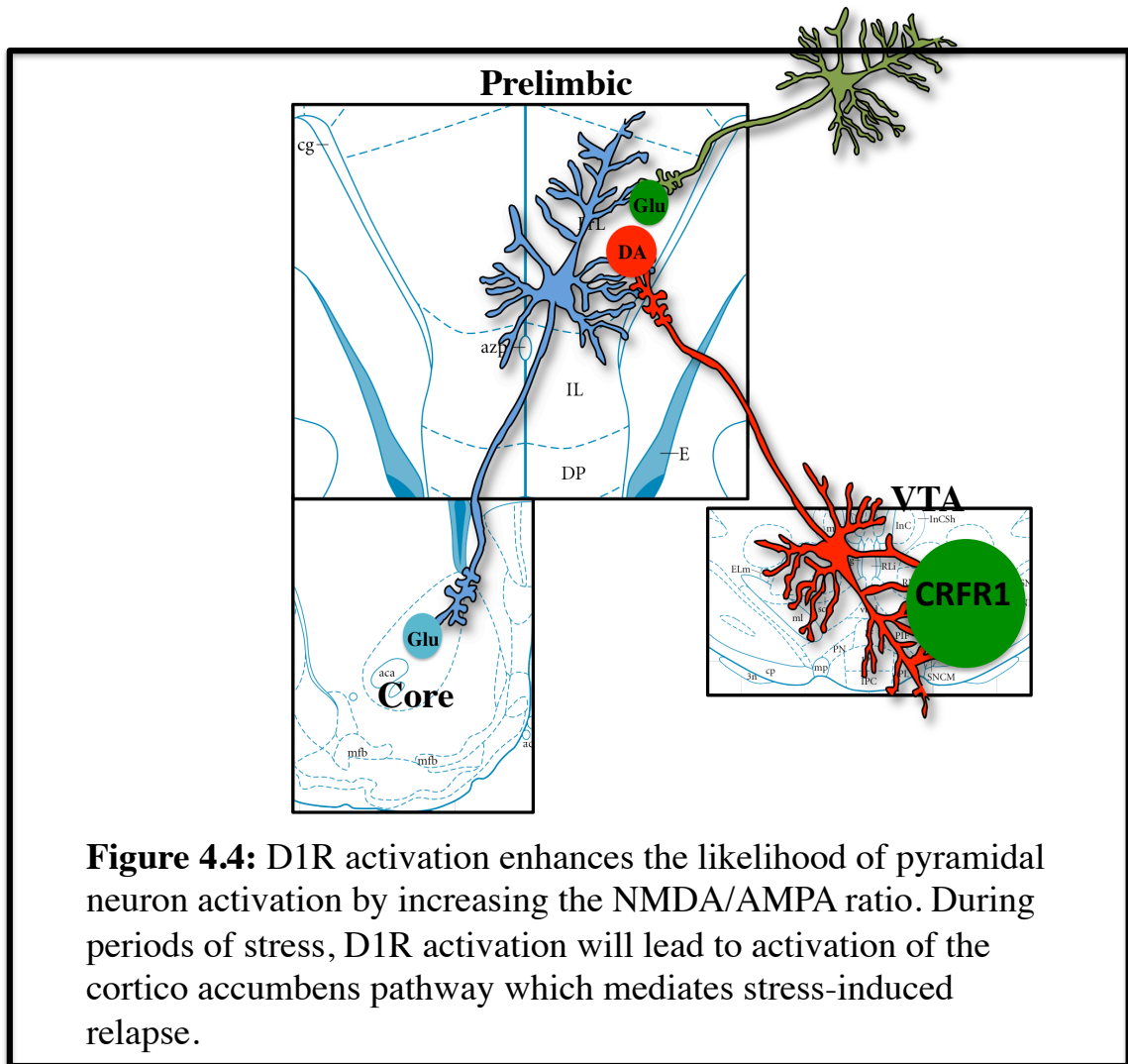
Studies have shown that dopamine receptor antagonism within the prelimbic cortex is necessary for stress-induced reinstatement (Capriles et al., 2003; McFarland et al., 2004) and that this may be due to activation of CRFR1 (Refojo et al 2011). However, no direct evidence has indicated that stress-induced reinstatement is dependent on CRFR1 activation of the mesocortical dopamine pathway or that the stress-induced activity within the prelimbic cortex is dependent on CRFR1 activation within the VTA. In chapter 4, we show that inhibition of CRFR1 within the VTA and D1 receptor inhibition within the prelimbic, but not infralimbic, cortex is sufficient in blocking footshock-induced drug seeking through a disconnection approach. While it has been known that stressors increase the activity of the prelimbic and the infralimbic cortex (Felice et al 2014, Morrow et al 2000) we have shown that a footshock stressor significantly augments Fos reactivity within the prelimbic cortex in long-access animals compared to saline and short-access animals, and this effect was not observed in the infralimbic cortex. Interestingly, the stress-induced increase in Fos activity was blocked by administration of an intra-VTA CRFR1 antagonist.



This suggests that during periods of stress, CRFR1 activation within the VTA recruits a subset of VTA neurons to enhance the activity of prefrontal cortex (Figure 4.4). This is similar to an early finding that showed that a restraint stress specifically increased Fos activity in DA neurons that project into the prefrontal cortex (Deutch et al 1991). Unfortunately, we cannot definitively state that VTA DA neurons are the sole driving force for the increase in prefrontal Fos reactivity. VTA GABAergic neurons may be driving the stress-dependent effect. In addition, studies have suggested that CRF may also decrease the activity of VTA DA neurons that project into the accumbens (Twining et al., 2015), possibly through an interaction between CRFR1 and GABA_B mediated inhibition by activating GIRK channels (Blacktop et al., 2015; Beckstead et al., 2009) (figure 4.3).

The role of the prefrontal and infralimbic cortex in relapse

Although shock increased Fos in the infralimbic cortex, the Fos response in the prelimbic cortex was much more robust and was more pronounced in long-access rats that displayed stress-induced reinstatement of cocaine seeking. This is not surprising because previous studies have suggested that prelimbic glutamate input into the nucleus accumbens core is involved in extinguished drug seeking to drugs, cues, and a footshock stressor (Capriles et al 2003, Cornish et al 1999, LaLumiere & Kalivas 2008, McFarland et al 2004). The infralimbic cortex sends a glutamatergic projection into the shell of the nucleus accumbens that is thought to be involved in extinction learning (LaLumiere et al 2010, LaLumiere et al 2012). Furthermore, it has been shown that the glutamatergic projection from the infralimbic cortex to the shell of the accumbens regulates suppression of drug seeking (Peters et al 2009, Peters et al 2008). Specifically, inactivation of the infralimbic cortex, or the shell of the nucleus accumbens reinstates drug seeking in the absence of drugs, cue, or stressful triggers (Peters et al 2008). This is surprising because we show that a footshock stressor increases the activity of the infralimbic cortex, though to a lesser degree than the prelimbic cortex.



The role of the infralimbic cortex as a stop pathway has been recently challenged (Moorman et al 2014). Studies have shown that both the infralimbic and prelimbic cortex display rapid, stimulus-evoked activity changes that are closely linked to contextually appropriate behaviors. This was shown to be independent of whether the behavior involved execution or inhibition (Moorman & Aston-Jones 2015). Other studies have shown that activity is increased in the infralimbic cortex following both cue- and context-induced cocaine, and heroin seeking (Moorman et al 2014). Therefore, our results may

suggest that within extended access cocaine use, the activity within the infralimbic cortex may be changed as well. However, our results from chapter 4 have indicated that D1R-mediated DA activity within the infralimbic cortex, as it relates to CRFR1-dependent activation of VTA DA neurons, is not involved in stress-induced reinstatement since a disconnection between VTA CRFR1 and infralimbic D1R did not block stress-induced reinstatement.

Conclusion

The impact of stress on cocaine relapse is very complex. Not only do we report a beta-2 AR-mediated CRF-dependent activation of a CRFergic afferent pathway from the bed nucleus of the stria terminalis to the ventral tegmental area is necessary for stress-induced relapse, we also report that animals with a history of extended access cocaine use are susceptible to reinstatement to a stressor by the recruitment of the mesocortical DA pathway to the prelimbic cortex. This recruitment may be partially due to an increase in CRFR1 expression within in the VTA that is specific to extended cocaine use. Human studies have already shown that clonidine can prevent cocaine craving during periods of stress in humans (Jobes et al 2011). While other studies have shown that polymorphisms in genes that encode the CRF receptors is associated with exacerbated stress responses and the propensity to develop drug addiction (Blomeyer et al 2008, Clarke & Schumann 2009, De Luca et al 2007, Enoch et al 2008, Treutlein et al 2006), we still do not understand the microcircuitry of CRF signaling within the VTA. Thus, future directions for investigating the mechanisms by which CRF controls stress-related drug use should focus on mapping CRF circuitry within the VTA to determine the mechanism by which

CRF may activate, or inhibit specific dopaminergic projections. This can be achieved by determining the cellular location of CRFR1 and CRFR2 receptors within the VTA in animals with a history of extended cocaine access. Currently, novel tools are being developed to determine the microcircuitry within the VTA.

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APPENDIX

Drug	Action
Antalarmin	Antagonist at CRFR1
Antisauvagine	Antagonist at CRFR2
Betaxolol	Antagonist at Beta-1AR
BRL 44408	Antagonist at Alpha-2AR (2 α)
Clenbuterlol	Agonist at Beta-2AR
Clonidine	Agonist at Alpha-2AR
CP 154,526	Antagonist at CRFR1
Dobutamine	Agonist at Beta-2AR
Guanfacine	Agonist at Alpha-2A (2 α)
ICI 118,551	Antagonist at Beta-2AR
Isoproterenol	Agonist at Beta-1AR and Beta-2AR
Kynurenic Acid	Antagonist at AMPA, NMDA, and Kianate Receptors
Prazosin	Antagonist at Alpha-1AR
Propranolol	Antagonist at Beta-1AR and Beta-2AR
SCH 23390	Antagonist at D1R
Yohimbine	Antagonist at Alpha2-AR (2 α , alpha1 α AR, 5HT: 1A,1,B,1D,1F,2B and D2)

List of abbreviations

Abbreviations	Name
AC	Anterior Commissure
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	Adrenergic Receptor
BNST	Bed Nucleus of the Stria Terminalis
CPP	Conditioned place preference
CRF	Corticotropin releasing factor
CRFR	Corticotropin releasing factor receptor
CTb	Cholera Toxin B
DA	Dopamine
EPSC	Excitatory Postsynaptic Current
GABA	Gamma -Aminobutyric Acid
IL	Infralimbic Cortex
IPSC	Inhibitory Postsynaptic Current
MSN	Medium spiny neurons
Nacc	Nucleus Accumbens
NE	Norepinephrine
NMDA	N-methyl-D-aspartate receptor
PFC	Prefrontal Cortex
PL	Prelimbic Cortex
SA	Self-Administration
VP	Ventral Pallidum
VTA	Ventral Tegmental Area