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THE ROLE OF THE BNST IN STRESS AND ALCOHOL-RELATED BEHAVIORS

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ABSTRACT

Alcohol Use Disorder (AUD) is a chronic and relapsing disease that affects around 14.5 million individuals in the United States, and about 16 million Americans use alcohol heavily. Stress is often cited as a key trigger to harmful alcohol use and relapse into drinking, and thus provides a major obstacle in the prevention and treatment of AUD. Two major molecules involved in the physiological stress response include corticotropin releasing factor (CRF) and norepinephrine (NE). An important brain region associated with behavioral responses to stress and alcohol-related behaviors is the bed nucleus of the stria terminalis (BNST), and CRF and NE in the BNST are implicated in mediating such behaviors. Further, the BNST is separated into numerous subregions that differ in their cytoarchitecture and function, and two major subregions of interest are the dorsal BNST (dBNST) and ventral BNST (vBNST). Evidence suggests that stress-related and drug-seeking behaviors are mediated via CRF and NE in the dBNST and vBNST, but through different pathways and mechanisms. For example, the beta1-adrenergic receptor (β1-AR) is implicated in dBNST control of excitatory neurotransmission via CRF; however, the vBNST mediates stress-related drug-seeking behavior via a mechanism involving the β2-AR specifically. This dissertation explores CRF and β-AR mediated glutamatergic transmission in the dBNST and vBNST after alcohol or stress exposure. BNST subregional differences and neurocircuitry changes after stress and alcohol exposure are uncovered. A novel mouse model of stress-enhanced drinking after a period of forced abstinence was also developed and used to further investigate BNST CRF and NE's role in stress-related alcohol use. Ultimately, investigating the neurocircuitry changes involved in stress and alcohol use is useful for the development of future prevention and treatment options for AUD.

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Chapter 1

Corticotropin Releasing Factor and Norepinephrine Related Circuitry Changes in the Bed Nucleus of the Stria Terminalis in Stress and Alcohol and Substance Use Disorders

Abstract

Alcohol Use Disorder (AUD) affects around 14.5 million individuals in the United States, with Substance Use Disorder (SUD) affecting an additional 8.3 million individuals. Relapse is a major barrier to effective long-term treatment of this illness with stress often described as a key trigger for a person with AUD or SUD to relapse during a period of abstinence. Two signaling molecules, norepinephrine (NE) and corticotropin releasing factor (CRF), are released during the stress response, and also play important roles in reward behaviors and the addiction process. Within the addiction literature, one brain region in which there has been increasing research focus in recent years is the bed nucleus of the stria terminalis (BNST). The BNST is a limbic structure with numerous cytoarchitecturally and functionally different subregions that has been implicated in drug-seeking behaviors and stress responses. This review focuses on drug and stress-related neurocircuitry changes in the BNST, particularly within the CRF and NE systems, with an emphasis on differences and similarities between the major dorsal and ventral BNST subregions.

1. Introduction

Substance Use Disorders (SUD) are a significant problem in the United States. According to the Substance Abuse and Mental Health Services Administration, SUDs affected 22.8 million people in the United States in the most recent 2019 estimates, with Alcohol Use Disorder (AUD) affecting about 14.5 million of those individuals. Additionally, the number of alcohol-related deaths is rising (Spillane et al., 2020; Vierboom 2020). Stress is also highly prevalent and continues to be a great health concern. When individuals attempt to cut back or quit using drugs or alcohol, stress often becomes a major obstacle to doing so (Sinha 2008). This situation is more relevant than ever in 2020-2021, with the COVID-19 pandemic leading to excessive stress, social isolation, and increased alcohol drinking and drug use (Boschuetz et al., 2020; Dubey et al., 2020; Killgore et al., 2021; Vanderbruggen et al., 2020; Wardell et al., 2020).

When a stressor is present, an organism reacts physiologically through multiple pathways including the autonomic nervous system (ANS), as well as through activation of the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 2016; Kemeny 2003; Smith and Vale, 2006). Autonomic nervous system signaling via sympathetic noradrenergic innervation of the adrenal medulla of the adrenal gland results in the release of epinephrine into the bloodstream in response to a stressor (Kemeny 2003). In the HPA axis, parvocellular neurons within the paraventricular nucleus of the hypothalamus (PVN) release corticotropin releasing factor (CRF) (sometimes referred to as corticotropin releasing hormone/CRH) into hypophyseal portal veins in response to signaling within connected neural pathways, indicating the perception of a stressor. CRF then acts in the anterior pituitary, causing the release of adrenocorticotropic hormone (ACTH) which then travels through the bloodstream and acts on receptors located on the cortices of the adrenal glands. The adrenal glands then release glucocorticoids (e.g., cortisol in humans, corticosterone in rodents) which act upon downstream target organs. Glucocorticoids also have

action in the hypothalamus and pituitary gland, which can encourage a negative feedback loop (Smith and Vale, 2006). While the pathways described are sufficient for generally explaining the physiological stress response, there are many other structures at play in this system that also modulate the behavioral response to stress, including extra-hypothalamic limbic structures such as the amygdala, hippocampus, anterior cingulate cortex, and the bed nucleus of the stria terminalis (BNST). Additionally, CRF and norepinephrine (NE) signaling also play a larger variety of roles than mentioned above and will be discussed further in this review.

The BNST is a structure comprising many different subregions, and that number can be between 12-18 subregions in the rodent brain, depending on who is defining it (Bota et al., 2012; Lebow and Chen, 2016). The BNST is part of the "extended amygdala" along with the central amygdala (CeA) and the nucleus accumbens (NAcc) shell (Alheid 2003). It plays many roles in numerous processes including feeding behavior, mood, and social attachment (Lebow and Chen 2016), but for this review we will focus on its role in the stress response and AUD/SUD. *For a detailed review on the comparison between the amygdala and the BNST's role in anxiety-related psychiatric disorders such as generalized anxiety disorder (GAD), social anxiety, and PTSD see Lebow and Chen (2016). For a discussion regarding the BNST vs. amygdala's role in anxiety vs. fear see companion reviews Gungor and Pare (2016) and Shackman and Fox (2016).*

The BNST acts as a relay between limbic regions such as the hippocampus, CeA, medial amygdala (MeA), and the PVN. It has been suggested that the BNST is one of the most important extra-hypothalamic relays to regulate the HPA axis during times of stress (Forray and Gysling, 2004). Investigating how stress and AUD/SUD, both separately and in combination, affect BNST neurocircuitry and subsequent behaviors will help identify treatment targets for these diseases and potentially for stress-related conditions such as post-traumatic stress disorder. This review will focus on the current state of research examining the role of dorsal and ventral BNST NE and CRF

in stress and SUDs, and will highlight potential future research needs in order to fill gaps and better understand stress-related alcohol and substance use.

2. BNST Anatomy

The BNST is a complex structure with many afferent and efferent projections (Dong et al., 2000; Dong et al., 2001a; Dong et al., 2001b; Dong and Swanson 2004; 2006a; 2006b; Lebow and Chen, 2016). This structure is comprised of numerous subregions that are cytoarchitecturally, chemoarchitecturally, and functionally diverse (Ju and Swanson 1989; Ju et al., 1989). The BNST can be subdivided using multiple approaches. One approach uses structural landmarks such as the anterior commissure to delineate subregions, and other approaches use cell types or incoming/outgoing projections to define regions. Subregions also typically differ in their primary neurotransmitter production and functionality. For example, the anterior regions of the BNST appear to be more relevant for stress and anxiety-like behaviors (Young and Tong, 2021) while posterior regions may be more important in maternal behaviors (Klampfl and Bosch, 2019). Ju and Swanson (1989) divided the anterior portion of the BNST into dorsal, lateral, and ventral areas containing 12 nuclei. Each nucleus contains distinct cell types projecting to, and receiving projections from, many brain regions such as the CeA and hypothalamus. Ju and Swanson (1989) used cytoarchitectural delineations of the anterior BNST to designate multiple areas including the anterolateral area, anteromedial area, fusiform nucleus, oval nucleus, and juxtacapsular nucleus. The anterolateral and anteromedial areas can be further subdivided into dorsal and ventral components. For this review, dorsal BNST (dBNST) will refer to the anterolateral dorsal area, anteromedial dorsal area, juxtacapsular nucleus, and oval nucleus combined; ventral BNST (vBNST) will refer to the anterolateral ventral, anteromedial ventral, and fusiform nucleus combined. These subregions can be easily located and identified by locating the anterior

commissure on a coronal section of a rodent brain; the triangular areas immediately above and below are the dBNST and vBNST, respectively (Figure 1-1). Since manuscripts may not explicitly define whether they are manipulating the dorsal/ventral BNST specifically, figures within the papers demarcating injection sites were used to determine this delineation. If no figures indicating the specific sites were provided, the coordinates were input into the Paxinos, George, and Watson 2006 atlas to determine the location (http://labs.gaidi.ca/rat-brain-atlas/or http://labs.gaidi.ca/mouse-brain-atlas/).

It is important to note, however, that using provided coordinates to determine the BNST

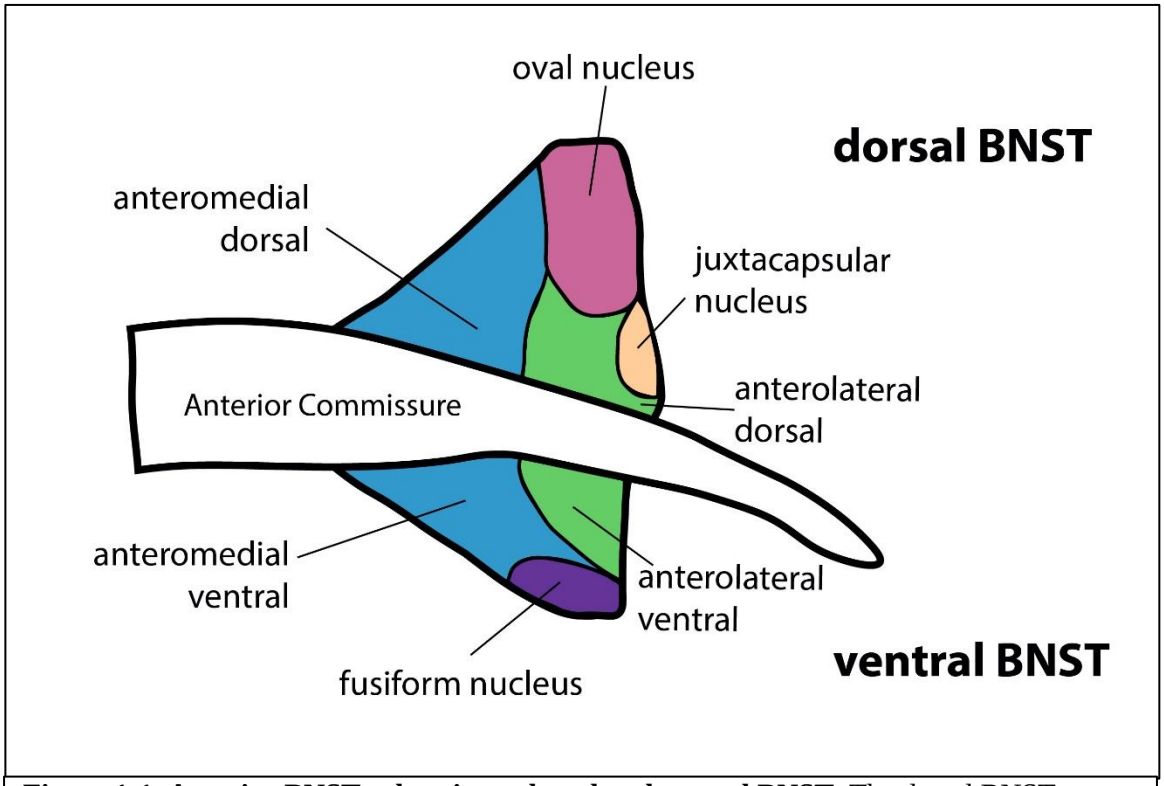

Figure 1-1. Anterior BNST subregions: dorsal and ventral BNST. The dorsal BNST includes the anterolateral dorsal area, anteromedial dorsal area, juxtacapsular nucleus, and oval nucleus. The ventral BNST consists of the anteromedial ventral area, the anterolateral ventral area, and the fusiform nucleus. This figure was adapted from the Adult Mouse Allen Brain Reference Atlas.

subregion might not accurately represent the actual injection site. Giardino and Pomrenze (2021) emphasize the discrepancy in stereotaxic coordinates reported as the vBNST within male mice on a C57BL/6J background with considerable variation in the A/P and M/L coordinates (1.24 mm

and 0.5 mm range, respectively). This variation in reported vBNST coordinates emphasizes the importance of verifying injection/probe placement sites and reporting those on a figure within the text. Since each brain is slightly different, and each group uses different tools and possibly defines bregma differently, this would help clear up ambiguity and allow for better comparisons across studies. Due to these types of discrepancies, delineation of the BNST by combining cell type or projection-specific characteristics along with spatial boundaries will be important in the future and may be more informative regarding the way these subregions modulate behavioral responses (Giardino and Pomrenze, 2021). *For a more thorough discussion of the anatomic literature of the dBNST and vBNST see the review by Giardino and Pomrenze (2021).* For the purpose of this review, we will still separate based on spatial delineations of the dBNST and vBNST since that is how most literature reports it to date.

The various BNST subregions also appear to have distinct and overlapping efferent and afferent connections. The oval nucleus (BNSTov) in the dBNST has many outgoing projections to areas such as the amygdala, hypothalamus, and lower brainstem structures such as the nucleus of the tractus solitarius (NTS). The BNSTov also has local projections throughout the BNST with dense projections to the fusiform nucleus (BNSTfu) in the vBNST. The BNSTfu has some similar target regions as the BNSTov such as the amygdala and the hypothalamus, but its projections are more complex (Dong et al., 2001b). The juxtacapsular nucleus (BNSTju) located in the lateral area of the dorsal subregion sends projections within the BNST, particularly to the subcommissural zone, caudal anterolateral area, and strial extension, along with dense projections to the caudal substantia innominata, ventromedial caudoputamen, medial CeA, and anterior basolateral amygdala (BLA) among other regions (Dong et al., 2000). One major source of inputs into the BNST is the CeA. CeA projections to the BNST tend to project primarily to the lateral and medial anterior regions of the BNST (Dong et al., 2001a), and are a primary source of CRF in the BNST (Sakanaka et al., 1986). *For a much more detailed characterization of these inputs,*

please see Dong et al. (2001a). The anterolateral region is sometimes subdivided into dorsal and ventral portions named the anterolateral area (BNSTal) and the subcommissural zone (BNSTsc), respectively. According to Dong (2004), they share the same projection distributions to areas within the BNST such as the rhomboid nucleus, the BNSTfu, the anterodorsal region, and anteroventral (Dong and Swanson, 2004). Many other brain structures also receive projections from the anterolateral regions, including the VTA, the CeA, and the hypothalamus, but the BNSTal/sc projections are unique from projections originating in the BNSTju, BNSTov, BNSTfu, and rhomboid nuclei. Based on the specific projections observed from the general anteromedial area of the BNST (BNSTam) to areas such as the CeA, PVN of the hypothalamus, VTA, and NAcc, it is inferred that this area is generally involved in maintaining energy homeostasis through coordination of behavioral, autonomic, and neuroendocrine responses (Dong and Swanson 2006a). Oddly, the region defined as the dorsomedial nucleus (BNSTdm) is part of the vBNST, located immediately ventral to the anterior commissure. This nucleus also contains unique projections from other anterior nuclei and is thought to be involved in regulating fluid balance homeostasis, particularly fluid drinking and sodium appetite (Dong and Swanson 2006b). As demonstrated in this description of dBNST and vBNST anatomy, the BNST is interconnected with many neural structures and performs a diversity of functions even within the dBNST and vBNST subdivisions.

2.1 Cell Types in BNST Subregions

The discrete subnuclei in the anterior dBNST and vBNST contain various cell types that display their own physiological properties (Hammack et al., 2007; Rodríguez-Sierra et al., 2013; Silberman et al., 2013). The cell types in the anterolateral group of the BNST (BNSTalg), primarily the anterolateral dBNST, have been characterized in rat (Dabrowska et al., 2013; Daniel

et al., 2017; Hammack et al., 2007; Hazra et al., 2011; Rodríguez-Sierra et al., 2013), mouse (Daniel et al., 2017; Silberman et al., 2013), and macaque (Daniel et al., 2017). First categorized in the rat, largely in the dorsolateral subregion of the BNST (dlBNST), the neurons were separated into three groups corresponding to their physiological properties: Type I, Type II, and Type III (Hammack et al., 2007). Type I neurons are "regular spiking", Type II are "low-threshold bursting", and Type III are "fast inward rectifiers" (Hammack et al., 2007; Rodríguez-Sierra et al., 2013). Rodriguez-Sierra et al. (2013) further investigated subregional differences of these cell types in the dorsomedial, dorsolateral, and anteroventral subregions of the Lewis rat. All three cell types were present, but there were subregional differences in physiology and even additional cell types—spontaneously active cells only seen in the anteroventral subregion and late-firing cells only seen in the dorsolateral subregion. Subsequent studies in the dlBNST of the rat identified correlate expression of I_h current, I_T calcium current, $I_{K(IR)}$ current, and the I_A current ion channel subunits with the electrophysiological properties of Type I, II, and III neurons (Hazra et al., 2011). Daniel et al. (2017) observed differences in BNSTalg (dlBNST) neurons across species. For example, out of Type I, II, and III cells, Type III was the most commonly observed in the mouse and macaque. Additionally, they also observed cells that did not fit neatly into these categories in the macaque as well as in the mouse, similar to other observations (Silberman et al., 2013). It is important to recognize that due to differences in morphological and electrophysiological properties in these cell types across species, the function of these cell types across species is likely variable, and further characterization and comparison are necessary.

Nguyen et al. (2016) examined the neuronal subtypes in the anterior dorsal and ventral subregions of the mouse BNST using a combination of genetic and immunohistochemical techniques. They determined that the dBNST contained a higher density of neurons than the vBNST with varying composition. The dBNST contained approximately 66% GABAergic and 14% glutamatergic neurons, whereas the vBNST had 32% GABAergic and 29% glutamatergic.

This contrasting neuronal composition highlights the functional variability and the importance of studying each subregion separately if possible. To further sub-divide the GABAergic cell types, Nguyen et al. (2016) examined the expression of parvalbumin (“rare/largely absent”), somatostatin (dBNST=17%, vBNST=4.4%), calretinin (dBNST=10%, primarily the ovBNST, vBNST=3.6%), and vasoactive intestinal peptide (“scarce”). There was also only a small amount of ChAT+ cells. Additionally, the authors assessed CRF expression and found that the dBNST had about 8% of the total cells expressing CRF, and the vBNST had about 13% (Nguyen et al., 2016).

Gungor et al. (2018) examined the morphological and physiological differences between mouse glutamatergic and GABAergic vBNST neurons. They identified two major types of morphologies: varicose neurons and spiny neurons. Varicose neurons accounted for 56% of all cells, with no dendritic spines but with dendritic varicosities like "beads on a necklace" (Gungor et al., 2018). Spiny neurons made up 34% of all vBNST cells with low dendritic spine density comprising a spectrum of shapes from "thin to stubby". Ultimately, the authors identified three main populations in the vBNST: glutamatergic varicose neurons, GABAergic varicose neurons, and GABAergic spiny neurons. The glutamatergic cells had a greater number of primary processes, longer dendrite length, and greater process complexity than the GABAergic cells, and glutamatergic neurons demonstrated characteristics associated with increased excitability relative to the GABAergic neurons (Gungor et al., 2018). For example, responding to depolarizing current steps, glutamatergic neurons fired quicker and more frequently than the GABAergic neurons, and spontaneous activity was more commonly observed in the glutamatergic neurons (Gungor et al., 2018). Taken with results from Nguyen et al. (2016) indicating that the vBNST contains approximately the same amount of GABAergic and glutamatergic neurons, this suggests that glutamatergic neurons may have a larger influence than GABAergic neurons on network activity within the vBNST, at least in basal conditions.

For much more detail describing the diverse neuronal populations in the BNST, see Beyeler and Dabrowska (2020) and Giardino & Pomrenze (2021). Further discussion about certain cell types, particularly BNST CRF neurons and their interactions with NE, will be provided in sections below.

2.2. Sex Differences

The BNST is known to be a highly sexually dimorphic brain region, and there are numerous examples of this. When looking at the anterior dBNST and vBNST in mice (Uchida et al., 2019) and rats (Funabashi et al., 2004), there are more CRF neurons in the dorsolateral region of females compared to males (Funabashi et al., 2004; Uchida et al., 2019). In mice, the BNSTov and surrounding dorsolateral region was the specific region identified as sexually dimorphic (Uchida et al., 2019). Interestingly, there was no difference in the number of CRF cells in the rest of the dBNST and vBNST between male and female mice. It was also found that the sexually dimorphic regions in the mouse contained a larger overall number of neurons in females (Uchida et al., 2019), which is consistent with previously reported data in the rat (Guillamón et al., 1988). In the rat, females had a larger number of neurons in the lateral area of the anterior BNST (Guillamón et al., 1988). This difference in the total number of CRF-expressing BNST neurons in females vs. males appears to be modulated by sex hormones in mice, as the dlBNST from ovariectomized females looked like that from intact males and had fewer CRF neurons than proestrus females (Uchida et al., 2019). Also, the dlBNST from orchiectomized males was not different from proestrus females (Uchida et al., 2019). Although there is still limited data on sex differences, observed consequences of CRF differences between the two sexes in the context of stress and substance use will be discussed further in later sections when possible.

3. Corticotropin Releasing Factor (CRF) and CRF Receptors

Corticotropin releasing factor (CRF) is a 41-residue protein that was originally isolated from sheep hypothalamus in 1981 by Vale and colleagues (Vale et al., 1981). CRF is a neuropeptide that is primarily attributed to the role of modulating the stress response, as well as other behaviors such as anxiety, arousal, feeding behavior, and sleep (Dedic et al., 2018). While the PVN is the first brain region often associated with CRF since it is critical to initiate the HPA axis response, CRF is highly expressed in other regions. Using whole-brain mapping of CRF neurons in the mouse brain, the largest density of CRF neurons (>20,000 cells/mm^3) were identified in the PVN, Barrington's nucleus, and the inferior olivary complex, consistent with prior studies (Peng et al., 2017). The BNST as a whole had a reported CRF neuron density of 10,000 to 20,000 cells/mm^3 (Peng et al., 2017). It is important to note, however, that the *entire* BNST was reported, as opposed to specific subregions which are known to have varying densities of CRF neurons. More discussion regarding CRF neurons in the BNST is presented in section 5.1. Other limbic structures that contain CRF neurons include the BLA, the CeA, and the hippocampus (Peng et al., 2017). Additionally, CRF has been shown to exert effects in the central nervous system (CNS) independent of the typical glucocorticoid responses resulting from activation of the HPA axis (Dedic et al., 2018), which speaks to its functionality. One related example of this is that intra-BNST infusions of CRF are able to produce reinstatement to drug-seeking behavior (Erb and Stewart 1999), a topic which will be discussed in further detail in subsequent sections of this review.

There are two major types of CRF receptors: CRFR1 and CRFR2. CRFR1 and CRFR2 are categorized as class B G-protein-coupled receptors (GPCRs) (Bale and Vale, 2004). Both

receptors are expressed in the CNS, however it is the CRFR1 that is widely expressed throughout the brain (Potter et al., 1994) in structures including the olfactory bulbs, the cingulate cortex, the VTA, and the BNST (Dedic et al., 2018; Reul and Holsboer, 2002). Both the type and the location of the CRF receptor will determine if activation is anxiogenic or anxiolytic (Dedic et al., 2018). *For a more detailed review on the distribution of CRFR1 receptors in the rodent brain and how the CRF family of neuropeptides and receptors affects the stress response in the CNS, see Reul and Holsboer (2002) and Dedic (2018*). Urocortin (UCN) 1, UCN2 (aka stresscopin-related peptide), and UCN3 (aka stresscopin) are also signaling molecules with affinity for the CRF receptor (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). More specifically, UCN1 has a stronger affinity than CRF for CRFR1 and CRFR2 by six times and 40 times, respectively (Vaughan et al., 1995), but a relatively equal affinity for both CRFR1 and CRFR2 (Vaughan et al., 1995). UCN2 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001) and UCN3 (Hsu and Hsueh, 2001; Lewis et al., 2001) are both selective for CRFR2 only. CRF itself has a much greater affinity for CRFR1 over CRFR2. The BNST expresses both CRFR1 and CRFR2, as well as CRF and UCN3 (Dedic et al., 2018; Lewis et al., 2001). While CRF and CRFRs have various specific functions throughout the CNS and periphery, the purpose of this review is to discuss them in the context of the BNST, stress, and substance use. *For a more in-depth review of CRF's role in addictive behaviors in general, please see Roberto et al. (2017).*

4. Norepinephrine (NE) and NE Receptors

Norepinephrine (NE) and epinephrine (also referred to as noradrenaline and adrenaline, respectively) are also major molecules involved in the stress response. The release of NE leads to

many downstream effects, some of which perpetuate the stress response. NE receptors are found throughout the brain including in the BNST. Although most brain NE is produced by the locus coeruleus (LC) (Schwarz and Luo, 2015), this is not the primary source of the BNST's NE. There are two noradrenergic pathways of innervation to the BNST: the dorsal noradrenergic bundle (DNB) and the ventral noradrenergic bundle (VNB). The VNB is the BNST's primary source of NE, connecting medullary regions A1 and A2 to the vBNST. The LC (A6) contributes less NE to the BNST (Forray and Gysling, 2004) and sends its NE projections through the DNB. Fox et al. (2016) reported in male Sprague-Dawley rats that medullary noradrenergic afferents from the brainstem project directly to both the ipsilateral and contralateral vBNST, with unilateral stimulation of the DNB, VNB, and LC resulting in comparable NE release in both hemispheres. NE release via LC and the DNB stimulation, however, was demonstrated to be via an indirect pathway requiring A2 activation. Notably, the vBNST has the largest density of noradrenergic terminals in the brain (Forray and Gysling, 2004), emphasizing the importance of NE modulation in BNST control of the stress response. Here we give a general background of the various adrenergic receptor subtypes, but specific effects of NE and the adrenergic receptor subtypes on BNST circuit function and related behaviors will be further discussed in later sections.

4.1 NE Receptors

Animal studies have investigated NE circuitry throughout the brain and have found the expression of adrenergic receptors (ARs) in many regions. ARs, like CRF receptors, are GPCRs. These receptors fall into the families of α1, α2, and β, with nine subtypes as follows: $\alpha1_A$, $\alpha1_B$, $\alpha1_D$, $\alpha2_A$, $\alpha2_B$, $\alpha2_C$, β1, β2, β3 (Pupo and Minneman, 2001). α1-ARs and β-ARs are generally excitatory, primarily coupling with Gαq and Gαs subunits, respectively, while α2-ARs are

generally inhibitory, coupling with Gαi and acting as autoreceptors in most brain regions (Birnbaum et al., 2004; Duman and Nestler 1995; Pupo and Minneman, 2001). Whether receptors are excitatory or inhibitory, however, may differ depending upon the tissue and level of stimulation (Daaka et al., 1997; Xiao 2001).

Epinephrine and NE bind with similar affinity to α1-ARs and α2-ARs (Pupo and Minneman, 2001), although some sources say that NE has the highest affinity for α2-ARs followed by α1-ARs, and the lowest affinity for β-ARs (Ramos and Arnsten, 2007). When NE is released into a synapse, clearance of excess extracellular neurotransmitter occurs primarily via the NE transporter (NET). The NET is also important in determining the amount of NE stored within neurons (Xu et al., 2000). Because the interaction between release and uptake of neurotransmitter determines extracellular concentrations (Wightman et al., 1988), the NET plays a vital role in the process of NE regulation. Additionally, it is important to note that each subtype of receptor has its own localization and associated functions.

4.1.1 α1-ARs

Typically, α1-ARs are Gαq coupled, which means the downstream effect of activation is to increase intracellular calcium (Birnbaum et al., 2004; Duman and Nestler 1995; Pupo and Minneman, 2001), although some have reported that α1-ARs may also utilize Gi/Go G proteins (Duman and Nestler 1995). α1-AR mRNA has been detected widely throughout the brain using *in situ* hybridization in areas including the cortex, olfactory bulb, thalamus, hypothalamus, hippocampus, amygdaloid complex, and BNST (Day et al., 1997). $\alpha 1_B$-ARs were detected in the same structures mentioned, with much greater expression in the thalamus. $\alpha 1_D$-ARs were abundant in the cortex, hippocampus, and the olfactory bulb, present in the amygdaloid complex and within the reticular nucleus of the thalamus, and absent in the hypothalamus and BNST (Day

et al., 1997). Overall, it appears that the $\alpha 1_A$-AR was the most highly-expressed α1-AR in the BNST, followed by the $\alpha 1_B$-AR, with no expression of the $\alpha 1_D$-AR (Day et al., 1997). It is important to note that this was the entire BNST as opposed to subregions, so the content of the dBNST or vBNST is not reported separately. However, one study has pointed to subregional differences in α1-AR density, with the vBNST showing a significantly lower α1-AR density than the dBNST of naïve rats (Wee et al., 2008).

4.1.2 α2-ARs

In general, α2-ARs are inhibitory autoreceptors, so they play a role in the regulation of NE signaling. α2-ARs are often Gαi coupled (Ramos et al., 2006). All three subtypes ($\alpha 2_A$, $\alpha 2_B$, and $\alpha 2_C$) are found in the brain, with the $\alpha 2_A$ and $\alpha 2_C$ found in the BNST (Scheinin et al., 1994; Wang et al., 1996). These two receptors are differentially regulated via various desensitization and phosphorylation paradigms by an assortment of G-protein coupled receptor kinases (Jewell-Motz and Liggett 1996). Additionally, knockout (KO) studies identified the $\alpha 2_A$-AR as the primary autoreceptor compared to the other subtypes (Trendelenburg et al., 2001).

The $\alpha 2_A$-AR is found throughout the brain in areas such as the LC, pontine nuclei, amygdala, hippocampus, olfactory system, hypothalamus, NTS (Scheinin et al., 1994; Wang et al., 1996), and the BNST (Shields et al., 2009). $\alpha 2_A$-ARs are often thought of as presynaptic autoreceptors, and in this role function to inhibit the presynaptic release of NE (Cedarbaum and Aghajanian 1977; Engberg and Eriksson 1991). Despite this, they are also found postsynaptically (Duka et al., 2000; Zhang et al., 2009), and there is mounting evidence that the $\alpha 2_A$-AR is also present on non-noradrenergic neurons. Their presence on non-noradrenergic neurons supports the idea that the $\alpha 2_A$-AR may also function as a heteroreceptor in numerous brain regions (Gilsbach et al., 2009), including the CeA (Delaney et al., 2007), NTS (Glass et al., 2001), hippocampus

(Milner et al., 1998), ventrolateral medulla (Milner et al., 1999) and the dBNST (Fetterly et al., 2019; Flavin et al., 2014; Harris et al., 2018; Perez et al., 2020; Shields et al., 2009). Some functions of the $\alpha 2_A$-AR are associated with are attention and arousal (Broese et al., 2012), sensitization to amphetamines (Lähdesmäki et al., 2004), and anxiety-like behavior (Lähdesmäki et al., 2002).

Although the $\alpha 2_B$-AR is abundant in peripheral tissues, it also plays a role in the CNS and has been identified in areas including the thalamus (Scheinin et al., 1994; Wang et al., 1996), olfactory tubercle, and cerebellum (Wang et al., 1996). In the rat brain, Luhrs et al. (2016) determined that the $\alpha 2_B$-AR comprises 20-30% of the total brain α-ARs and were present in the largest quantities in the thalamus and cortex, followed by the cerebellum and then the striatum. The $\alpha 2_B$-AR was not detected in the BNST (Scheinin et al., 1994; Wang et al., 1996).

The $\alpha 2_C$-AR is most abundant in the brain relative to other tissues and is found in the amygdaloid complex, olfactory system, hippocampus, and striatum (Scheinin et al., 1994; Wang et al., 1996). The $\alpha 2_C$-AR is associated with aggression, startle responses (Sallinen et al., 1998), locomotor sensitization to psychostimulants (Luhrs et al., 2016), and depression (Sallinen et al., 1999). There are limited studies on the effects of $\alpha 2_C$-AR activation in BNST. As such, the $\alpha 2_A$-AR will be the predominant subtype to be discussed in later sections of this review.

4.2 β-ARs

β-ARs are generally considered to be Gαs coupled (Pupo et al., 2001; Evans et al., 2010; Ordway et al., 1987; Duman and Nestler, 1995), but have the ability to couple Gαs or Gαi (Abramson et al., 1988) and may do so under different conditions or within different tissues. It has also been demonstrated that although GPCRs are commonly described as coupling to only

one specific type of Gα subunit, this is not always true as they are usually flexible in their Gα subunit coupling and can activate numerous signaling pathways (Evans et al., 2010).

4.2.1 β1-ARs

β1-ARs are typically Gαs coupled (Ordway et al., 1987). In the rat brain, β1-ARs have been detected in areas including the thalamus, hippocampus, amygdala, cingulate cortex, and BNST (Rainbow et al., 1984), and have been identified in the human brain in many of the same structures (Joyce et al., 1992). Many studies have indicated the critical importance of β1-AR in the BNST for stress and AUD/SUD, and these receptors will be described in much more detail later in this review.

4.2.2 β2-ARs

The β2-AR is also a GPCR that is typically Gαs coupled (Ordway et al., 1987; Hausdorff et al., 1989); however, there is evidence that it may switch its Gα subunit coupling from Gαs to Gαi in response to desensitization leading to activation of the inhibitory signaling cascade (Daaka et al., 1997). In the rat brain, β2-ARs have been detected in many areas including the thalamus, cerebellum, hippocampus, amygdala, and cingulate cortex, and BNST (Rainbow et al., 1984), and have been identified in many of the same structures in the human brain (Joyce et al., 1992). This receptor in the BNST appears to play a critical role in stress-induced reinstatement of drug-seeking behaviors, which will be discussed in sections 6 and 7.

4.2.3 β3-ARs

The β3-AR is localized in numerous areas throughout the body, including the heart, adipose tissue, retina, kidney, bladder, and the brain, and depending on the tissue, the β3-AR may be Gαi or Gαs coupled (Schena and Caplan, 2019). β3-ARs are expressed in the mouse (Claustre et al., 2008; Jhaveri et al., 2010) and rat (Claustre et al., 2008; Summers et al., 1995) hippocampus and have also been detected in the amygdala, anterior cingulate cortex, and entorhinal cortex among other regions (Claustre et al., 2008). In the human brain, β3-AR mRNA has been detected in the insula and brainstem (Rodriguez et al., 1995). Although β3-ARs have been shown to modulate anxiety-like behavior, predominantly via modulation of the BLA (Silberman et al., 2010), the β3-AR is the least-studied adrenergic receptor in terms of the BNST.

Throughout this review, many studies use pharmacological compounds to investigate the role of CRFRs and ARs. For a quick reference of each compound and its target see **Table 1-1.**

Table 1-1. List of CRF and NE receptor targeted compounds used in studies throughout the review.

Drug	Target	Function
Methoxamine	α1-AR	Agonist
Benoxathian	α1-AR	Antagonist
Prazosin	α1-AR	Antagonist
Clonidine	α2-AR	Agonist
Guanfacine	$\alpha 2_A$-AR	Agonist
ST-91	α2-AR	Agonist
UK-14,304	α2-AR	Agonist
Atipamezole	α2-AR	Antagonist
Idazoxan	α2-AR	Antagonist
RX821002	α2-AR	Antagonist
Yohimbine	α2-AR	Antagonist
Isoproterenol	β-AR	Agonist
Propranolol	β-AR	Antagonist
Timolol	β-AR	Antagonist
Dobutamine	β1-AR	Agonist

Betaxolol	β1-AR	Antagonist
Clenbuterol	β2-AR	Agonist
ICI 118,551	β2-AR	Antagonist
Urocortin 1	CRFR	Agonist
$_{D}$-Phe $CRF_{(12-41)}$	CRFR	Antagonist
Antalarmin	CRFR1	Antagonist
CP 154,562	CRFR1	Antagonist
CP376395	CRFR1	Antagonist
GSK876008	CRFR1	Antagonist
NBI 27914	CRFR1	Antagonist
NBI 35965	CRFR1	Antagonist
R121919	CRFR1	Antagonist
SSR125543	CRFR1	Antagonist
Urocortin 3	CRFR2	Agonist
Anti-sauvagine 30	CRFR2	Antagonist
Astressin2B	CRFR2	Antagonist

5. CRF in the BNST

5.1 CRF neurons in the BNST

The BNST includes many CRF neurons, and their density varies among the BNST subregions and subnuclei. More specifically, CRF-immunoreactive neurons are primarily located in the anterior portion of the BNST, with the highest densities in the BNSTov (Ju et al., 1989; Dabrowska et al. 2013; Dabrowska et al., 2016; Giardino et al., 2018), BNSTfu (Ju et al., 1989; Dong et al., 2001b; Dabrowska et al. 2013), BNSTju (Giardino et al., 2018) and the magnocellular nucleus, with a lower distribution in the undefined anterolateral region (Ju et al., 1989). The BNSTov is also a major contributor of CRF in the brain (Dabrowska et al., 2016). Nguyen et al. (2016) examined the anterior dBNST and vBNST subregions and found that about 8% and 13% of neurons were CRF+, respectively. CRF neurons in the BNST are primarily

GABAergic, but the proportion does vary among BNST subregions. Nguyen et al. (2016) observed that almost all CRF+ cells located in the dlBNST were GABAergic (~98%), while ~79% of dorsomedial BNST (dmBNST) CRF neurons were GABAergic, and ~70% of vBNST CRF neurons were GABAergic. Dabrowska et al. (2013) also observed that 94% of CRF+ neurons in the BNSTov were GABAergic, while none were glutamatergic. Studies have demonstrated that the location of CRF neurons in the BNST is different between rats and mice (Beyeler and Dabrowska, 2020). CRF neurons cluster in the BNSTov and BNSTfus in rats (Dabrowska et al., 2013) but may be more randomly distributed among the dBNST and vBNST in mice (Silberman et al., 2013). This is an important consideration when comparing study results across species.

Mainly explored in the rat dlBNST, neurons were separated into three groups corresponding to their electrophysiological properties: Type I (regular spiking), Type II (low-threshold bursting), and Type III (fast inward rectifiers) (Hammack et al., 2007). Dabrowska et al. (2013) found that 19/20 Type III cells from the rat BNSTalg were CRF+, 7/20 Type II cells were CRF+, and 3/20 Type I cells were CRF+ validating previous studies deeming the Type III cells "putative CRF" neurons in rats. However, in the mouse, dBNST CRF neurons have been shown to vary among all three phenotypes and even display other electrophysiologically-determined phenotypes (Silberman et al., 2013). It has been suggested that this difference in characterization from mouse to rat may be due to the CRF-*tomato* reporter line incorrectly identifying neurons as CRF-expressing (Daniel and Rainnie, 2016); while ectopic expression is always a possibility with transgenic lines, the CRF reporter used in Silberman et al. (2013) has been thoroughly validated and displays high fidelity for CRF neurons in numerous brain regions including the BNST, particularly relative to other CRF reporter lines (Chen et al., 2015). It is more likely that there are strain and species differences, as other studies have also identified differences in the mouse and the macaque (Daniel et al., 2017).

CRF neurons in the BNST may be interneurons or projection neurons, and the projection neurons can be further delineated by their projection targets. For example, one study showed that efferent BNST CRF neuron projections synapsed in the lateral hypothalamus (LH), MeA, and other hypothalamic and midbrain structures (Giardino et al., 2018). Silberman et al. (2013) determined that dBNST CRF neurons sending projections to the VTA or the medial hypothalamus are mostly distinct populations from one another. Many incoming projections onto CRF neuron populations in the dlBNST come from within the BNST itself, with major extra-BNST sources originating in the MeA, medial preoptic area, PVN, and CeA (Giardino et al., 2018). Incoming CRF projections, however, will be discussed further later in this section.

Recent studies examining the effects of activating BNST CRF neurons and their resulting behavioral outcomes have noted that activation is associated with aversive behavior. Salimando et al. (2020) uncovered the importance of GluN2D subunit-containing NMDA receptors in excitatory modulation of dlBNST CRF neurons, with 77.2% of CRF neurons also expressing the NMDA receptor subunit GluN2D (Salimando et al., 2020). Using constitutive GluN2D KO mice and conditional dlBNST-targeted GluN2D KO mice, the authors observed increased excitatory neurotransmission onto BNST-CRF neurons. Behaviorally, the constitutive GluN2D KOs had increased anxiety-like and depressive-like behavior compared to WT. With the dlBNST-specific GluN2D KO, there was an increase in depressive-like, but not anxiety-like, behavior (Salimando et al., 2020). In another study investigating dlBNST CRF neuron stimulation, two-choice incentive motivation tests were used where rats were left to choose between two options: sucrose+intra-dlBNST CRF neuron laser stimulations or sucrose without laser stimulation (Baumgartner et al., 2021). In the dlBNST, activation of CRF-neurons was avoided and suppressed the desire for sucrose (Baumgartner et al., 2021). Using laser self-stimulation, no rats exhibited self-stimulation of BNST CRF neurons, and laser stimulation of the dlBNST CRF neurons induced place avoidance. Stimulation of BNST CRF neurons was also associated with a

substantial increase in c-fos expression in brain regions associated with pain, aversion, fear, and satiety, and mesocorticolimbic regions (Baumgartner et al., 2021). These results suggest that dlBNST CRF neuron activation contributes to an aversive state in rodents.

Some effects of dlBNST CRF neuron stimulation may be pathway-specific. For example, CRF neurons from the dlBNST shown to project to the LH are responsive to a predator odor stimulus, but not a mouse scent stimulus, indicating the role for the CRF neurons in response to an aversive, but not a desirable, stimulus (Giardino et al., 2018). Additionally, optogenetic stimulation of dBNST CRF neurons as well as BNST CRF-LH projection neurons led to a real-time place aversion; although stimulation of all VGAT+ (GABA) neurons led to real-time place preference (Giardino et al., 2018). This result is particularly interesting because it supports the idea that GABAergic neuronal populations in the dBNST that project to the LH play different roles depending on if they contain CRF or not. Future research will be needed to determine if stimulation of dBNST and vBNST CRF projections to the same or differing brain regions produces similar aversive behavioral responses.

Overall, CRF neurons in the BNST are primarily GABAergic. These neurons have been extensively explored and categorized in the dBNST, with considerably fewer studies focusing on the vBNST subregion. Interestingly, stimulation of dlBNST CRF neurons has been associated with aversive behaviors (Salimando et al., 2020; Baumgartner et al., 2021), a result that seems unique to this extended amygdala structure (Baumgartner et al., 2021). Future sections of this review will discuss BNST CRF neurons and their activity in the contexts of stress and substance use.

5.1.1 CRF Receptors and CRF Signaling in the BNST

In addition to local BNST CRF neurons, another main source of CRF in the BNST is the CeA. The CeA sends dense CRF projections to the rat dlBNST (Pomrenze et al., 2015; Pomrenze et al., 2019) and vBNST (Pomrenze et al., 2015), and CeA CRF neurons were found to synapse onto neurons in the dBNST and the vBNST (Beckerman et al., 2013). In the rat, CeA CRF neuron axons were colocalized in a region with many dlBNST CRF neurons, with projections from the CeA synapsing in the ipsilateral BNST (Pomrenze et al., 2019). These observations indicate that CRF-containing neurons from the CeA terminate in both the dorsal and ventral subregions of the BNST and may preferentially target areas containing CRF neurons, at least in the dlBNST.

CRF has been shown to increase spontaneous excitatory postsynaptic current (sEPSC) frequency in a CRFR1, but not CRFR2, dependent manner in the dlBNST of mice (Kash et al., 2008). CRF also increased miniature excitatory postsynaptic current (mEPSC) frequency, but not amplitude. Additionally, application of the CRFR1 antagonist NBI27914 by itself led to a decrease in sEPSCs, suggesting that CRFR1s are involved in tonically regulating glutamatergic signaling in the dlBNST (Kash et al., 2008). VTA-projecting dBNST neurons were shown to be concentration-dependently affected by bath application of CRF, with increased CRF concentration leading to increased sEPSC frequency without causing a change in amplitude (Silberman et al., 2013). This indicates that CRF is likely modulating increased glutamatergic signaling onto VTA-projecting dBNST neurons. This effect was also determined to be CRFR1 dependent (Silberman et al., 2013). Overall, these results emphasize the importance of CRF and the CRFR1 in regulating glutamatergic transmission in the dBNST and the regulation of dBNST outputs.

In Long-Evans rats, direct intra-dBNST administration of CRF produced anxiety-like behavior and conditioned place aversion (CPA) (Sahuque et al., 2006). This result is similar to

observations described in section 5.1, where CRF neuron activation resulted in place avoidance (Baumgartner et al., 2021). Co-administration of the CRFR1 antagonist CP154,562, but not the CRFR2 antagonist anti-sauvagine 30, was able to block the CRF-induced anxiety-like behavior, and both the CRFR1 and CRFR2 antagonists were able to prevent the conditioned aversion to the CRF-paired chamber (Sahuque et al., 2006). Overall, these results implicate CRFR1 in modulating CRF-related anxiety-like behavior in the dBNST and both CRFR1 and CRFR2 in CRF-related aversion behaviors.

CRF in the BNST has been shown to interact with other neuropeptides and neurotransmitters, including oxytocin, PACAP, GABA, dopamine, serotonin. (General interactions with glutamate were discussed previously, and interactions with NE will be discussed later in this review).

Dabrowska et al. (2011) showed that oxytocin likely affects the activity of most Type III dlBNST (CRF) neurons which contain oxytocin receptor mRNA, with a major source of oxytocin coming from CRFR2-expressing projections originating the PVN and synapsing on CRF-expressing neurons in the BNSTov. CRFRs also affect the release of oxytocin in the dlBNST (Martinon and Dabrowska 2018). In male rats, when urocortin 3 (a CRFR2 agonist) was injected into the dlBNST, this had no effect on levels of oxytocin; however, when Astressin 2B, a CRFR2 antagonist, was injected into the dlBNST, this significantly increased oxytocin levels at 30 minutes post-injection. The CRFR2 antagonist effect was blocked by co-administration of either a CRFR1 antagonist (NBI 35965) or CRF. At 90-minutes post-infusion, the CRFR2 antagonist also led to an increase in oxytocin levels, an effect which was unchanged by the presence of CRF or the CRFR1 antagonist. Application of CRF itself also led to a significant increase in oxytocin levels relative to controls at 90-minutes post-infusion, an effect which was not blocked by co-infusion of the CRFR1 antagonist but was blocked by the CRFR2 antagonist (Martinon and Dabrowska 2018). These results paint a complicated picture of the interaction between CRF,

CRFRs, and oxytocin levels in the dlBNST, which needs to be explored further; but provides strong evidence that these neurotransmitter systems interact dynamically, likely impacting the regulation of stress-related behaviors. Additionally, parental behaviors are known to be regulated by oxytocin signaling (Lopatina et al., 2013). While maternal behavior is out of scope for this review, it is important to recognize that there is an extensive body of literature on CRF activation in the BNST and its effect on maternal care (Klampfl and Bosch, 2019; Klampfl et al., 2014; Klampfl et al., 2016). For much more detail regarding oxytocin's role in adaptive fear and anxiety as well as the interaction between oxytocin and CRF in the dlBNST, please see a recent review by Janeček and Dabrowska (2019), and for more information on oxytocin's role in addiction, please see a recent review by Sanna and De Luca (2021).

Pituitary adenylate cyclase polypeptide (PACAP) is also a molecule that interacts with CRF in the BNST. In general, there is evidence to support that PACAP signaling occurs upstream of CRF (Tsukiyama et al., 2011; Miles et al., 2019). In the dlBNST specifically, one study identified that PACAP+ terminals synapse onto CRF neurons (Kozicz et al., 1997), which suggests that PACAP has the potential to directly modulate dlBNST CRF neuron function. PACAP in the BNST has also been shown to play a role in stress responses as well as in alcohol and cocaine use. For example, chronic stress increased PACAP expression in the CRF neuron-dense BNSTov (Roman et al., 2014). Acute footshock stress also increased BNST PACAP expression, and intra-BNST PACAP administration increased the acoustic startle response (ASR) in rats (Seiglie et al., 2019). Further, intra-BNST PAC1 receptor antagonism was able to block footshock-stress induced ASR sensitization (Seiglie et al., 2019). Chronic alcohol exposure also led to an increase in dBNST PACAP expression, and intra-BNST PAC1 receptor antagonism attenuated increased alcohol consumption and withdrawal-associated anxiety-like behavior in ethanol-dependent rats (Ferragud et al., 2020). Self-administration of cocaine also increased dBNST PACAP expression, and PACAP signaling was shown to be necessary for footshock-

induced reinstatement (Miles et al., 2018). PACAP was also able to trigger reinstatement itself (Miles et al., 2018). Overall, these studies show that there is a role for BNST PACAP in stress and substance use, which is particularly important to acknowledge in this review since PACAP likely modulates downstream CRF signaling.

CRF signaling has also been shown to interact with GABAergic transmission in the dBNST. Using electrophysiological methods, Nagano et al. (2015) found that CRF reduces inhibitory transmission onto dlBNST CRF neurons in an action-potential dependent mechanism, potentially suggesting a self-propagating mechanism of CRF further inducing CRF release. Systemic administration of THIP (a "superagonist" for extrasynaptic $GABA_A$ receptors containing the delta subunit) increased c-fos expression in the CRF neuron-dense BNSTov (de Miguel et al., 2019), suggesting that activating extrasynaptic GABA receptors may also activate CRF neurons. THIP-induced CPA was also dependent upon CRFR1 (de Miguel et al., 2019), indicating a role for CRF in this mechanism. Further experiments with a CRF neuron-specific $GABA_A\alpha1$ subunit KO suggest that GABA receptors on CRF neurons modulate inhibition of CRF release and are involved in regulating anxiety-like behavior (Gafford et al., 2012). There is also evidence to support that knocking out $GABA_A\alpha1$ subunits in CRF neurons affects the function of benzodiazepines in the BNST, likely by preventing their inhibitory effects on CRF neuron CRF release (Gafford et al., 2012). Further experiments suggest that $GABA_A\alpha1$ subunits may also be important in BNST CRF cell signaling and plasticity (Gafford et al., 2012). Taken together, these studies suggest that there is a dynamic interaction between GABAergic signaling, CRF signaling, and CRF neurons in the BNST, and these interactions may affect avoidance and anxiety-like behaviors.

Dopamine is another neurotransmitter present in the BNST that interacts with CRF. When looking at the dlBNST of adult, male rats, Meloni et al. (2006) found a high density of

TH+ fibers and CRF+ neurons in the BNSTov, with most of these incoming TH+ projections originating in primarily dopaminergic regions such as the periaqueductal grey (Meloni et al., 2006), suggesting that many dopaminergic projections enter the dlBNST and interact with CRF neurons. Kash et al. (2008) also observed that TH+ fibers were in close proximity to CRF neurons in the mouse dlBNST. In the dlBNST of adult male mice, dopamine increased sEPSC frequency and amplitude in a D1-like receptor and a D2-like receptor dependent manner (Kash et al., 2008), indicating that dopamine acts via D1-like receptors and D2-like receptors in the dlBNST to increase glutamatergic signaling. Looking at dBNST CRF neurons specifically, application of 1uM dopamine led to a depolarization of CRF neurons in mice (Silberman et al., 2013). Altogether, these observations support the hypothesis that dopamine in the dBNST acts to increase glutamatergic signaling potentially via increased excitability of CRF neurons.

Marcinkiewcz et al. (2016) explored the relationship between serotonin and CRF signaling in the BNST. They observed that ~70% of dBNST and ~43% of vBNST CRF neurons expressed 5-HT_{2C}Rs, and they also identified multiple populations of CRF neurons responsive to serotonin. One set of BNST CRF neurons depolarized in response to serotonin via the 5-HT_{2C} receptor and did not project to the VTA; however, VTA-projecting BNST CRF neurons were hyperpolarized by serotonin through a mechanism utilizing a different serotonin receptor. The authors also found that exposure to footshock stress increased activation of dBNST- and vBNST-projecting serotonergic neurons (originating from the dorsal raphe nucleus), which could have a direct effect on 5-HT_{2C}R-expressing CRF neurons in the dBNST and vBNST (Marcinkiewcz et al., 2016). Ultimately, it was determined that serotonin acts via 5-HT_{2C}Rs to activate a BNST CRF-neuron-specific inhibitory circuit to inhibit projections to the VTA and LH that are typically anxiolytic. This inhibition of an anxiolytic circuit contributed to an increased anxiety-like phenotype in mice, and the anxiogenic effect was mediated by CRFR1s (Marcinkiewcz et al.,

2016). Overall, this study describes a role for BNST serotonin and CRF signaling in the modulation of anxiety-like behavior.

Less is known about CRF interactions with neuropeptide and neurotransmitter systems in the vBNST. In the vlBNST of male mice, CRF was shown to increase GABAergic transmission via CRFR1, but these effects were counteracted by neuropeptide Y (NPY) (Kash and Winder, 2006). Results also suggest that CRF acts to increase the effects of GABA signaling via a postsynaptic mechanism (Kash and Winder, 2006); findings that are quite different from CRF effects in the dBNST where CRF was shown to decrease inhibitory transmission onto dlBNST CRF neurons in a manner which is action-potential dependent (Nagano et al., 2015). Further experiments suggest that NPY acts via the Y2R to modulate presynaptic calcium influx and inhibits presynaptic GABA release. Additionally, NPY and CRF were able to produce their respective effects in the same cell, indicating that these peptides are able to act on the same set of neurons in the vlBNST (Kash and Winder, 2006). Lastly, UCN1 application did not alter evoked excitatory postsynaptic currents (eEPSCs), indicating the effects of CRF/UCN1 are likely relevant to GABAergic, not glutamatergic, signaling (Kash and Winder, 2006), but CRF effects on vBNST glutamatergic transmission have not been fully explored.

To summarize, the BNST is home to a large population of CRF neurons and also receives a significant CRF input from the CeA. In general, CRF interacts with numerous other neuropeptides and neurotransmitters in the BNST, including glutamate, PACAP, GABA, oxytocin, NE (more on NE later), dopamine, and serotonin. Most studies have investigated BNST CRF interactions in the dorsal subregion, but there is little literature that acknowledges the ventral subregion in terms of general CRF signaling, despite evidence for differential CRF-related mechanisms. More studies examining baseline CRF behavior in the ventral subregion would be useful for understanding the various actions of CRF in the BNST.

5.2 CRF Signaling in the BNST Related to Stress

CRF is a critical substrate of the stress response. Although it may be intuitive to assume that CRF increases in response to a stressor in general, the direction in which CRF expression changes depends on the particular stressor and BNST subregion. Additionally, the types of stressors and how they are implemented across studies can differ greatly, which also likely influences CRF expression. In this section we will review acute and chronic stressors, and how they recruit or are influenced primarily by CRF signaling in the BNST. For a summary of studies described below regarding c-fos and CRF expression in response to only stressors see **Table 1-2**, and for a summary of behavioral experiments with stressors **se**e **Table 1-3.**

Table 1-2. Summary of CRF and c-fos expression changes in response to stress exposure. Results are sorted by stress type then by BNST subregion. This table includes results from sections 5.2 and 7.1. Abbreviations: EPM: elevated plus maze; CUS: chronic unpredictable stress; CMS: chronic mild stress; cort: corticosterone; CeA: Central Amygdala

Reference	Stress Type	Stressor	Subregion	Expression Change
Butler et al. 2016	Acute	Predator Odor	vBNST	Increased c-fos+ CRF neurons
Butler et al. 2016	Acute	Predator Odor	dlBNST	No change in c-fos
Butler et al. 2016	Acute	Predator Odor	dmBNST	No change in c-fos
Butler et al. 2016	Acute	EPM	vBNST	Increased c-fos+ CRF neurons Increased c-fos density
Butler et al. 2016	Acute	EPM	dlBNST	Increased c-fos density Did not increase c-fos+ CRF neurons
Butler et al. 2016	Acute	EPM	dmBNST	Increased c-fos density and Increased c-fos+ CRF neurons
Fetterly et al. 2019	Acute	Restraint stress	dBNST	C-fos increased in CRF neurons; Injection of guanfacine ($\alpha 2_A$-AR agonist, 1 mg/kg) prior to RS blocked this effect Guanfacine injection decreased c-fos+ CRF neurons in naïve animals
Watts et al. 1995	Acute	Hypertonic saline	dBNST (oval nucleus)	CRF mRNA decreased
Watts et al. 1995	Acute	Hypertonic saline	vBNST (fusiform nucleus)	CRF mRNA increased
Shepard et al. 2006	Acute	EPM	dlBNST	CRF mRNA increased
Shepard et al. 2006	Acute	EPM	vlBNST	CRF mRNA increased
Shepard et al. 2006	Acute	EPM+CeA cort implant	dlBNST	CRF mRNA increased (even further than non-implant)
Shepard et al. 2006	Acute	EPM+ CeA cort implant	vlBNST	CRF mRNA increased (but not any further than non-implant)
Ventura-Silva et al. 2012	Chronic	CUS	vBNST (dorsomedial nucleus)	Downregulation of CRFR1 mRNA Upregulation of CRF mRNA
Ventura-Silva et al. 2012	Chronic	CUS	vBNST (fusiform nucleus)	Downregulation of CRFR1 mRNA Downregulation of CRF mRNA
Ventura-Silva et al. 2012	Chronic	CUS	vBNST (dorsomedial nucleus)	C-fos higher than control at baseline (before anxiogenic stimulus)
Ventura-Silva et al. 2012	Chronic	CUS	vBNST (fusiform nucleus)	C-fos higher than control at baseline (before anxiogenic stimulus)

Ventura-Silva et al. 2020	Chronic	CUS + CeA scramble inj	BNST (anterior)	CRF expression decreased CRFR1 expression decreased CRFR2 expression unchanged
Ventura-Silva et al. 2020	Chronic	CUS + CeA CRF knockdown	BNST (anterior)	CRF expression trended toward a decrease CRFR1 expression unchanged CRFR2 expression unchanged
Ventura-Silva et al. 2020	Chronic	CUS + CeA CRF knockdown	dBNST (anterodorsal)	No difference in c-fos expression among groups (naïve+scramble; naïve+knockdown; CUS+scramble; CUS+knockdown)
Ventura-Silva et al. 2020	Chronic	CUS + CeA CRF knockdown	vBNST (dorsomedial nucleus)	No difference in c-fos expression among groups (naïve+scramble; naïve+knockdown; CUS+scramble; CUS+knockdown)
Ventura-Silva et al. 2020	Chronic	CUS + CeA CRF knockdown	vBNST (fusiform nucleus)	Increase in c-fos expression in CUS+scramble relative to naïve+scramble and naïve+knockdown; CUS+knockdown attenuated this effect
Kim et al. 2006	Chronic	Chronic mild stress	dBNST	Increased CRF mRNA Increase in CRF mRNA was attenuated with systemic administration of tianeptine (5-HT reuptake enhancer) Tianeptine also decreased CRF mRNA in the non-stressed rats
Kim et al. 2006	Chronic	Chronic mild stress	vBNST	CMS did not have an effect on CRF mRNA expression CRF mRNA was decreased with systemic administration of tianeptine (5-HT reuptake enhancer, other targets as well) Tianeptine also decreased CRF mRNA in the non-stressed rats
Daniel et al. 2019	Chronic	Chronic shock stress	dBNST (oval nucleus)	Increase in CRF mRNA expression in Type III cells (but not Type I or II)
Makino et al. 1994	Chronic	Subcutaneous cort injections for 14 days	dlBNST	High dose (5mg/kg/day) increased CRF mRNA Low dose (1 mg/kg/day) had no effects
Makino et al. 1994	Chronic	Subcutaneous cort injections for 14 days	vBNST	Neither high dose nor low dose had effects on CRF mRNA expression
Makino et al. 1994	Chronic	200mg cort 60-day release pellet	dlBNST	Increased CRF mRNA observed at one week

Makino et al. 1994	Chronic	200mg cort 60-day release pellet	vBNST	Increased CRF mRNA observed at two weeks
Sink et al. 2013	Chronic	CRF over-expression	BNST (not specified)	Decrease in membrane expression of the CRFR1
Regev et al. 2011	Chronic	CRF over-expression in the dlBNST	dlBNST	Decrease in CRFR1 mRNA expression
Albrechet-Souza et al. 2017	Chronic	10-day episodic social defeat protocol	BNST (not specified)	Increased CRF mRNA No change in CRFR1 or CRFR2 mRNA
Makino et al. 1994	Chronic-"Opposite"	Adrenalectomy	dlBNST	No effect on CRF mRNA expression
Makino et al. 1994	Chronic-"Opposite"	Adrenalectomy	vBNST	No effect on CRF mRNA expression
Santibanez et al. 2005	Chronic-"Opposite"	Adrenalectomy	dlBNST	Decreased CRF mRNA expression Decreased CRF-like immunoreactivity
Santibanez et al. 2005	Chronic-"Opposite"	Adrenalectomy	vlBNST	No change in CRF mRNA expression Decreased CRF-like immunoreactivity

Table 1-3. Summary of behavioral experiments involving stressors without drug exposure. All studies list the specific BNST subregion investigated; however the behavioral intervention or observation is not BNST-specific unless otherwise noted. Results are sorted by stress type then BNST subregion. This table includes results from sections 5.2, 6.1, and 7.1. Abbreviations: EPM: Elevated Plus Maze; OFT: Open Field Test; LD: Dark-Light Transfer Test; AS: Acoustic Startle; PPI: Prepulse Inhibition

Reference	Stress Type	Stressor	Subregion	Behavior	Result
Sink et al. 2013a	Acute	Intra-BNST infusion of calcitonin gene-related peptide (400 ng)	BNST (not specified)	EPM	Increased anxiety-like behavior; blocked with systemic administration of GSK876008 (CRFR1 antagonist) (10 mg/kg; orally administered)
Sink et al. 2013a	Acute	Intra-BNST infusion of calcitonin gene-related peptide (400 ng)	BNST (not specified)	Acoustic startle response	Increased anxiety-like behavior; blocked with intra-BNST administration of CP376395 (CRFR1 antagonist) (2μg/side) or siRNA knockdown of CRF in the BNST
Fendt et al. 2005	Acute	Predator Odor	vBNST	Freezing behavior	Increased freezing behavior; blocked with an intra-vBNST injection of clonidine (α2-AR agonist) (100μM)
Cecchi et al. 2002	Acute	Immobilization	vBNST	Social interaction	Decreased social interaction time; unaffected by intra-vBNST benoxathian (α1-AR antagonist) (2.0 nmol) or Betaxolol + ICI (β-AR antagonists) (1.0 nmol each)
Cecchi et al. 2002	Acute	Immobilization	vBNST	EPM	Decreased open arm time and entries; attenuated by intra-vBNST benoxathian

					(α1-AR antagonist) (2.0 nmol) or Betaxolol + ICI (β-AR antagonists) (1.0 nmol each)
Pomrenze et al. 2019	Acute	Immobilization	dlBNST	OFT, EPM	Activation of dlBNST-projecting CeA neurons in the dlBNST attenuates stress-induced anxiety-like behavior measured in the EPM and OFT
Jasnow et al. 2004	Acute	Social Defeat	dlBNST	Submissive-defensive behavioral observations	dlBNST infusion of $_{D}$-Phe $CRF_{(12-41)}$ (CRFR antagonist) (100 or 250 ng) decreases submissive-defensive behavior
Sink et al. 2013	Chronic	CRF overexpression in the BNST	BNST (not specified)	EPM	No different than non-CRF overexpressing rats
Sink et al. 2013	Chronic	CRF over-expression in the BNST	BNST (not specified)	Defensive withdrawal	No different than non-CRF overexpressing rats
Regev et al. 2011	Chronic	CRF over-expression in the dlBNST	dlBNST	OFT, LD, AS, EPM	No different than non-CRF overexpressing mice
Wang et al. 2020	Chronic	Chronic restraint stress (3 weeks)	dBNST	EPM	Ablation of dBNST CRF neurons followed by chronic restraint stress led to less anxiety-like behavior
Rajbhandari and Bakshi 2020	Chronic	Intra-dBNST infusion of CRF (200 ng/0.5 µL) or NE (20µg/0.5µL) for three days	dBNST	Prepulse Inhibition	dBNST NE infusion lowered PPI, subsequent dBNST CRF "challenge" (200 ng/0.5 µL) also lowered PPI dBNST CRF infusion did not alter PPI, subsequent dBNST NE (0.3µg/0.5 µL) "challenge" also did not alter PPI

5.2.1 Acute Stress

There are a wide variety of acute stressors used across studies. Additionally, different outcomes are measured across studies including c-fos expression for neuronal activation, CRF mRNA expression levels, and changes in anxiety-like behavior. The following described studies utilize one or more of the following: predator odor, EPM, acute restraint/immobilization stress, hypertonic saline, anxiogenic infusion of calcitonin gene-related peptide, and social defeat. Results within the same nucleus may differ across stressors; however, persistent results despite differences in the method of acute stressor utilized provides greater confidence in the observed effect relative to BNST CRF function.

There are multiple experiments demonstrating differential BNST subregion responses to acute stressors. As an example of how an acute stressor may impact CRF neurons in various BNST subregions differently, Butler et al. (2016) showed that exposure to predator odor led to a significant increase in the percentage of c-fos+ CRF neurons in the vBNST, but not the dlBNST or dmBNST, of male rats. Using EPM exposure as another acute stressor led to increased overall c-fos density in all BNST subregions, with a significant increase in percentage of c-fos+ CRF neurons in the vBNST and dmBNST, but not the dlBNST (Butler et al., 2016). Ultimately, these results suggest that diverse acute stressors differentially activate CRF neurons in multiple BNST subregions. Also, vBNST CRF neurons were reactive in the greatest proportion to predator odor and EPM (Butler et al., 2016), suggesting a greater role for the vBNST in acute stress responses to these stressors. This is an important consideration, as the studies mentioned in section 5.1 on CRF modulation of BNST neurocircuitry have predominantly examined the dBNST. Differential BNST subregion responses to acute stressors can also be observed in terms of CRF expression changes. After one night of drinking hypertonic saline, CRF mRNA significantly decreased in the

BNSTov, but significantly increased in the BNSTfu (Watts et al., 1995). In response to EPM-induced stress, however, CRF mRNA was increased in both the dlBNST and vlBNST of rats (Shepard et al., 2006). Interestingly, corticosterone implantation into the CeA led to an even further CRF mRNA increase in only the dlBNST (Shephard et al., 2006). Overall, these results indicate that the dBNST and vBNST may have differential CRF-related responses depending upon the acute stressor, and that the subregions may respond differently from one another which may be in part due to subregional variation in the CeA's modulation of BNST CRF.

Utilizing restraint stress, Fetterly et al. (2019) demonstrated that c-fos expression increases significantly in CRF neurons in the dBNST of mice, a result that partially coincides with the EPM-exposure described in the previous paragraph. Additionally, restraint stress led to an increase in the percentage of CRF+ cells that co-express Pkcdelta in the dBNST of female mice, but not male mice, and this effect was blocked by ovariectomy (Fetterly et al., 2019). This observation is interesting for two reasons: CRF and Pkcdelta are typically thought to represent distinct neuronal populations in the BNST, and this result uncovers a sex-specific response to an acute stressor. In both males and females, however, restraint stress led to an increase in the percentage of c-fos mRNA+ cells in CRF neurons that did not co-express Pkcdelta (Fetterly et al., 2019). More needs to be done to investigate these observed sex differences, and these differences suggest a role for hormonal involvement in stress-related neurological changes in the dBNST.

Another study examined the role of dBNST CRF in behavioral responses to an acute stressor. Sink et al. (2013a) infused calcitonin gene-related peptide (CGRP) into the rat dlBNST, and this increased anxiety-like behavior in the EPM and also increased the acoustic startle response. Systemic administration of a CRFR1 antagonist attenuated the increased EPM anxiety-like behavior, and intra-BNST administration of a CRFR1 antagonist blocked the CGRP-induced increase in startle. Lastly, siRNA knockdown of CRF in the BNST also blocked the CGRP-associated increased startle response (Sink et al., 2013a). Ultimately, these experiments

demonstrate that dBNST CRF signaling plays a critical role in acute stress-induced anxiety-like behavior.

CRF projections from the CeA to the BNST may also play a critical role in acute stress responses. Rat dlBNST-projecting CRF neurons from the CeA were shown to synapse in a dlBNST nucleus with a high concentration of CRF neurons (Pomrenze et al., 2019). These dlBNST-projecting CRF neurons are not only an important source of CRF, but also play a significant role in the modulation of anxiety-like behavior. Activation of the CeA-derived CRF inputs to the dlBNST increased anxiety-like behavior, an effect which was dependent upon CRFR1 (Pomrenze et al., 2019). Additionally, inhibition of the CeA-originating CRF inputs decreased immobilization stress-induced anxiety-like behavior (Pomrenze et al., 2019). Another study showed similar results and demonstrated that CRF projections from the CeA to the dlBNST, at least in part, modulate the submissive behavior of hamsters after acute social defeat stress (Jasnow et al., 2004). Taken together, these studies suggest that there is a critical role for CeA-dlBNST CRF projections in mediating anxiety-like and submissive behavior in rodents, particularly following an acute stressor, and this effect is likely mediated by CRFR1.

Many of the previously described studies demonstrated that there are not only subregional differences in responses to acute stressors, but the responses also vary depending upon the specific stressor. Numerous studies provided evidence that CRFR antagonists block stress-induced anxiety-like behavior despite the fact that various acute stressors were used. The persistence of this effect provides strong support for the critical role of CRFRs, particularly CRFR1s, in the dlBNST. More studies are needed to further investigate the vBNST's CRF-related response to acute stressors.

5.2.2 Chronic Stress

Chronic stressors are more long-term and may be more intense than acute stressors, and are often repeated over extended time points (multiple days to multiple weeks). Just like acute stressors, chronic stressors vary in their nature and execution across studies. Further, the outcomes measured may differ and include indicators such as c-fos expression for neuronal activation, CRF mRNA expression, and changes in levels of anxiety-like behavior. The following studies utilize chronic stressors including chronic unpredictable stress, chronic mild stress, chronic shock stress, chronic social defeat stress, or overexpression of CRF in specific brain regions to mimic a chronically stressed state.

Chronic unpredictable stress (CUS) was used to investigate CRF-related changes in subnuclei of the vBNST in rats. Ventura-Silva et al. (2012) utilized CUS and identified that CRF expression, but not CRFR1 mRNA expression, differs in the direction of change depending upon the vBNST subnuclei examined. The authors also demonstrate that CUS increases c-fos activation in the vBNST, and exposure to acoustic startle increases c-fos in the vBNST, but to a lesser extent if previously exposed to CUS (Ventura-Silva et al., 2012). Ultimately, this suggests that CUS leads to altered CRF-related circuitry in the vBNST and affects basal neuronal activation in specific vBNST nuclei, altering the ability for a subsequent anxiogenic stimulus to activate this region. These changes in CRF and CRFR1 in the vBNST likely contribute to impaired stress responses often seen after chronic stress exposure. Examining the close relationship between CeA CRF expression and the BNST, Ventura-Silva et al. (2020) looked at the effects of knocking down CRF expression in the CeA on CRF gene expression and cellular activation in the anterior BNST in response to CUS. The authors found that CUS decreases CRF and CRFR1 expression in the anterior BNST, with knockdown of CRF in the CeA blocking the decrease in CRFR1 expression. Additionally, the authors found that the only BNST subregional

change in c-fos+ cells occurred in the BNSTfu, with the CeA CRF knockdown attenuating the increase (Ventura-Silva et al., 2020). Overall, these results show that CUS has subregion-specific effects in the BNST, and some of these stress-induced effects are related to CeA CRF expression.

In 2006, Kim et al. determined that chronic mild stress (CMS) led to an increase in CRF mRNA in the dBNST, but not the vBNST. Systemic treatment with tianeptine (a serotonin reuptake enhancer that interacts with several other receptor systems) throughout the CMS paradigm attenuated this increase in the dBNST and decreased CRF mRNA in the vBNST. In non-stressed rats, tianeptine treatment decreased CRF mRNA in both the dBNST and vBNST (Kim et al., 2006). Collectively, these observations present a role for serotonin in modulating the response to CMS, and this mechanism may be mediated by CRF expression levels in particular BNST subregions.

In male mice, Hu et al. (2020) showed that chronic variable mild stress (CVMS) led to hyperactivity of dBNST neurons in the BNSTov via a PKA and CRFR1-dependent mechanism. Further, this CVMS-related CRF-driven hyperactivity of BNSTov neurons was behind what the authors referred to as “maladaptive behaviors” such as decreased sucrose preference, less time in the open arm of the EPM, decreased time/distance in the center of the OF, and increased latency to feed in the novelty suppressed feeding (NSF) test (Hu et al., 2020). Complementary to these results, another study found that ablation of dBNST CRF neurons before exposing mice to chronic restraint stress for three weeks led to mice that displayed less anxiety-like behavior in the EPM than controls (Wang et al., 2020). Taken together, these two studies suggest that chronic stress may recruit dBNST CRF neurons to promote anxiety-like behavior.

In whole-cell electrophysiology recordings primarily from the BNSTov in the dBNST of rats exposed to chronic shock stress, Daniel et al. (2019) observed that Type III cells (previously shown to express CRF mRNA (Dabrowska et al., 2013; Hammack et al., 2007)) had significant changes in their basic physiological properties in response to the stressor in the following ways: a

lower input resistance, a quicker membrane time constant, longer action potential rise time, and decreased firing rate. Although all types of cells in the non-stressed condition expressed CRF mRNA, only Type III cells increased their CRF mRNA expression after chronic shock stress and expressed more CRF mRNA than other types of cells following the stress (Daniel et al., 2019). Overall, it appears that the Type III neuronal population in the BNSTov of the rat BNST is a predominantly CRF neuron population uniquely susceptible to changes associated with chronic stress exposure.

In Swiss mice, chronic social defeat stress as well as an intra-right medial prefrontal cortex injection of a nitric oxide donor (NOC-9) both induced anxiety-like behaviors. The anxiety-like behavior, however, was blocked by intra-BNST administration of AP-7 (an NMDA receptor antagonist) or CP376395 (a CRFR1 antagonist) (Faria et al., 2020). Since a large proportion of CRF neurons in the dlBNST also express NMDA receptors (Salimando et al., 2020), it is possible that the NMDA receptor antagonist exerted its anxiety-reducing effects, at least in part, by blocking NMDA receptor activation on BNST CRF neurons. These findings indicate that BNST NMDA receptors, possibly on CRF neurons, and CRFR1s are involved in the modulation of chronic stress-induced anxiety-like behaviors.

Mimicking a chronic stress response, Makino et al. (1994) administered corticosterone chronically either via injection for 14 days or via a pellet implant for 60 days. They found that injected corticosterone can increase dlBNST CRF mRNA, but not vBNST; however, the corticosterone pellet increased CRF mRNA in both subregions. This suggests that CRF expression in the dlBNST may be affected by chronic repetitive stressors, and CRF expression in both the dlBNST and vBNST are affected by chronic continuous stressors.

Another method to mimic chronic stress effects is by lentiviral vector-mediated overexpression of CRF in the BNST. Sink et al. (2013b) observed no difference in anxiety-like behavior when male rats overexpressed CRF in the BNST, and CRF overexpression did not affect

basal or post-footshock blood corticosterone levels relative to controls. Similarly, overexpression of CRF in the dlBNST of adult male mice did not affect basal or acute stressor-induced anxiety-like behavior, and plasma corticosterone levels did not differ between the control and the CRF overexpression groups at baseline or post-acute stress (Regev et al., 2011). CRF overexpression did lead to a decrease in the membrane expression (Sink et al., 2013b) and mRNA expression (Regev et al., 2011) of CRFR1 in the BNST, however, suggesting compensatory mechanisms may be occurring in response to the CRF overexpression that may affect other stress-related behaviors. The results from both studies parallel one another, and the consistency of results across numerous anxiety-like behavioral tests provides strong evidence that CRF overexpression, at least in the dlBNST, is not enough to produce these behaviors in mice and rats.

Looking at what might be considered the opposite of chronic stress, adrenalectomy in adult male rats leads to a decrease in CRF mRNA in the dlBNST but not the vlBNST; however, looking at CRF-like immunoreactivity, adrenalectomy decreased signal in both the dlBNST and the vlBNST (Santibañez et al., 2005), suggesting that while the transcription is not altered in the vlBNST after adrenalectomy, translation may be. Interestingly, however, adrenalectomy did not affect CRF mRNA expression levels in either subregion in another study (Makino et al., 1994). These findings indicate that adrenalectomy may be affecting CRF levels in the BNST, but the variation in results between studies, as well as between mRNA and protein expression, warrants further exploration.

Altogether, BNST CRF appears to play an important role in stress responses and subsequent anxiety-like behavior, although the outcomes from acute and chronic stress studies show variability in responses among the BNST subregions and subnuclei, and these changes may also be related to the specific stressors themselves. More studies are needed to investigate the reactivity of specific subnuclei to these stressors. Further analysis into the differences in BNST CRF signaling mediated by different stress exposure paradigms is also warranted. For further

information about how stress interacts with CRF and other neurotransmitter systems in the BNST, see a recent review by Daniel and Rainnie (2016). In the next section of this review, we will discuss CRF in the BNST in relation to alcohol or drug use in a stressor-free context.

5.3 CRF Signaling in the BNST Related to Alcohol or Drug Use

CRF has been implicated in alcohol and other substance use disorders. The way that CRF in the BNST interacts with specific drugs may vary depending upon the BNST subregion, as well as the class of drug. The following section will discuss BNST CRF in the context of substance use disorder including nicotine, alcohol, cocaine, and opioids. Similarities and differences among various drug classes and the BNST CRF system will be discussed. Results including c-fos and CRF expression are incorporated into **Table 1-4**, and behavioral results are in **Table 1-5**.

One of the lesser-studied classes of drugs when exploring CRF in the BNST is nicotine. In male rats, nicotine exposure via subcutaneous injection did not alter the amount of CRF mRNA detected in the dBNST or the vBNST. This same trend was seen in adolescent rats, and there was also no difference in CRF mRNA between adolescent and adult rats (Kupferschmidt et al., 2010). The results of this study suggest that acute nicotine exposure has no direct effect on CRF expression in the dBNST or vBNST subregions. For a recent review detailing CRFs role in alcohol and nicotine addiction, including regions outside of the BNST, see Simpson et al. (2020).

More studies have focused on the role that BNST CRF and CRF neurons play in alcohol dependence. Recent studies reveal the importance of BNST microcircuits in mediating various aspects of the development of alcohol use disorders. For instance, studies indicate that there is a relationship between the VTA and BNST CRF neurons in alcohol consumption. Rinker et al. (2016) demonstrated a role for VTA-projecting dlBNST CRF neurons in modulating drinking

behavior through a mechanism involving CRFR1 and CRFR2 in the VTA. Corresponding with these results, another study showed that inhibiting GABAergic dlBNST-VTA projections that largely co-expressed CRF led to decreased binge-like alcohol intake (Companion and Thiele, 2018). Altogether, the results from these studies suggest that inhibition of GABAergic CRF neurons projecting from the dlBNST to the VTA leads to a decrease in binge-like alcohol intake, emphasizing the importance of these BNST CRF projections in alcohol use. GABAergic vBNST projections to the VTA also display importance in mediating reward behaviors more generally (Jennings et al., 2013), suggesting that both dBNST and vBNST projections to the VTA may modulate reward-seeking and intake behaviors.

In addition to the importance of the CRF BNST-VTA circuit in alcohol intake, the CRF CeA-BNST circuit has also been implicated in alcohol drinking in rodents. Optogenetic inhibition of CeA CRF projections to the vBNST in alcohol-dependent rats decreased alcohol self-administration, withdrawal signs, and overall withdrawal severity (de Guglielmo et al., 2019). In a slice preparation, optically inhibiting the CeA-BNST CRF projections led to a decrease in action potential firing of BNST neurons. Bath application of the CRFR1 antagonist R121919 without optical inhibition decreased firing rate as well, but presence of the CRFR1 antagonist occluded the effect of optically-evoked inhibition (de Guglielmo et al., 2019). These results suggest that CRF projections from the CeA to the vBNST play a role in the rewarding aspects of alcohol intake and stimulate alcohol intake behaviors in alcohol-dependent rats, and may also be involved in modulating withdrawal symptoms. Additionally, the CeA-vBNST CRF projections likely act via the CRFR1 to activate CRF neurons in the vBNST and may be related to alterations in activity of BNST-CRF projections to the VTA.

Neuropeptide Y (NPY) has been shown to play a role in alcohol intake and interact with BNST CRF neurons. In adult mice, activation of the inhibitory NPY receptor Y1R in the dBNST was shown to decrease binge alcohol intake, while activation of Y2R increased alcohol intake

(Pleil et al., 2015). Electrophysiological experiments demonstrated that Y1R activation specifically inhibits dBNST CRF neurons (Peil et al., 2015). Chemogenetically inhibiting dBNST CRF neurons of mice led to decreased binge drinking and decreased anxiety-like behavior, with activation of the BNST CRF neurons having no effect. NPY Activation of CRF neurons along with intra-BNST infusion of the Y1R agonist LeuPro NPY, however, prevented this agonist's ability to decrease binge alcohol intake (Pleil et al., 2015). Taken together, these results support the idea that acting via the Y1R, NPY induces inhibitory effects on BNST CRF neurons to decrease binge alcohol drinking behavior. Further, Pleil et al. (2015) also showed that Y1R function also changes in response to chronic alcohol consumption in monkeys. Seeing that NPY, particularly via the Y1R, is associated with alcohol in both mice and monkeys makes it an even more promising target to investigate further.

The effects of cocaine on BNST CRF signaling have also been studied. Physiologically, cocaine appears to directly alter activity of neurons in the dlBNST by enhancing NMDA-receptor dependent short-term potentiation (STP) through a mechanism involving dopamine receptors and CRFR1 (Kash et al., 2008). Cocaine has also been shown to affect the interaction of BNST CRF with β1-ARs (Nobis et al., 2011) an effect which will be discussed later in this review. Although these previously-mentioned studies provide evidence for a role of cocaine affecting CRF signaling in the BNST, others have not come to the same conclusion while emphasizing a greater role for CRF in other brain regions. In rats, intra-vBNST CRFR1/2 antagonism was unable to alleviate anxiety-like behaviors resultant of cocaine administration, while CRFR1/2 antagonism in the VTA did reduce anxiety-like behaviors (Ettenberg et al., 2015). These limited observations not supportive of CRF in the BNST's involvement in cocaine-mediated changes may be due to the various modes of cocaine administration as well as different measured outcomes. It is also possible that chronic cocaine administration in the above model resulted in modulation of CRFR1/2 expression in the BNST, making exogenous antagonists less effective, but does not rule

out a role for the CRF BNST-VTA pathway in mediating cocaine-related behaviors similar to that discussed above with alcohol-related behaviors. Further research in this area is warranted.

Opioids have been shown to modulate BNST CRF signaling, and they are the final class of drugs to be discussed in this section. Using electron microscopy, Jaferi and Pickel (2009) found that many neurons in addition to CRFR-containing neurons express the μ-OR in the dlBNST. They also found that CRFR-containing terminals form excitatory synapses with cells expressing CRFR and/or μ-OR, whereas the μ-OR-containing terminals form inhibitory synapses with other types of dendrites/spines. Overall, their results provide evidence that opioids can act directly on CRF neurons in the dlBNST (Jaferi and Pickel, 2009). Another study by Jaferi et al. (2009) used EM immunolabeling to look at the effects of morphine injections on dlBNST CRFR1/2 and CRF expression and localization. The authors of the study suggest that morphine either increases internalization of the CRFR or decreases the trafficking of the CRFR to the membrane, and does not have an effect on CRF axonal availability (Jaferi et al., 2009). This level of synaptic detail provided by EM is useful for understanding the delicate dynamics of pre- and post-synaptic receptor expression as well as the internal cellular dynamics associated with morphine administration. Similar studies with other drugs could reveal important information regarding the bioavailability of CRFR for subsequent therapeutic targeting.

Multiple studies have examined the effects of morphine on neuronal activation and CRF expression. Using phosphorylated CREB (pCREB) expression as a marker of neuronal activation, it was determined that mice that exhibited morphine conditioned place preference (CPP) had increased levels of pCREB in most dBNST CRF neurons, suggesting that exposure to a conditioned context associated with morphine administration may affect circuitry involving dBNST CRF neurons (García-Carmona et al., 2013). Another study explored the effects of an acute morphine injection in morphine-naïve and morphine-tolerant rats (Milanés et al., 1997). In morphine-naïve rats an acute injection of morphine produced a significant increase in BNST CRF

protein, but morphine-tolerant rats injected with morphine displayed a significantly decreased CRF (Milanés et al., 1997). It is important to point out that distinct BNST subregions were not specified in these studies; regardless, these studies provide evidence for a role of BNST CRF neuronal activation and CRF expression in response to morphine and morphine dependence.

Overall, CRF in the BNST has been investigated in the context of numerous drugs including nicotine, cocaine, alcohol, and opioids. Although CRF and CRFR mRNA expression in the dBNST and vBNST may not always correlate with the observed drug behaviors or drug treatments, there is consistent evidence for the role of CRF and CRFRs in the BNST in response to drug administration and drug-seeking. Moreover, a few studies uncovered sex-differences that warrant further exploration. Finally, while some studies in this section may have touched upon withdrawal, withdrawal is often considered a type of stressor and will be discussed specifically in the next section.

Table 1-4. Summary of CRF and c-fos expression changes in response to drug administration. Results are sorted by drug and then by BNST subregion. This table includes results from sections 5.3 and 6.2. Abbreviations: i.p.: intraperitoneal; s.c.: subcutaneous; i.v.: intravenous; i.c.v.: intracerebroventricular; CIE: Chronic Intermittent Ethanol

Reference	Drug	Drug Administration	Subregion	Expression Change
Kupferschmidt et al. 2010	Nicotine	Acute injection (0.4 or 0.8 mg/kg; s.c.)	dBNST	CRF mRNA expression was unchanged in both adult and adolescent rats
Kupferschmidt et al. 2010	Nicotine	Acute injection (0.4 or 0.8 mg/kg; s.c.)	vBNST	CRF mRNA expression was unchanged in both adult and adolescent rats
Centanni et al. 2019	Alcohol	Chronic alcohol drinking (10%)	dBNST	Increase in dBNST c-fos expression as a whole and in CRF neurons in both male and female mice experiencing forced abstinence
Funk et al. 2006	Alcohol	CIE to induce dependence	dlBNST	CRF immunoreactivity at 2 hours withdrawal from CIE did not differ between dependent and nondependent rats
Rinker et al. 2016	Alcohol	Drinking in the Dark	dlBNST	One drinking in the dark cycle did not alter CRF mRNA expression
Olive et al. 2002	Alcohol	6.7% alcohol diet	vBNST	During withdrawal from the alcohol diet, CRF levels as measured via microdialysis, significantly increased Subsequent return to the alcohol diet decreased CRF levels back to baseline If given a control diet during withdrawal, CRF levels increased further
Connelly and Unterwald 2020	Cocaine	Chronic injections (10mg/kg; i.p.) three times daily for 14 days	dBNST	Rats were sacrificed at various timepoints after their last cocaine injection: 30 mins, 24 hrs, 48 hrs, 7 days, or 14 days Males (cocaine-injected): Significant increase in CRF mRNA expression (relative to saline-injected) at 30 mins

				Females (cocaine-injected): Significant increase in CRF mRNA expression (relative to saline-injected) at 24 hrs Neither males nor females had a difference in CRFR1 mRNA expression relative to their saline-injected controls There were also sex differences in the amount of CRF and CRFR1 mRNA expression at the 30 minute time point: saline-injected and cocaine-exposed females had significantly greater CRF mRNA relative to saline (but not cocaine) males Additionally, at that same time point, both saline-injected and cocaine-exposed females had significantly greater CRFR1 mRNA relative to saline and cocaine males
Erb et al. 2005	Cocaine	Injections for 7 consecutive days (Day 1 and 7: 15 mg/kg; i.p.) (Day 2-5: 30 mg/kg; i.p.)	dBNST	After 11 or 12 days of withdrawal and an i.c.v. infusion of CRF, there was no change in c-fos mRNA expression
Erb, Funk, Le 2005	Cocaine	Injections for 7 consecutive days (Day 1 and 7: 15 mg/kg; i.p.) (Day 2-5: 30 mg/kg; i.p.)	vBNST	After 11 or 12 days of withdrawal and an i.c.v. infusion of CRF, there was no change in c-fos mRNA expression
Colussi-Mas et al. 2005	Amphetamine	Acute injection (1 or 4 mg/kg; s.c.)	dBNST (oval nucleus)	C-fos-immunoactivity was highly concentrated in the BNSTov; some c-fos-ir was dotted throughout the anterodorsal and ventral BNST

Milanes et al. 1997	Morphine	Acute injection (30 mg/kg, i.p.)	BNST (subregion not specified)	Morphine-naïve rats: Significant increase in CRF protein relative to saline-injected controls Morphine-tolerant rats: Significant decrease in CRF protein relative to morphine-tolerant saline-injected rats, and decreased CRF protein relative to morphine-injected naïve rats
Garcia-Carmona et al. 2013	Morphine	Injections (6 mg/kg; i.p.) on days 1,3,5 for CPP training	dBNST	Most dBNST CRF neurons expressed pCREB (a marker of neuronal activation) after morphine CPP

Table 1-5. Summary of behavioral experiments involving drug administration without stressors. All studies list the specific BNST subregion investigated; however, the behavioral intervention or observation is not BNST-specific unless otherwise noted. Results are sorted by drug and then BNST subregion. This table includes results from sections 5.3 and 6.2. Abbreviations: i.p.: intraperitoneal; s.c.: subcutaneous; i.v.: intravenous; CPP: conditioned place preference; CIE: chronic intermittent ethanol; DID: drinking in the dark

Reference	Drug	Drug Administration	Subregion	Pharmacological Intervention	Behavioral Test	Result
Marcinkiewcz et al. 2009	Nicotine	Chronic (osmotic mini pumps; 9 mg/kg/day)	Lateral BNST (posterior)	Intra-lateral BNST administration of $_{D}$-Phe $CRF_{(12-41)}$ (CRFR1/2 antagonist) (5-500ng)	Reward threshold through intracranial self-stimulation after mecamylamine (nAChR antagonist, 3mg/kg, s.c.) to induce withdrawal	Mecamylamine (NAChR antagonist) (3 mg/kg; s.c.) was able to increase the reward threshold.
Pleil et al. 2015	Alcohol	Voluntary drinking-DID	dBNST	Chemogenetic activation of dBNST CRF neurons (Gs DREADD)	Binge drinking behavior (DID) and Open Field Test	Activating dBNST CRF neurons did not change binge drinking behavior, nor did it alter anxiety like behavior
Pleil et al. 2015	Alcohol	Voluntary drinking-DID	dBNST	Chemogenetic inhibition of dBNST CRF neurons (Gi DREADD)	Binge drinking behavior (DID) and Open Field Test	Inhibiting dBNST CRF neurons led to decreased binge drinking behavior and decreased anxiety-like

						behavior in the OFT
Huang et al. 2010	Alcohol	4.5% Alcohol diet	dBNST	Intra-dBNST administration of CRF (0.5μg) before alcohol diet	Social interaction	Decreased social interaction (increased anxiety-like behavior) was observed Systemic administration of SSR125543 (CRFR1 antagonist) (10 mg/kg) blocked this affect
Huang et al. 2010	Alcohol	4.5% Alcohol diet	dBNST	Intra-dBNST administration of SSR125543 (CRFR1 antagonist) (10μg/0.5μL) prior to restraint stress exposure, before alcohol diet	Social interaction	Decreased social interaction (increased anxiety-like behavior) observed with restraint stress was blocked by the CRFR1 antagonist
Huang et al. 2010	Alcohol	4.5% Alcohol diet	vBNST	Intra-vBNST administration of CRF (0.5 μg) before alcohol diet	Social interaction	No change in social interaction behavior was observed

Funk et al. 2006	Alcohol	CIE to induce dependence; Oral self-administration	dlBNST	Intra-dlBNST infusion of $_{D}$-Phe $CRF_{(12-41)}$ (CRFR1/2 antagonist) (0.125, 0.25, or 0.5 μg/μL)	Lever pressing for oral self-administration of alcohol after 2 hours of withdrawal from CIE	Dependent rats self-administered more alcohol than nondependent rats. Intra-dlBNST infusion of the CRFR1/2 antagonist did not change the amount of lever pressing in either group
Rinker et al. 2016	Alcohol	Voluntary drinking-DID	dlBNST	Chemogenetic inhibition of VTA-projecting dlBNST CRF neurons	Binge-like alcohol consumption	Inhibiting VTA-projecting dlBNST CRF neurons significantly decreased binge-like alcohol intake
Companion and Thiele 2018	Alcohol	Voluntary drinking-DID	dlBNST	Intra-VTA CNO to chemogenetically inhibit GABAergic dlBNST-VTA projection neurons	Binge-like alcohol consumption	Inhibiting the VTA-projecting dlBNST GABAergic neurons* significantly decreased binge-

						like alcohol intake *87% of the GABAergic neurons co-expressed CRF
de Guglielmo et al. 2019	Alcohol	CIE to induce dependence; Oral self-administration	vBNST	Optogenetic inhibition of CeA CRF projections to the vBNST	Lever pressing for oral self-administration of alcohol	Inhibiting the vBNST-projecting CeA CRF neurons reduced alcohol self-administration to the baseline level observed before dependence was induced
Gyawali et al. 2020	Fentanyl	i.v. self-administration (2.5μg/kg/infusion)	dBNST	Intra-dBNST infusion of R121919 (CRFR1 antagonist) (1μg/0.3μL/hemisphere)	Incubation of seeking behavior	Non-dependent rats: Bilateral infusion of R121919 blocked incubation of increased seeking behavior for fentanyl at 30 days post-abstinence of fentanyl self-administration

Gyawali et al. 2020	Fentanyl	i.v. self-administration (2.5μg/kg/infusion)	dBNST	Intra-dBNST infusion of R121919 (CRFR1 antagonist) (1μg/0.3μL/hemisphere)	Fentanyl-seeking behavior	Non-dependent and dependent rats: Bilateral infusion of R121919 blocked fentanyl-seeking behavior during 5-days post-opioid self-administration (acute withdrawal)
Colussi-Mas et al. 2005	Amphetamine	Injection (1 mg/kg; s.c.)	BNST	Intra-BNST infusion of timolol (β-AR antagonist) (2.5μg/side)	Behavioral sensitization	Behavioral sensitization was blocked by the b-AR antagonist
Wee et al. 2008	Cocaine	i.v. self-administration (6 hours, "long-access"; 1 mg/kg/injection for 3 days then 0.5 mg/kg/injection)	BNST	Systemic injections of prazosin (α1-AR antagonist) (1 or 3 mg/kg; i.p.), UK14304 (α2-AR agonist) (0.03, 0.1 or 0.3 mg/kg; i.p.), or betaxolol (β1-AR antagonist) (0.3, 1.0, 3.0, or 10 mg/kg; s.c.)	Self-administration break-point (progressive ratio)	Prazosin decreased break-point for cocaine; the α2-AR agonist and β1-AR antagonist had no effect
Wee et al. 2008	Cocaine	i.v. self-administration	BNST	Systemic injections of prazosin (α1-AR antagonist)	Self-administration break-point (progressive ratio)	The α1-AR antagonist, α2-AR agonist, or

		(1 hour, "short access"; 1 mg/kg/injection for 3 days then 0.5 mg/kg/injection)		(1 or 3 mg/kg; i.p.), UK14304 (α2-AR agonist) (0.03, 0.1 or 0.3 mg/kg; i.p.), or betaxolol (β1-AR antagonist) (0.3, 1.0, 3.0, or 10 mg/kg; s.c.)		β1-AR antagonist all had no effect on break-point
Davis et al. 2008	Cocaine	Injection (20 mg/kg; i.p.)	dlBNST	Systemic injections of yohimbine (α2-AR antagonist) (5mg/kg; i.p.)	Cocaine CPP extinction	Yohimbine impaired CPP extinction
Davis et al. 2008	Cocaine	Injection (20 mg/kg; i.p.)	dlBNST	Systemic injections of atipamezole (α2-AR antagonist) (3mg/kg; s.c.)	Cocaine CPP extinction	Atipamizole did not affect cocaine CPP extinction
Xu et al. 2000	Cocaine or Amphetamine	Injections: Cocaine (20 mg/kg; i.p.) Amphetamine (1 mg/kg; i.p.)	vBNST	*No pharmacological intervention* Norepinephrine transporter knockout (global) mouse	Locomotor sensitization	Increased locomotor sensitization
Ettenberg et al. 2015	Cocaine	i.v. self-administration (1mg/kg/infusion)	vBNST	Intra-vBNST infusion of $_{D}$-Phe $CRF_{(12-41)}$ (CRFR1/2 antagonist) (5 or 25ng/0.5 μL)	Approach-avoidance conflict behavior of rats running for i.v. cocaine	CRFR1/2 antagonism in the vBNST was unable to alleviate anxiety-like behaviors resultant of cocaine-administration

Wenzel et al. 2014	Cocaine	Infusion (1 mg/kg/0.1 mL)	vBNST	Intra-vBNST infusion of betaxolol and ICI 118,551 (β1-AR and β2-AR antagonists) (1nmol/0.5 μL)	Runway self-administration	vBNST infusion of β-AR antagonists decreased start latency, run time, and retreat frequency
Wenzel et al. 2014	Cocaine	Injection (1mg/kg/0.1 mL for 4.3s)	vBNST	Intra-vBNST infusion of betaxolol and ICI 118,551 (β1-AR and β2-AR antagonists) (1nmol/0.5 μL)	Place conditioning	vBNST infusion of β-AR antagonists prevented the development conditioned place aversion to cocaine's delayed anxiogenic effects; no change in conditioned place preference for cocaine

5.4 CRF Signaling in the BNST Related to Stress with Alcohol or Drug Use

In this section, we will discuss how stress interacts with alcohol or drug use, and how these interactions involve BNST CRF. For a summary of results investigating c-fos and CRF expression mentioned in this section see **Table 1-6**; for a summary of behavioral results see **Table 1-7**. Withdrawal from drugs or alcohol is often considered a stressor itself, so it will be discussed in this section. Additionally, stress- and drug-induced reinstatement, prominent animal models for relapse to substance use disorders, will be addressed in the context of BNST CRF alone. Reinstatement involving NE will be discussed in later sections of this review.

Table 1-6. Summary of CRF and c-fos expression changes in experiments combining stressors and drug administration.
Results are sorted by type of stress. This table includes results from sections 5.4, 6.3, and 7.3. Abbreviations: i.p.: intraperitoneal; s.c.: subcutaneous; i.v.: intravenous

Reference	Stress Type	Stressor	Drug	Drug Administration	Subregion	Expression Change
Houshyar et al. 2003	Acute	Restraint stress	Morphine	Injections of morphine in escalating doses (10-40 mg/kg; s.c.; 2 times/day for 4 days)	dBNST (oval nucleus)	CRF mRNA significantly decreased in the: morphine-injected group morphine-injected+restraint stress group saline-injected+restraint stress group relative to the saline-injected controls morphine-injected+stress did not further decrease CRF mRNA expression compared to morphine-injection alone
Shalev et al. 2001	Acute	Intermittent footshock	Heroin	Self-administration (0.1 mg/kg/infusion, i.v.)	dBNST	CRF mRNA was significantly increased on days 1 and 6 into withdrawal relative to the non-stress group
Shalev et al. 2001	Acute	Intermittent footshock	Heroin	Self-administration (0.1 mg/kg/infusion, i.v.)	vBNST	No changes in CRF mRNA expression relative to non-stress group
Aston-Jones et al. 1999	Acute	Naltrexone-induced withdrawal	Morphine	Subcutaneous morphine pellets (150 mg total)	dBNST	Significant increase in c-fos expression in the dBNST, particularly the dlBNST; blocked by systemic propranolol (β-AR antagonist)

Aston-Jones et al. 1999	Acute	Naltrexone-induced withdrawal	Morphine	Subcutaneous morphine pellets (150 mg total)	vBNST	Significant increase in c-fos expression; blocked by systemic propranolol (β-AR antagonist)
McReynolds et al. 2014	Acute	Forced Swim	Cocaine	Injections (15 mg/kg; i.p.)	BNST	CRF mRNA expression was greater in mice exposed to FSS Pretreatment with ICI-118,551 (β2-AR antagonist) blocked stress-induced CRF mRNA increase
Qi et al. 2016	Chronic	Overexpression of CRF in the Lateral BNST	Nicotine	Osmotic minipumps (3.6 mg/kg/day) to induce dependence	Lateral BNST (posterior)	Overexpression of CRF led to a 175% increase in CRFR1 mRNA and a 412% increase in CRFR2 mRNA

Table 1-7. Summary of behavioral experiments combining stressors with drug administration. All studies list the specific BNST subregion investigated; however the behavioral intervention or observation is not BNST-specific unless otherwise noted. Results are sorted by acute stress, chronic stress, or withdrawal, then by drug type and BNST subregion. This table includes results from sections 5.4, 6.3, and 7.3. Abbreviations: i.p.: intraperitoneal; s.c.: subcutaneous; i.v.: intravenous; CPA: conditioned place aversion; CPP: conditioned place preference; KO: knockout

Reference	Stress Type	Stressor	Drug	Drug Administration	Subregion	Pharmacological Intervention	Behavioral Test	Result
Wang et al. 2006	Acute	Intermittent footshock	Morphine	CPP training: (10mg/kg; s.c.)	dBNST (posterior)	Intra-BNST infusion of CP-154,526 (CRFR1 antagonist) (1.0μg)	Stress-induced reinstatement to morphine CPP	CRFR1 antagonism blocked footshock-induced reinstatement to CPP
Delfs et al. 2000	Acute	Naltrexone-precipitated withdrawal	Morphine	Morphine pellets (150 mg total)	BNST	Intra-BNST microinjections of betaxolol (β1-AR antagonist) plus ICI 118,551 (β2-AR antagonist) (0.1 or 1.0 nmol)	CPA to Morphine Withdrawal	Intra-BNST injection of the β-AR antagonists blocked CPA
Delfs et al. 2000	Acute	Naltrexone-precipitated withdrawal	Morphine	Morphine pellets (150 mg total)	BNST	Intra-BNST microinjections of ST-91 (α2-AR agonist) (0.025 or 0.25 nmol)	CPA to Morphine Withdrawal	Intra-BNST injection of the β-AR antagonists blocked CPA
Aston-Jones et al. 1999	Acute	Naltrexone-precipitated withdrawal	Morphine	Morphine pellets (150 mg total)	BNST	Intra-BNST microinjections of betaxolol	CPA to Morphine Withdrawal	Intra-BNST injection of the β-AR

						(β1-AR antagonist) plus ICI 118,551 (β2-AR antagonist) (1.0 nmol each/0.5 μL/side)		antagonists blocked CPA
Wang et al. 2001	Acute	Intermittent footshock	Morphine	Injection (10 mg/kg; i.p.)	vBNST	Intra-BNST injection of clonidine (α2-AR agonist) (0.1 or 1 μg)	Continued footshock stress-induced morphine CPP	Intra BNST injection of the a2-AR agonist blocked stress-induced reinstatement to morphine CPP
Shalev et al. 2001	Acute	Intermittent footshock	Heroin	Self-administration (0.1 mg/kg/infusion; i.v.)	BNST	None	Stress-induced reinstatement to cocaine-seeking	Intermittent footshock induced reinstatement at 6, 12, 25 and 66 days into withdrawal
Erb and Stewart 1999	Acute	Intermittent footshock	Cocaine	Self-administration (0.5 mg/kg/infusion; i.v.)	BNST (not specified)	Intra-BNST administration of D-Phe $CRF_{(12-41)}$ (CRFR antagonist) (10 or 50 ng)	Stress-induced reinstatement to cocaine-seeking	Intra-BNST CRFR antagonism blocked stress-induced reinstatement
Leri et al. 2002	Acute	Intermittent footshock	Cocaine	Self-administration (0.5 mg/kg/infusion; i.v.)	BNST	Intra-BNST infusions of betaxolol (β1-AR antagonist) plus ICI 118,551 (β2-AR antagonist)	Stress-induced reinstatement to cocaine seeking	Intra-BNST infusions of the β-AR antagonists dose-dependently

						(0.25, 0.5, or 1 nmol/0.5 μL)		decreased reinstatement
McReynolds et al. 2014	Acute	Forced Swim	Cocaine	Injections (15 mg/kg; i.p.)	BNST	ICI-118,5551 (β2-AR antagonist) (1 mg/kg; i.p.)	Stress-induced reinstatement to cocaine CPP	Blocking β2-ARs prevents stress-induced reinstatement to cocaine CPP
McReynolds et al. 2014	Acute	Forced Swim	Cocaine	Injections (15 mg/kg; i.p.)	BNST	Antalarmin (CRFR1 antagonist) (10 mg/kg; i.p.)	Stress-induced reinstatement to cocaine CPP	Blocking CRFR1 prevents stress-induced reinstatement to cocaine CPP
Perez et al. 2020	Acute	Forced Swim	Cocaine	Injection (15 mg/kg; i.p.)	dlBNST	*No pharmacological intervention* $\alpha 2_A$-AR KO mouse	Stress-induced reinstatement to cocaine CPP	KO of $\alpha 2_A$-ARs blocks stress-induced reinstatement to cocaine CPP
Perez et al. 2020	Acute	Forced Swim	Cocaine	Injection (15 mg/kg; i.p.)	dlBNST	*No pharmacological intervention* $\alpha 2_A$-AR KO mouse (heteroreceptor-specific)	Stress-induced reinstatement to cocaine CPP	KO of $\alpha 2_A$-AR heteroreceptors blocks stress-induced reinstatement to cocaine CPP
Perez et al. 2020	Acute	Forced Swim	Cocaine	Injection (15 mg/kg; i.p.)	dlBNST	Low dose guanfacine ($\alpha 2_A$-AR agonist) (0.14mg/kg; i.p.)	Stress-induced reinstatement to cocaine CPP	Activating $\alpha 2_A$-ARs blocks stress-induced reinstatement to cocaine CPP

Vranjkovic et al. 2014	Acute	Footshock	Cocaine	Self-administration (1.0 mg/kg/infusion; i.v.)	vBNST	Intra-vBNST infusion of ICI-118,551 (β2-AR antagonist) (1 nmol/277 ng)	Stress-induced reinstatement to cocaine-seeking	Blocking vBNST β2-ARs blocked stress-induced reinstatement to cocaine-seeking
Vranjkovic et al. 2014	Acute	Footshock	Cocaine	Self-administration (1.0 mg/kg/infusion; i.v.)	vBNST	Intra-vBNST infusion of betaxolol (β1-AR antagonist) (1 nmol/307 ng)	Stress-induced reinstatement to cocaine-seeking	Blocking vBNST β1-ARs had no effect on stress-induced reinstatement to cocaine-seeking
Albrechet-Souza et al. 2017	Chronic	10-day episodic social defeat	Alcohol	Ethanol (20%) two-bottle free-choice	BNST (not specified)	Intra-BNST administration of CP376395 (CRFR1 antagonist) (0.25 μg or 0.5 ug)	Drinking behavior	Stress-exposed mice drank more than controls Intra-BNST CRFR1 antagonism at 0.25μg decreased alcohol intake in the control but not the stressed mice
Albrechet-Souza et al. 2017	Chronic	10-day episodic social defeat	Alcohol	Ethanol (20%) two-bottle free-choice	BNST (not specified)	Intra-BNST administration of	Drinking behavior	Intra-BNST CRFR2 antagonism

						astressin2B (CRFR2 antagonist) (0.25 or 0.5 μg)		increased alcohol intake in both control and stressed mice
Wang et al. 2001	Chronic	Intermittent footshock every three days for 28 days	Morphine	Injection (10 mg/kg; i.p.)	vBNST	Intra-BNST injection of clonidine (α2-AR agonist)	Continued footshock stress-induced morphine CPP	Intra BNST injection of the a2-AR agonist blocked stress-maintained morphine CPP
Wang et al. 2006	Other	Withdrawal	Morphine	CPP training: (10mg/kg s.c.) Reinstatement priming dose: (1 mg/kg s.c.)	dBNST (posterior)	Intra-BNST infusion of CP-154,526 (CRFR1 antagonist) (1.0μg)	Morphine-induced reinstatement to morphine CPP	CRFR1 antagonism did not alter morphine-induced reinstatement to CPP
Leri et al. 2002	Other	Withdrawal	Cocaine	Self-administration (0.5 mg/kg/infusion; i.v.)	BNST	Intra-BNST infusions of betaxolol (β1-AR antagonist) plus ICI 118,551 (β2-AR antagonist) (0.25, 0.5, or 1 nmol/0.5 μL)	Cocaine-induced reinstatement to cocaine seeking	Intra-BNST infusions of the β-AR antagonists were unable to block reinstatement
Erb and Stewart 1999	Other	Withdrawal	Cocaine	Self-administration (0.5 mg/kg/infusion; i.v.)	BNST (not specified)	None	CRF-induced reinstatement to cocaine-seeking	Intra-BNST CRF infusion (100 or 300 ng) induced stress-induced reinstatement

Vranjkovic et al. 2014	Other	Withdrawal	Cocaine	Self-administration (1.0 mg/kg/infusion; i.v.)	vBNST	Intra-vBNST infusion of antalarmin (CRFR antagonist) (1.32 nmol/500ng)	Drug-induced reinstatement to cocaine-seeking	Intra-vBNST clenbuterol (β2-AR agonist) (36 pmol/10ng) induced reinstatement. CRFR antagonism blocked this effect.
Vranjkovic et al. 2014	Other	Withdrawal	Cocaine	Self-administration (1.0 mg/kg/infusion; i.v.)	vBNST	None	Drug-induced reinstatement to cocaine-seeking	Intra-vBNST dobutamine (β1-AR agonist) (1 nmol/301 ng) did not induce reinstatement
Vranjkovic et al. 2014	Other	Withdrawal	Cocaine	Self-administration (1.0 mg/kg/infusion; i.v.)	vBNST	Intra-vBNST infusion of ICI 118,551 (β2-AR antagonist) (1 nmol/277 ng)	CRF-induced reinstatement to cocaine-seeking	Intra-vBNST CRF (63 pmol/ 300 ng) induced reinstatement. β2-AR antagonism did not block this effect

5.4.1 Withdrawal and Other Stressors

The interaction between nicotine withdrawal and BNST CRF signaling and expression has been studied in rats. In one study, rats that were made nicotine-dependent using osmotic minipumps were given mecamylamine (nicotinic acetylcholine receptor antagonist) to precipitate nicotine withdrawal. Mecamylamine administration increased the brain reward threshold as measured through intracranial self-stimulation, but blocking CRFR1 and CRFR2 in the lateral BNST did not affect mecamylamine's ability to do so (Marcinkiewcz et al., 2009). These observations suggest that CRF signaling via the CRFR1 and CRFR2 in the lateral BNST of nicotine-dependent rats is not involved in setting the reward threshold during nicotine withdrawal (Marcinkiewcz et al., 2009). Using similar methods as the previous study, Qi et al. (2016) determined that CRF overexpression in the lateral BNST may be beneficial for modulating reward threshold during nicotine withdrawal, but is unable to ameliorate withdrawal-induced anxiety-like behavior. Interestingly, overexpression of CRF led to a 175% increase in CRFR1 mRNA and 412% increase in CRFR2 mRNA, ultimately increasing the CRFR2/CRFR1 ratio (Qi et al., 2016), which may provide insight into the mechanism in which CRF overexpression is able to attenuate some aspects nicotine withdrawal. One caveat of the previously-described studies, however, is that the "lateral BNST" targeted may be more posterior than the regions typically targeted in other studies discussed in this review, so investigating the dorsal and ventral anterior subregions might be beneficial in future studies. Overall, more studies investigating the role of BNST CRF in nicotine use and dependence may be useful.

CRF in the BNST in relation to stress and alcohol has been more well-studied than nicotine. Initial studies focused on the role of BNST CRF signaling in alcohol withdrawal-

induced negative affect and craving. It was found that intra-dBNST injections of CRF, but not intra-vBNST CRF injections, sensitized anxiety during alcohol withdrawal in rats (Huang et al., 2010). Additionally, administration of a CRFR1 antagonist before intra-dBNST CRF administration blocked the CRF-sensitized alcohol withdrawal-induced anxiety (Huang et al., 2010). Such increases in withdrawal-induced anxiety-like behaviors would be expected to increase subsequent alcohol intake behaviors; however, alcohol withdrawal interactions with BNST CRF signaling are not always consistent. If rats were fed an alcohol-containing diet for two weeks, lateral BNST extracellular CRF levels increased after the alcohol diet was removed. Eight hours after the removal, the alcohol diet was returned to one group and their BNST CRF levels returned to baseline, but rats who did not return to the alcohol diet continued to show an increase in their BNST CRF levels (Olive et al., 2002). In another study, rats were made dependent upon alcohol through chronic intermittent ethanol (CIE) exposure and given the opportunity to self-administer alcohol after 2 hours of withdrawal. The dependent rats orally self-administered significantly more alcohol than nondependent rats as expected, but intra-dlBNST infusion of $_{D}$-Phe-CRF$_{(12-41)}$ (CRFR1/2 antagonist) did not affect the amount of lever pressing in either group (Funk et al., 2006). Additionally, CRF immunoreactivity in the dlBNST at 2hr withdrawal did not differ between the dependent and nondependent alcohol groups (Funk et al., 2006). Therefore, it appears that mode of alcohol intake and length of withdrawal may be factors in the role that BNST CRF plays in subsequent alcohol-related behaviors.

Pati et al. (2020) identified a role for CRF neurons following chronic intermittent ethanol (CIE) in the regulation of a previously characterized anxiety-related GABAergic BNST microcircuit (Marcinkiewcz et al., 2016). Marcinkiewcz et al. (2016) observed that BNST CRF neurons not projecting to the VTA or LH use GABAergic signaling to inhibit VTA-projecting and LH-projecting BNST neurons, ultimately increasing anxiety-like behavior. In a follow-up study investigating CIE's effects on this circuit, Pati et al. (2020) found that dBNST and vBNST

CRF neurons not projecting to the LH or VTA displayed increased excitability at 72 hours of withdrawal following completion of CIE exposure. Additionally, while VTA-projecting and LH-projecting non-CRF BNST neurons displayed increased evoked excitability, these neurons also displayed increased sIPSC frequency after withdrawal. Overall, this suggests that 72-hour withdrawal from CIE leads to increased GABAergic inhibition of the VTA-projecting and LH-projecting BNST neurons through the non-projecting CRF neurons, and ultimately leads to more anxiety-like behavior and further drinking (Pati et al., 2020). One limitation of this study, however, was the fact that they recorded from dBNST and vBNST neurons without separating the two, and since there are subregional differences it would be interesting to identify any possible subregion-specific effects. Further, like with most studies, it would be important to investigate this in female animals as well, as their lab has previously shown sex-differences in alcohol behaviors after CIE (Jury et al., 2016).

In the dBNST of male and female mice experiencing forced abstinence from chronic alcohol drinking, there was an increase in neuronal activation as a whole, and an increased percentage of c-fos+ CRF neurons (Centanni et al., 2019). Additionally, dBNST CRF neurons from those mice exhibited an increased sEPSC frequency (Centanni et al., 2019). In another study, VTA-projecting dBNST neurons of CIE-exposed mice recorded after 4-5 hours of withdrawal had a greater basal sEPSC frequency compared with control mice (Silberman et al., 2013). CRF application was unable to further increase the sEPSC frequency (Silberman et al., 2013), suggesting that CIE withdrawal induced increased CRF function in the dBNST such that further modulation by exogenous CRF was occluded. This CIE-induced increase in basal sEPSCs of VTA-projecting dBNST neurons was blocked when mice were pretreated with an injection of NBI27914, a CRFR1 antagonist. The findings from this study suggest that CIE acts to alter CRF transmission *in vivo* in the dBNST and is dependent upon the action of the CRFR1 (Silberman et al., 2013). This work is further supported by recent findings indicating that dBNST CRF neuron

resting membrane potentials are in a depolarized state at 4 hours of withdrawal from CIE (Snyder et al., 2019). Interestingly, 72 hours of withdrawal from CIE led to an increased sIPSC frequency in the non-CRF VTA/LH-projecting BNST neurons but did not alter sEPSC frequency (Pati et al., 2020). This difference in sEPSC and sIPSC properties observed in VTA-projecting BNST neurons between various withdrawal durations (i.e., 4 hours vs. 72 hours) is indicative of a time-dependent effect. These studies taken together show that chronic alcohol exposure, whether voluntary or passive, affects basal glutamatergic transmission of dBNST neurons and also involves the CRF system. Additionally, the timing of withdrawal may also determine the activity of non-CRF VTA-projecting BNST neurons, which may be important when explaining withdrawal-related alcohol-seeking behaviors.

Not all studies, however, show an interaction between stress, BNST CRF, and alcohol intake. When mice were exposed to episodic social defeat stress, they expressed higher levels of BNST CRF mRNA, although stress did not alter CRFR1 or CRFR2 expression (Albrechet-Souza et al., 2017). These stress-exposed mice drank more alcohol than non-stressed mice in the weeks following the stress-exposure but intra-BNST administration of CP376395, a CRFR1 antagonist, decreased voluntary alcohol intake in the control but not the stressed mice (Albrechet-Souza et al., 2017). Conversely, administration of astressin 2B, a CRFR2 antagonist, *increased* alcohol consumption in both the control and stressed groups (Albrechet-Souza et al., 2017). These findings indicate there may be intricate differences between types of stressors, BNST CRF, and alcohol-related behaviors, perhaps related to distinct BNST microcircuits that are activated by stress/alcohol interactions. This hypothesis should be addressed in future studies.

A limited number of studies have looked at BNST CRF and withdrawal from cocaine. In one study, male rats chronically exposed to cocaine showed elevated CRF mRNA in the dBNST 30 minutes post-final cocaine injection relative to saline-injected controls, while it took 24 hours for female rats to show increased dBNST CRF mRNA (Connelly and Unterwald, 2020).

Additionally, neither male nor female rats had elevated CRFR1 mRNA at any time point (30 mins, 24 hrs, 48 hrs, 7 days, or 14 days) post-final cocaine exposure (Connelly and Unterwald, 2020). Interestingly, at 30-minutes post-final injection, both saline-injected and cocaine-exposed females had significantly greater CRF mRNA relative to saline-exposed (but not cocaine-exposed) males. At that same time point, both saline-injected and cocaine-exposed females had significantly greater CRFR1 mRNA relative to saline and cocaine males (Connelly and Unterwald, 2020). This study demonstrates that CRF expression in the dBNST is altered during periods of cocaine withdrawal, and the time-course and expression levels vary between sexes, providing evidence supporting the need for further research investigating changes in both males and females. In another study, rats previously exposed to cocaine injections for 7 consecutive days, then not exposed for 11-12 days and challenged with an intracerebroventricular infusion of CRF, showed no change of c-fos mRNA expression in the dBNST or the vBNST (Erb et al., 2005). Together, these studies suggest that CRF mRNA expression levels change in the dBNST during withdrawal, but this effect may be separate from neuronal activation.

Opioids are another class of drugs that recruit the CRF system during withdrawal, and BNST CRF in response to fentanyl and morphine withdrawal has been briefly studied. CRFR1 in the dBNST has been shown to be important for cue-associated fentanyl-seeking behavior in rats during periods of abstinence (Gyawali et al., 2020). Injection of the CRFR1 antagonist R121919 into the dBNST of nondependent rats blocked incubation of increased seeking behavior measured at 30 days post-abstinence of fentanyl self-administration. Additionally, in both dependent and nondependent rats, dBNST infusion of the CRFR1 antagonist decreased fentanyl-seeking behavior during acute withdrawal (5-days post opioid self-administration) (Gyawali et al., 2020). This study suggests that the CRFR1 in the dBNST is important for modulating fentanyl-seeking behaviors in both early and protracted abstinence of rats. In rats receiving injections of morphine in escalating doses, CRF mRNA in the BNSTov and the BNSTfu was measured 12 hours post the

final injection (Houshyar et al., 2003). CRF mRNA significantly decreased in both nuclei in the morphine group, the morphine+restraint stress group, and the saline+stress group relative to saline-injected controls. Stress in addition to the morphine injection, however, did not further decrease CRF mRNA expression than from that seen in the stress or morphine alone groups (Houshyar et al., 2003). These results suggest that morphine and stress both affect CRF expression in the dBNST and vBNST, but together their effect on CRF expression is not compounded. Ultimately, these two studies demonstrate a role for CRF during withdrawal from opioids.

5.4.2 Stress-induced Reinstatement

CRF has been found to be extremely important for the phenomenon of stress-induced reinstatement to drug-seeking, which is a commonly used animal model of stress-related relapse. Rodents exhibiting stress- or drug-induced reinstatement are typically trained to self-administer a drug or are conditioned to prefer a drug-paired chamber in a CPP apparatus. After the training period, there is a period of extinction in which the rodents learn that self-administration behavior or entering the drug-paired context is no longer providing the reward of the drug. For stress-induced reinstatement, rodents are exposed to a stressor such as a footshock and their self-administration or place preference behavior is re-assessed. For drug-induced reinstatement, typically the drug the animal was previously exposed to during the training period is given as a trigger and the aforementioned behavioral tests are run. If the animal increases their drug-seeking behaviors, then it is said that reinstatement has occurred. This section will discuss stress-induced reinstatement in the context of CRF and the BNST. Future sections will examine stress-induced reinstatement involving NE both with and without CRF.

Studies examining the role of BNST CRF in stress-induced reinstatement to drug-seeking have primarily utilized footshock as the inducing stressor. Erb and Stewart (1999), demonstrated that a microinjection of a CRFR antagonist into the BNST of male rats was able to block footshock-induced reinstatement to cocaine, and intra-BNST administration of CRF was also able to successfully induce cocaine reinstatement (Erb and Stewart 1999). Intra-BNST infusion of the CRFR1 antagonist CP-154,526 was also able to block intermittent footshock reinstatement to morphine CPP in male rats (Wang et al., 2006), and systemic antagonism of CRFR1 blocked swim stress-induced reinstatement to cocaine CPP in mice (McReynolds et al., 2014). Interestingly, however, blocking CRFR1 in the BNST was unable to prevent morphine-induced reinstatement to morphine place preference (Wang et al., 2006). Together these studies find that the CRFRs in the BNST are necessary for stress-induced reinstatement to either cocaine or morphine, but not drug-induced reinstatement to morphine. Shalev et al. (2001) found that the degree to which footshock is able to induce reinstatement to heroin-seeking depends on the time into withdrawal. Further, in the footshock group compared to the no-shock group, CRF mRNA was increased in the dBNST at days 1 and 6, but no change was seen in the vBNST (Shalev et al., 2001), suggesting that these subregions respond differently to acute stressors during heroin withdrawal.

Altogether, the studies detailed throughout this section generally implicate CRF and CRF signaling in the BNST, particularly via CRFR1, in stress-related drug-seeking behaviors. The following sections will discuss NE in the BNST in stress, alcohol or drug use, and both combined. Future topics will also include findings of CRF and NE interacting with one another.

6. Norepinephrine in the BNST

Norepinephrine (NE) is an important neurotransmitter involved in the stress response. Noradrenergic projections are found in the BNST, with the densest projections entering the vBNST (Forray and Gysling, 2004). In this section we will discuss BNST noradrenergic signaling in the context of stress, drug use, and both of those combined. While NE does interact with CRF signaling in the BNST, studies addressing those interactions will be discussed in the last major section of this review.

NE release is one way to measure NE's influence in the BNST, and NE release varies greatly between the dBNST and vBNST. In vBNST minislices from male rats, potassium-induced NE release was shown to be modulated by the α2-AR, with activation inhibiting NE release and antagonism increasing NE (Forray et al., 1995). When looking at the dmBNST and vBNST of mice using *in vivo* voltammetry, evoked release of NE was greater in the vBNST than the dBNST (Herr et al., 2012). Investigating this further, systemic administration of the α2-AR antagonist idazoxan increased the maximum evoked NE release and clearance half-life in both the dmBNST and vBNST, with the dmBNST showing less maximal NE and a shorter NE clearance half-life (Herr et al., 2012). Systemic administration of the NET inhibitor DMI after administration of the α2-AR antagonist further increased the NE concentration and clearance half-life in the vBNST (Herr et al., 2012). Overall, these results suggest that α2-ARs play a role in modulating NE release in the dmBNST and vBNST, and that the vBNST has the potential to receive greater NE signaling than the dmBNST. This subregional distinction is likely very important in behavioral stress responses and interactions with various drug classes.

Multiple studies have shown that NE alters activity of neurons in the BNST in naïve animals. In BNST slices from naïve male mice, application of the α1-AR agonist methoxamine was able to induce long-term depression (LTD) in both the dlBNST and the vlBNST (McElligott

and Winder, 2008), an effect which was shown to be maintained via a postsynaptic mechanism (McElligott et al., 2010). Investigating this mechanism in the dlBNST specifically, NE produced similar effects as the α1-AR agonist, and this effect was confirmed to be α1-AR mediated. Interestingly, L-type voltage-gated calcium channels, but not NMDA receptor function or mGluR function, were necessary for the α1-AR mediated LTD to occur (McElligott and Winder, 2008). Using two models of affective disorders, $\alpha 2_A$-AR KO mice and NET KO mice, revealed that both did not exhibit the α1-AR mediated LTD (McElligott and Winder, 2008). Ultimately, α1-AR mediated LTD in the BNST may be important in mechanisms underlying affective disorders and corresponding behaviors, at least in these mouse models. In anesthetized rats, Casada and Dafny (1993) microiontophoretically applied NE to the BNST and found that NE decreased the firing rate of 70% of the neurons investigated and increased firing rate in only 2%. Of all neurons that responded to NE, 91% were located in the ventral subregion of the BNST (Casada and Dafny 1993), suggesting that the vBNST may be more susceptible to NE modulation. Gungor et al. (2018) examined how mouse vBNST glutamatergic and GABAergic neurons differ in their response to NE. NE application decreased excitatory postsynaptic potential (EPSP) amplitudes in both cell types, but the decrease was less drastic and shorter-lasting in the glutamatergic cells. α1-AR and α2-AR agonism both decreased the EPSP amplitudes of glutamatergic and GABAergic neurons. Conversely, β-AR agonism did not significantly change the EPSP amplitudes in either cell type, although there was a trend toward increased amplitude in the glutamatergic cells (Gungor et al., 2018). Overall, the authors found that NE primarily exhibits inhibitory control over glutamatergic and GABAergic vBNST neurons, an effect that is mediated by α1-ARs and α2-ARs. Activation of β-ARs, however, may elicit an excitatory effect in glutamatergic neurons, but not GABAergic neurons (Gungor et al., 2018). Given that the authors identified that glutamatergic neurons display more intrinsic excitability than the GABAergic neurons (described

in detail in section 2.1), this may make glutamatergic neurons more sensitive to excitatory effects of NE and contribute to the physiological responses of the vBNST to stress.

Multiple studies have also demonstrated that NE modulates glutamatergic signaling in the BNST through an α2-AR mediated mechanism. In the vBNST, perfusion of UK-14,304 (α2-AR agonist) decreased *in vivo* glutamate release, with RX821002 (α2-AR antagonist) having an opposite effect (Forray et al., 1999). A similar result was found using whole-cell patch-clamp and field recordings when application of UK-14,304 decreased glutamatergic signaling in both the dBNST and the vBNST (Egli et al., 2005). Further exploring this effect using $\alpha2_A$-AR KO mice, Egli et al. (2005) determined that $\alpha2_A$-ARs are the specific α2-AR subtype mediating the decrease in dBNST glutamatergic signaling. In the vBNST, however, the $\alpha2_A$-AR subtype appears to play only a partial role in mediating glutamatergic transmission (Egli et al., 2005). Shields et al. (2009) also determined that the $\alpha2_A$-AR subtype is responsible for modulating glutamatergic signaling in the dBNST. They observed that the $\alpha2_A$-AR is expressed on terminals of glutamatergic neurons, and concluded that guanfacine ($\alpha2_A$-AR agonist) was likely acting via a heterosynaptic mechanism to decrease glutamatergic transmission in the dBNST (Shields et al., 2009). Flavin et al. (2014) investigated the role of $\alpha2_A$-ARs in modulating excitatory glutamatergic input to the dBNST from the parabrachial nucleus (PBN). Activating dBNST-projecting PBN terminals produced EPSPs in 52.9% of recorded neurons, but $\alpha2_A$-AR activation was able to decrease the size of the resulting EPSPs (Flavin et al., 2014). Ultimately, the results from these studies emphasize the importance of α2-ARs in modulating glutamatergic signaling in the BNST, with a particular emphasis on heterosynaptic $\alpha2_A$-ARs on glutamatergic terminals in the dBNST.

Studies also found evidence that activation of $\alpha2_A$-ARs can increase excitatory signaling and neuronal activation in the dBNST. In a continuation of the experiments described previously, Flavin et al. (2014) showed that agonism of $\alpha2_A$-ARs while optically stimulating non-PBN presynaptic neurons in the dBNST led to an unexpected further increase in field potential size,

suggesting that $\alpha 2_A$-ARs may instead promote excitatory signaling from non-PBN presynaptic terminals and/or decrease GABAergic signaling. Additionally, systemic guanfacine administration increased c-fos immunoreactive neurons in the dBNST, but not the vBNST, of mice (Savchenko and Boughter, 2011). Further, Harris et al. (2018) found that guanfacine injection led to a large increase in dBNST c-fos expression in $\alpha 2_A$-AR-expressing neurons and a modest increase in c-fos expression in non-$\alpha 2_A$-AR-expressing neurons of WT mice; however, no increase in c-fos expression after guanfacine treatment was seen in either full or heteroreceptor-specific $\alpha 2_A$-AR KO mice. Harris et al. (2018) also observed that chemogenetic inhibition of dBNST neurons led to increased c-fos expression equivalent to that observed with guanfacine treatment alone, an effect which was not further enhanced by co-administration of guanfacine. Together, these experiments suggest that guanfacine can increase excitatory transmission and neuronal activation in the dBNST via postsynaptic, heterosynaptic $\alpha 2_A$-ARs.

There is also a role for $\alpha 2_A$-ARs in the modulation of inhibitory dBNST transmission, with multiple studies supporting the possible excitatory effect of $\alpha 2_A$-AR activation. Flavin et al. (2014) found that activating PBN terminals in the dBNST resulted in IPSPs in 47.1% of recorded neurons, and they determined that the IPSPs were likely a result of feedforward inhibition from local GABA neurons in the BNST activated by glutamatergic PBN inputs (Flavin et al., 2014). Recording from the "PBN-inhibited" neurons, they also found evidence that $\alpha 2_A$-AR activation may decrease IPSP amplitude in a subset of those neurons (Flavin et al., 2014). Shields et al. (2009) identified that $\alpha 2_A$-AR activation in the dBNST dose-dependently decreases inhibitory signaling and determined that the $\alpha 2_A$-AR may be involved in the modulation of inhibitory transmission through postsynaptic actions (Shields et al., 2009). In the vBNST, however, Dumont and Williams (2004) saw no effect on vBNST sIPSCs after α2-AR agonist or antagonist application (Dumont and Williams 2004) and there was no effect on *in vivo* vBNST GABA levels after α2-AR agonist administration (Forray et al., 1999).

In addition to α-AR experiments described above, other studies looked at the influence of β-AR activation on excitatory transmission in the BNST. Activation of β-ARs using isoproterenol or application of NE itself resulted in an increased glutamatergic response as measured by evoked field potentials in the dBNST, an effect largely driven by the β2-AR (Egli et al., 2005). Nobis et al. (2011) found similar results in the dlBNST, where bath application of isoproterenol increased sEPSC frequency; however, further results indicated that isoproterenol exerted its effects via the β1-AR instead of the β2-AR. Additionally, isoproterenol application was able to increase the amplitudes of eEPSCs with a decrease in PPR suggesting that isoproterenol acts via a presynaptic glutamatergic mechanism (Nobis et al., 2011). Interestingly, Egli et al. (2005) found that none of the β-AR mediated effects they saw in the dBNST were observed in the vBNST, although another study found evidence that activation of β-ARs may elicit an excitatory effect in vBNST glutamatergic neurons (Gungor et al., 2018). Of note, there are further studies linking β-AR to stress and drug responding in the dBNST and vBNST that will be detailed in subsequent sections.

Altogether, the above studies emphasize that NE has the potential to greatly influence BNST function. As might be expected, there are subregional differences in how NE affects signaling; for example, there are significantly fewer noradrenergic inputs into the dBNST than the vBNST, and $\alpha 2_A$-ARs appear to be the primary α2-AR subtype modulating glutamatergic inputs in the dBNST. A collection of studies above also presents an evolving story around heterosynaptic $\alpha 2_A$-ARs modulating neurotransmission and neuronal activation in the dBNST, with activation of $\alpha 2_A$-ARs having the potential to produce both activation and inhibition. Additionally, results implicating different β-AR subtypes modulating glutamatergic transmission depending upon the method of measurement (i.e., evoked field recordings vs. sEPSCs) in the dBNST warrant further exploration into this mechanism. The following section will focus on the interaction between stress and BNST NE signaling.

6.1 NE Signaling in the BNST Related to Stress

Stress has been shown to influence NE release and function in the BNST. In this section, we will focus on studies that demonstrate how BNST NE is altered after a stressor, how manipulation of BNST NE affects behavioral responses to stress, and how stress affects cellular responses to NE.

Multiple studies demonstrate that NE release in the BNST increases in response to stress exposure. For example, Pacak et al. (1995) observed that NE levels in the lateral BNST of rats during a two-hour restraint stress peaked within the first 30 minutes and steadily declined but remained elevated 90 minutes after termination of the stressor. Using a shorter version of restraint, Cecchi et al. (2002) showed that 5 minutes of immobilization stress increased NE in the rat vBNST, an effect which subsided after 30 minutes post-stress. In response to predator odor exposure, NE release in the vBNST also significantly increased (Fendt et al., 2005). Further, this predator odor increase in NE levels was blocked by intra-vBNST administration of clonidine, an α2-AR agonist (Fendt et al., 2005). Taken together, these studies consistently demonstrate that various stressors trigger increased norepinephrine release in the BNST, particularly in the vBNST subregion, and this increase can be modulated by α2-ARs in the vBNST. Variations in NE signaling in response to stressors also have behavioral consequences. Fendt et al. (2005) observed that the predator odor stressor that led to increased vBNST NE also produced increased freezing behavior and, like the increase in NE, this behavioral effect was attenuated by intra-vBNST administration of the α2-AR agonist. Interestingly, injections of clonidine into the dBNST and other adjacent brain regions were unable to block freezing behavior, emphasizing the specificity of this mechanism to the vBNST (Fendt et al., 2005). Cecchi et al. (2002) showed that immobilization stress led to increased anxiety-like behavior in both the social interaction test and EPM. Intra-vBNST infusion of an α1-antagonist (benoxathian) or a mix of β1-AR and β2-AR

antagonists did not affect behavior in the social interaction test; however, intra-vBNST administration of the α-AR antagonist or the β-AR antagonists attenuated the stress-induced anxiety-like behavior in the EPM (Cecchi et al., 2002). Additionally, acute immobilization also increased plasma ACTH, and intra-vBNST microinjection of the α1-AR antagonist, but not the β1/β2-AR antagonists, attenuated the stress-induced ACTH increase, indicating that the stress-related increase in NE acts via the α1-AR and β-ARs in the vBNST to increase anxiety-like behavior, but only via α1-AR to increase plasma ACTH levels (Cecchi et al., 2002). Ultimately, these studies emphasize that increased NE signaling in the vBNST post-stress exposure is responsible, at least in part, for producing stress-induced anxiety-like behavior.

Stress also affects NE-related synaptic signaling, but varies in its effects in the vlBNST vs. the dlBNST. As referred to in earlier sections, methoxamine, an α1-AR agonist, was previously shown to induce LTD in the dlBNST and vlBNST of mice (McElligott and Winder, 2008). After chronic exposure to restraint stress, methoxamine was unable to produce LTD in the vlBNST, although a transient period of depression was observed (McElligott et al., 2010). In the dlBNST, however, α1-AR LTD was present after restraint stress, but it was attenuated significantly compared to non-stressed mice (McElligott et al., 2010). This study demonstrates that stress differentially affects α1-AR LTD in the vlBNST compared to the dlBNST, but further examination into the differences between acute and chronic stress on BNST NE signaling are likely needed.

Together these studies emphasize the role of NE in the dBNST and vBNST after stress. Stressors reliably increase the release of NE in the BNST, but how stress affects subregions can differ significantly. This drastic difference in response to NE in the dBNST vs. vBNST is likely due in part to the prominent noradrenergic projections to the ventral, but not the dorsal, BNST. Further, the studies detailed above acknowledge the role of the vBNST specifically in modulating post-stress anxiety-like behavior. Summaries of the behavioral results in this section are included

in Table 1-3. The next section will talk about NE in the BNST and its interactions with alcohol or drug use.

6.2 NE Signaling in the BNST Related to Alcohol or Drug Use

NE signaling has been implicated in mediating both the rewarding and negative affective states associated with substance use disorders. In the BNST, studies have investigated how various drugs alter NE release and modulation of glutamatergic transmission. In this section we will discuss how drugs including nicotine, alcohol, amphetamine, and primarily cocaine, interact with BNST NE. Later sections will detail the interactions between BNST NE *and* stress in substance use disorders.

Elevated extracellular NE in the BNST is associated with exposure to many drug classes. Jadzic et al. (2019) demonstrated that systemic administration of numerous drugs —nicotine, cocaine, amphetamine, morphine, and ethanol— all increased NE levels in what appears in their figures to be the vBNST in rats. Using microdialysis, Fuentealba et al. (2000) found that extracellular levels of vBNST NE were elevated in morphine-dependent rats, and intra-vBNST infusion of a selective α2-AR agonist, UK-14,304, decreased NE levels from baseline. Xu et al. (2000) observed that NET KO mice exhibited a sensitivity to increased locomotor effects of acute cocaine and amphetamine exposure. Additionally, in the vBNST of NET KO mice, NE reuptake was at least 6 times slower, and stimulated NE release was decreased by 60%. These changes in NE release an uptake dynamics in response to cocaine and amphetamine exposure produced a net increase in extracellular NE levels, likely contributing to the sensitization to stimulant effects (Xu et al., 2000). Macey et al. (2003) investigated the effects of cocaine self-administration on NET expression in the BNST of rhesus monkeys. In naïve monkeys, NET expression varied among BNST subnuclei, with the highest densities in the lateral dBNST and the vBNST. After 5 days of

cocaine self-administration, there was no significant difference in NET density; however, after 100 days of cocaine there was a significantly higher NET density in the lateral nuclei of the dBNST, and also in the vBNST (Macey et al., 2003). Ultimately, these studies show that NE levels throughout various BNST subregions are likely altered in response to drug administration, but also that the resulting behavioral effects of drugs may be mediated by the dynamics of NE release and uptake.

Looking at how amphetamine affects BNST neuronal activation in rats, Colussi-Mas et al. (2005) observed that a subcutaneous injection of amphetamine led to a dense cluster of c-fos+ cells in the BNSTov, and activated cells were also scattered throughout the rest of the dBNST and the vBNST (Colussi-Mas et al., 2005). The authors also observed a dense presence of dopamine beta-hydroxylase (DBH)+ fibers covering C-fos+ cells in the vBNST; however, the BNSTov had almost no DBH+ fibers and the rest of the dBNST had a moderate amount (Colussi-Mas et al., 2005). These findings suggest that amphetamine activates BNST neurons, and NE may interact with amphetamine-sensitive BNST neurons particularly in the vBNST and dBNST neurons outside of the BNSTov. Further, both systemic and intra-BNST administration of timolol (β1/β2-AR antagonist) blocked behavioral sensitization to amphetamine (Colussi-Mas et al., 2005). Overall, these results suggest that amphetamine has different effects on specific BNST subregions, with significant impacts on cell activation in the BNSTov of the dBNST. Since the BNSTov is a subregion with dense CRF-neurons, CRF might be involved in this process, although it is difficult to say since the cell types were not identified. Additionally, β-ARs in the BNST likely play a significant role in the development of amphetamine-induced behavioral sensitization, and ultimately may modulate some of the addictive properties of amphetamine (Colussi-Mas et al., 2005).

In the context of substance use disorders, cocaine has been the focus of most studies investigating the BNST NE's cellular and behavioral effects independently of CRF or stress

(including withdrawal). Wenzel et al. (2014) looked at the effects of blocking vBNST β-ARs in rats on anxiogenic effects of cocaine. They used two measures of cocaine-associated anxiety-like behavior to assess anxiogenic effects: the runway self-administration test using self-administration of cocaine, and place conditioning, pairing rewarding effects of cocaine with one chamber (CPP) and negative effects with another (CPA). Intra-vBNST infusion of betaxolol and ICI 118,551 (β1-AR and β2-AR antagonists, respectively) during runway administration and place conditioning significantly reduced anxiety-like behavior in both tests (Wenzel et al., 2014). Ultimately, these results suggest that vBNST β-ARs play a role in anxiety-like behaviors associated with cocaine use. Wee et al. (2008) demonstrated that rats given long access (6 hours) to cocaine self-administration displayed a significantly higher break-point for cocaine self-administration in a progressive ratio schedule than rats given short access (1 hour). Further behavioral experiments uncovered a likely role for the α1-AR, but not the α2-AR or β1-AR, in modulating cocaine self-administration in a progressive ratio schedule in long-access rats (Wee et al., 2008). The authors also found that α1-AR expression levels in the dBNST and vBNST are significantly decreased in response to long-access to cocaine exposure (Wee et al., 2008). Overall, these results suggest that BNST α1-ARs might play a role in this behavioral response; however, similar cocaine-α1-AR interactions are not always seen in mice. In naïve mice, methoxamine (α1-AR agonist) induced LTD in the dlBNST and the vlBNST, but in mice exposed to an acute injection of cocaine, the ability of methoxamine to evoke LTD was unchanged (McElligott and Winder, 2008). This suggests that acute cocaine exposure does not alter α1-AR mediated LTD. Collectively, these studies mainly demonstrate that cocaine exposure affects noradrenergic signaling in the BNST, influencing anxiety-like behaviors via β-ARs and self-administration behaviors via α1-ARs, although acute cocaine exposure does not affect α1-AR mediated LTD.

Studies examining cocaine interactions with α2-ARs in the BNST have been mixed. Yohimbine is often used as a pharmacologic stressor to induce reinstatement of drug-seeking behavior and this is thought to occur via α2-AR antagonism and increases in extracellular NE (Shaham et al., 2000). While some studies initially showed that yohimbine can impair extinction of cocaine-associated CPP, these studies also showed that other α2-AR antagonists like atipamezole did not modulate CPP behaviors (Davis et al., 2008). Interestingly, a later study found evidence that yohimbine may be acting via the orexin 1 receptor to impair cocaine CPP extinction and elicit depression of excitatory transmission in the dlBNST (Conrad et al., 2013). More recent studies demonstrated that knocking out $\alpha 2_A$-ARs completely, or selectively on non-noradrenergic neurons (heteroreceptors), did not alter the acquisition or extinction process to cocaine CPP, but did alter reinstatement behaviors (Perez et al., 2020). Overall, α2-AR mediated signaling in models of substance use disorders is complex based on the ability of α2-ARs to modulate NE signaling via autoreceptor function and glutamatergic transmission via heteroreceptor populations.

Overall, there is significant evidence to support the claim that BNST NE is highly implicated in both cellular and behavioral responses to misused drugs. Studies show that NE levels and dynamics within the BNST are associated with various drug-related behavioral responses. Additionally, subregional differences in adrenergic receptor expression and response to drug administration are something to be further researched. Lastly, most studies have focused on the use of cocaine and other stimulants when looking solely at NE signaling in the BNST, so more studies on other drugs such as opioids and alcohol would be beneficial. Many studies have examined BNST NE signaling in the context of CRF modulation which will be discussed in Section 7. Summaries of studies in this section involving c-fos expression and behavior can be found in Table 1-4 and Table 1-5, respectively. In the next section BNST NE in the context of stress combined with drug use will be discussed.

6.3 NE Signaling in the BNST Related to Stress with Alcohol or Drug Use

In this section we will discuss how stress and drug exposure affect BNST NE signaling, and some proposed mechanisms by which NE modulates stress-related drug-seeking behaviors. Additionally, noradrenergic signaling in the BNST in relation to withdrawal will be discussed. The major drugs discussed here are morphine and cocaine, since they are the most well-studied in the context of stress paired with drug use and BNST NE specifically.

Multiple studies have investigated BNST NE signaling in the context of morphine withdrawal and stress-related morphine use. Fuentealba et al. (2000) found that naloxone-precipitated withdrawal further increased vBNST NE from elevated levels observed following chronic morphine exposure, and intra-vBNST UK-14,304 (α2-AR agonist) infusion significantly decreased vBNST NE levels in both groups (Fuentealba et al., 2000). Studies exploring morphine withdrawal have also identified an important role for BNST β-ARs and α-ARs in morphine-withdrawal-associated CPA and physical withdrawal symptoms. CPA associated with naltrexone-precipitated withdrawal in morphine-dependent rats was blocked with intra-BNST microinjections of β1/β2-AR antagonists or an α2-AR agonist when administered prior to conditioning (Delfs et al., 2000). Additionally, intra-BNST injections of the β-AR antagonists or an α2-AR agonist also decreased some signs of physical withdrawal (Delfs et al., 2000). Similar results were observed by Aston-Jones et al. (1999), when intra-BNST injection of β-AR antagonists dose-dependently reduced CPA and frequency of withdrawal behaviors (Aston-Jones et al., 1999). Propranolol (β-AR antagonist) administration also attenuated increases in c-fos expression in the dBNST and vBNST associated with naltrexone-induced withdrawal (Aston-Jones et al., 1999). Additionally, microinjection of clonidine (α2-AR agonist) into the BNST was able to attenuate continued footshock stress-induced morphine CPP, and was also able to block footshock-induced reinstatement to morphine CPP (Wang et al., 2001). Taken together, these

results highlight a role for BNST adrenergic receptors in morphine-withdrawal-induced CPA, behavioral symptoms of withdrawal, stress-induced maintenance of morphine CPP, and stress-induced relapse to morphine, although better delineation of BNST subregion specificity for these behaviors is still needed.

It is important to consider strain differences when studying stress- and drug-related BNST NE signaling. For example, Fox et al. (2015) observed differences in the way morphine withdrawal affected NE in the vBNST of Sprague-Dawley and Wistar-Kyoto rats. In Sprague-Dawley rats, a strain with fewer anxiety-like characteristics than the Wistar-Kyoto rats, they showed that morphine withdrawal led to a downregulation of vBNST NE uptake and decreased $\alpha 2_A$-AR autoreceptor regulation (Fox et al., 2015). Wistar-Kyoto rats, a strain shown to have a more depressive-like phenotype and increased HPA axis function compared to Sprague Dawley rats (Carr and Lucki, 2010; Pardon et al., 2002), only exhibited decreased $\alpha 2_A$-AR function with no effect on NE uptake (Fox et al., 2015). This observed strain difference in vBNST NE modulation during morphine withdrawal is an interesting observation, and highlights the need to conduct further studies in different strains to understand how withdrawal may affect their noradrenergic response differently.

Cocaine withdrawal and reinstatement have also been shown to involve BNST NE signaling, and multiple studies have explored the role of β-ARs in this mechanism. Brown et al. (2009) reported that intracerebroventricular (icv) administration of NE was able to induce reinstatement to cocaine-seeking in rats. Following up with that study, Brown et al. (2011) assessed c-fos mRNA expression in the dBNST and vBNST in drug-naïve rats in response to icv NE administration at the same doses used to produce reinstatement, and they found that there was an increase in c-fos mRNA 45-minutes post-injection in the dBNST but no change in the vBNST. It is possible that the lack of change in the vBNST, however, may be due to the rats being drug-naïve and stress-naïve at the time of NE administration (Brown et al., 2011). Systemic

administration of ICI-118,551 (β2-AR antagonist) (Vranjkovic et al., 2012; McReynolds et al., 2014) or betaxolol (β1-AR antagonist) (Vranjkovic et al., 2012) blocked forced swim stress (FSS)-induced reinstatement to cocaine CPP, and clenbuterol injection (β2-AR agonist) was able to reinstate cocaine CPP on its own without a stressor (Vranjkovic et al., 2012). This suggests that the β2-AR is involved in mediating reinstatement behavior. Leri et al. (2002) observed that intra-BNST infusion of betaxolol and ICI-118,551 together decreased footshock stress-induced reinstatement to cocaine. Interestingly, these infusions were unable to block cocaine-induced reinstatement behavior, suggesting the importance of BNST β-ARs in stress-related cues in reinstatement as opposed to drug-induced reinstatement (Leri et al., 2002). Looking at the vBNST specifically, intra-vBNST microinjection of ICI-118,551 blocked footshock-induced reinstatement while betaxolol did not, indicating that stress-induced reinstatement is mediated by β2-ARs, but not β1-ARs in the vBNST (Vranjkovic et al., 2014). Further, intra-vBNST injection of clenbuterol (β2-AR agonist) was able to induce reinstatement on its own, but injection of dobutamine (β1-AR agonist) could not (Vranjkovic et al., 2014). These results indicate that in the vBNST, β2-AR activation is necessary and sufficient to reinstate cocaine-seeking behavior, and this system is necessary for stress to reinstate cocaine-seeking behaviors.

Perez et al. (2020) investigated the role of $\alpha 2_A$-ARs in stress-induced reinstatement. They found that $\alpha 2_A$-AR heteroreceptors, as opposed to $\alpha 2_A$-AR autoreceptors, are the specific $\alpha 2_A$-ARs mediating forced swim stress-induced reinstatement of cocaine CPP (Perez et al., 2020). This group previously observed that 1mg/kg guanfacine treatment increased c-fos-reported dBNST activity (Harris et al., 2018). Although this high dose of guanfacine has the potential to block stress-induced reinstatement, it also leads to sedation; therefore, the authors determined it was beneficial to investigate the effects of a lower dosage of guanfacine (Perez et al., 2020). When mice were administered a low dose of guanfacine shown to not directly activate dBNST neurons, stress-induced reinstatement was blocked (Perez et al., 2020). Additionally,

chemogenetically activating G_i-coupled receptors on dBNST cells that express CaMKIIa (to model $\alpha2_A$-AR heteroreceptor signaling) was enough to reinstate the cocaine CPP behavior (Perez et al., 2020). These findings suggest that $\alpha2_A$-AR autoreceptors and heteroreceptors may have opposing effects in the dBNST, with autoreceptors preventing reinstatement by decreasing the amount of NE released, whereas activation of heteroreceptors may encourage reinstatement (Perez et al., 2020). Overall, it can be concluded that $\alpha2_A$-AR heteroreceptors play a significant role in stress-induced reinstatement to cocaine CPP.

The studies discussed in this section reinforce the idea that BNST NE levels are sensitive to drug use and withdrawal or other stressors, and noradrenergic signaling through both α-ARs and β-ARs is important for the maintenance of CPP, CPA, and stress-induced reinstatement to opioids and stimulants. It is important, however, to gain more insight into how BNST subregions and rodent strains may differ in their NE responses to stress and drug use. Additionally, exploring other classes of drugs in this context is necessary. Results from this section are included in Table 1-6 (c-fos expression) and Table 1-7 (behavioral observations). The interaction between NE and CRF in the BNST and the role that this interaction plays in stress-induced reinstatement will be discussed in the following sections.

7. Interaction Between NE and CRF Signaling in the BNST

NE and CRF are highly expressed and active within the BNST, as previous sections have detailed. We previously described each of their roles separately in the BNST and how each interacts with stress and various aspects of alcohol and substance use disorder. This section will review the interactions between NE and CRF signaling in the BNST, and later sections will discuss how these interactions influence, and are influenced by, stress and drug exposure.

A few studies have examined the interactions between NE and CRF signaling in the BNST using electrophysiological methods. In the dlBNST, Nobis et al. (2011) demonstrated that bath application of isoproterenol (non-selective β-AR agonist) increased sEPSC frequency but not amplitude via a β1-AR-specific mechanism, and further results showed that isoproterenol acts via a presynaptic glutamatergic mechanism. Application of the CRFR1-specific antagonist NBI27914 inhibited the isoproterenol-induced increase in sEPSC frequency, indicating that the β1-AR mediated effect requires functional CRFR1 (Nobis et al., 2011). Conversely, when urocortin (CRFR agonist) was applied, an increase in sEPSC frequency was observed, but betaxolol (β1-AR antagonist) was unable to block this increase (Nobis et al., 2011). This finding is extremely interesting since it indicates that while the noradrenergic-mediated increase in sEPSCs requires CRFR1 function, CRF signaling does not require intact β1-AR signaling to produce its effects. Ultimately, this suggests that CRF signaling is downstream from β1-AR activation in the dlBNST circuit mediating the increase in sEPSCs. Nobis et al. (2011) also determined that isoproterenol and dopamine modulate different glutamatergic synapses, although their actions to increase sEPSC frequency are both mediated by CRFR1.

Electrophysiological studies have also examined the effects of NE signaling in the dBNST on CRF neurons specifically. For example, bath application of isoproterenol, a non-selective β-AR agonist, led to a significant depolarization of dBNST CRF neurons (Silberman et al., 2013). Additionally, Fetterly et al. (2019) recorded eEPSCs from dBNST CRF cells and found that bath application of NE, methoxamine (α1-AR agonist), or isoproterenol (β-AR agonist) inhibited EPSC amplitude, but it did not alter the PPR. Guanfacine ($\alpha 2_A$-AR agonist) also inhibited eEPSC amplitude; however, unlike the α1-AR or β-AR agonists, it increased the PPR, which points to a presynaptic mechanism. The authors also found that blocking the function of $\alpha 2_A$-ARs completely blocks NE's inhibitory effects on excitatory signaling on CRF neurons in the dBNST (Fetterly et al., 2019). Using c-fos as a measure of neuronal activation, the authors

also saw that an injection of guanfacine decreased the percentage of dBNST CRF neurons expressing c-fos (Fetterly et al., 2019). Additionally, they observed that a set of excitatory inputs onto dBNST CRF neurons originate from the parabrachial nucleus (PBN), and ultimately determined that NE can inhibit EPSCs in CRF neurons when the PBN afferents are stimulated. Further, guanfacine (the $\alpha 2_A$-AR agonist) produced similar effects to NE, suggesting that this result is $\alpha 2_A$-AR mediated. Lastly, inhibiting PBN inputs led to a reduced eEPSC amplitude in dBNST CRF neurons (Fetterly et al., 2019). Taken together, the results from these studies suggest that NE modulates PBN excitatory inputs onto CRF neurons in the dlBNST while also directly activating dBNST CRF neurons.

The above studies emphasize the dynamics between NE and CRF signaling in the BNST. For example, CRFR1 activation in the dlBNST is likely within a circuit downstream from β1-AR activation that increases sEPSC frequency. Additionally, NE has an excitatory effect on dBNST CRF neurons, and also mediates excitatory signaling from the PBN. The studies focusing on CRF and NE signaling in the BNST have largely targeted the dBNST, however, so more research needs to focus on other subregions as well.

7.1 NE and CRF Signaling Interactions in the BNST Related to Stress

NE and CRF are both critical molecules in the physiologic stress response and their activity is altered in response to stress. In this section we will discuss the interaction between CRF and NE in the BNST after stress exposure.

Exposure to stressors affects future NE and CRF function in the BNST. In one study, rats were given intra-dBNST infusions of either NE or CRF for three days and prepulse inhibition (PPI) was measured. Next, rats were then given a “challenge” dose of either NE or CRF (whichever one they had not been infused with initially) and PPI was measured again. During the

first three days, each intra-dBNST infusion of NE lowered PPI. In the rats with repeated NE exposure, the CRF "challenge" dose decreased PPI as well. Conversely, CRF infusions for rats in the initial trials did not affect PPI, and administration of the "challenge" dose of NE on a later test day also had no effect (Rajbhandari and Bakshi, 2020). These results suggest that NE release in the dBNST, perhaps in response to a stressor, can change the ability for dBNST CRFR activation to affect PPI (Rajbhandari and Bakshi, 2020). Interestingly, adrenalectomy in adult male rats resulted in increased extracellular NE levels in the lateral BNST (Santibanez et al., 2005). However, adrenalectomy decreased CRF-like immunoreactivity in the dlBNST and vlBNST, and the number of cells expressing CRF mRNA in the dlBNST was also decreased (Santibañez et al., 2005). This suggests that glucocorticoids have different effects on the CRF system than they do on extracellular BNST NE levels. Further examination into how extracellular NE and glucocorticoids interact with BNST CRF neurons in acute and chronic stress will be of interest in future studies.

Manipulation of the NE system has also been shown to affect stress-related dBNST CRF neuron activation. For example, following an acute restraint stress, c-fos expression significantly increased in dBNST CRF neurons of mice, an effect which was attenuated when the mice were injected with guanfacine (an $\alpha 2_A$-AR agonist) prior to restraint. Additionally, activating $\alpha 2_A$-ARs decreased the percentage of dBNST CRF neurons expressing c-fos in both non-stress-exposed and stress-exposed mice (Fetterly et al., 2019). Related to the findings of Harris et al. (2018), Fetterly et al. (2019) also found that guanfacine increased c-fos expression in non-CRF cells. These experiments demonstrate an interaction between noradrenergic signaling and CRF neuron activation in the dBNST but also how noradrenergic signaling may modulate other neuronal populations in the dBNST that may further impact CRF neuron activity. Additionally, chemogenetic inhibition of PBN inputs to the BNST prior to restraint stress mimicked the effect of guanfacine to reduce BNST CRF neuron c-fos expression (Fetterly et al., 2019). This suggests

that the noradrenergic regulation of CRF-cell activation during stress may be at least partially mediated by activation of $\alpha 2_A$-ARs on the terminals of PBN-dBNST projections.

The ability of β-AR agonists to depolarize CRF neurons in the dBNST (Silberman et al., 2013) and the impact of acute stress on this response was further examined in Snyder et al. (2019). Snyder et al. (2019) showed that NE could depolarize CRF neurons and that this occurred via a β-AR dependent mechanism. dBNST CRF neurons in mice exposed to acute restraint stress had a significantly elevated resting membrane potential (RMP), and NE was unable to further depolarize these cells as it was in naïve mice, suggesting NE-induced depolarization of dBNST CRF neurons had already occurred *in vivo* (Snyder et al., 2019). Further, NE was still able to inhibit eEPSC amplitude in the CRF cells from naïve and restraint stress-exposed mice (Snyder et al., 2019), a process which had been shown to be α-AR dependent (Fetterly et al., 2019). Overall, restraint stress occluded NE's β-AR mediated effects to depolarize dBNST CRF neurons, but did not alter α-AR dependent inhibitory mechanisms (Fetterly et al., 2019), suggesting complementary roles of β-AR and α-AR signaling in controlling dBNST CRF neuron activity following acute stress. Further work examining the consequences of chronic stress on β-AR and α-AR modulation of dBNST CRF neurons and any subregional differences in such effects should be explored in future studies. Results from this section measuring c-fos or behavior are included in summary Table 1-2 and Table 1-3, respectively.

7.2 NE and CRF Signaling Interactions in the BNST Related to Alcohol or Drug Use

In this section, the interaction between CRF and NE signaling in the BNST will be discussed in the context of drug administration without stress or withdrawal. There is only one study detailed here, because most research focusing on BNST NE and CRF interaction are often in the context of stress/withdrawal modulation of drug-seeking behaviors. It is important that the

interaction between NE-CRF in the absence of stress is highlighted in order to completely understand how NE-CRF interactions are potentially altered in alcohol and substance use disorders.

Nobis et al. (2011) found differences in dlBNST neurons' response to noradrenergic or CRF signaling after chronic cocaine exposure. Shortly after the final injection, electrophysiological recordings were taken. Basal sEPSC frequency did not differ between cocaine-injected and saline-injected groups; however, each group differed in their response to bath application of urocortin 1 (CRFR agonist) and isoproterenol (β-AR agonist). dlBNST neurons from saline-injected mice displayed an increase in sEPSC frequency during application of urocortin or isoproterenol. Conversely, dlBNST neurons from cocaine-injected mice were not responsive to either agonist (Nobis et al., 2011). Additionally, a one-time cocaine injection, however, did not block the ability of urocortin or isoproterenol to increase sEPSC frequency (Nobis et al., 2011). These results indicate that chronic cocaine exposure affects dlBNST neuron responsiveness to CRFR or β-AR activation. Interestingly, the effect of chronic cocaine exposure was reversed after 10 days of withdrawal (Nobis et al., 2011), but withdrawal will be discussed in the next section. Overall, these findings are unique in that they examine impact of cocaine on BNST circuits shortly after administration. Understanding the differences between effects of drug administration in the short-term and after long-term withdrawal may uncover novel mechanisms not previously explored.

7.3 NE and CRF Signaling Interactions in the BNST Related to Stress with Alcohol or Drug Use

CRF and NE in the BNST play a substantial role in responses to drugs and stress. In this section, the interaction between the two in the context of stress and alcohol or drug use will be discussed. This section includes discussion on withdrawal as well as stress-induced reinstatement.

When investigating the effects of alcohol on NE and CRF in the BNST in mice, 4hr withdrawal, but not 4-day withdrawal, from chronic intermittent ethanol (CIE) exposure led to a significantly more depolarized RMP in dBNST CRF neurons than naïve animals (Snyder et al., 2019). Although NE was able to depolarize dBNST CRF neurons from naïve mice in a β-AR dependent manner, this effect was occluded after 4hr withdrawal from CIE, suggesting that NE release and subsequent depolarization of BNST CRF neurons had occurred during that 4hr withdrawal period. After 4-day withdrawal, however, NE was able to depolarize the dBNST CRF neurons in the same way as naïve mice. Further, NE was able to inhibit eEPSC amplitude in the CRF cells in naive and CIE-exposed mice (Snyder et al., 2019), a mechanism which was previously shown to be α-AR dependent (Fetterly et al., 2019). Taken together, these results indicate that 4hr withdrawal from CIE occluded NE's β-AR mediated effects to depolarize dBNST CRF neurons, but did not change the α-AR mediated ability of NE to decrease eEPSC amplitude.

Most of the other studies looking at stress and drugs with NE and CRF focus on cocaine. Using electrophysiology, Nobis et al. (2011) demonstrated that chronic cocaine exposure, but not a one-time cocaine injection, prevented the ability for a CRFR agonist or β-AR agonist to increase sEPSC frequency in dlBNST neurons in mice. After 10 days of withdrawal from cocaine exposure, this effect of chronic cocaine administration was reversed (Nobis et al., 2011). A "challenge" injection of cocaine 10-days into withdrawal, however, was able to trigger the same physiological observations seen immediately after the chronic cocaine administration (Nobis et

al., 2011). This result suggests that although the physiology of neurons in the dlBNST seemingly returned to baseline during abstinence, chronic cocaine administration may prime the dlBNST to reenter the physiological state after one acute exposure during abstinence from chronic cocaine use. This provides insight into important circuitry changes that may make an individual more susceptible to subsequent drug use after abstinence.

Multiple studies highlight the interaction between NE and CRF signaling in cocaine-related reinstatement behaviors. In previously-described experiments, it was demonstrated that blocking β2-AR function (Vranjkovic et al., 2012; McReynolds et al., 2014) or CRFR1 function attenuates FSS-induced reinstatement to cocaine CPP (McReynolds et al., 2014), and intra-vBNST β2-AR antagonism blocked footshock-induced reinstatement to cocaine-seeking (Vranjkovic et al., 2014). It was also demonstrated that systemic (Vranjkovic et al., 2012) and intra-vBNST (Vranjkovic et al., 2014) clenbuterol (β2-AR agonist) injection led to reinstatement of cocaine-associated behaviors. Interestingly, systemic (Vranjkovic et al., 2012) or intra-vBNST (Vranjkovic et al., 2014) administration of antalarmin (CRFR1 antagonist) prior to clenbuterol blocked the ability of the β2-AR agonist to induce reinstatement (McReynolds et al., 2014). These results indicate that reinstatement via β2-AR is dependent upon the availability of functional CRFR1 receptors. Additionally, pretreatment with a β2-AR antagonist blocked FSS-induced increase in CRF expression in the BNST of mice previously exposed to cocaine (McReynolds et al., 2014). Collectively, these results uncover the importance of β2-ARs in regulating BNST CRF expression during cocaine withdrawal and subsequent FSS exposure, as well as the necessity of CRFR1 function for β2-AR activation to produce cocaine reinstatement. Lastly, Vranjkovic et al. (2014) determined that vBNST β2-ARs regulate VTA-projecting vBNST CRF neurons, thereby modulating CRF release in the VTA, and these projections are important for stress-induced reinstatement to cocaine (Vranjkovic et al., 2014).

Overall, these studies highlight two dynamics: one dynamic between noradrenergic signaling and CRF, and another between α-AR and β-AR functions in stress and drug use. While both α-ARs and β-ARs play a role in the stress response, in stress-induced reinstatement it appears that β-ARs, particularly β2-ARs in the vBNST, predominate. Additionally, the effects of NE in many of these studies were shown to be dependent upon CRFR function, while CRF may be able to exert its effects without intact β-AR function. These findings suggest that CRF neurons sit downstream of NE signaling and more specifically that β-ARs on CRF neurons are a critical target for NE signaling in stress-induced reinstatement behaviors. More studies need to be done investigating the interactions between CRF and NE in stress-related drug behaviors, particularly in drugs other than cocaine. Experiments from this section measuring c-fos or behavioral tests are included in Table 1-6 and Table 1-7.

8. Summary

The most recent SAMHSA National Survey on Drug Use and Health (2019) reported that Substance Use Disorder affects 20.4 million individuals in the United States, and of those 14.5 million suffer from Alcohol Use Disorder. Stress is a common trigger to drug use and relapse (Sinha 2008), and two substrates of the stress response are corticotropin releasing factor (CRF) and norepinephrine (NE). The BNST is a limbic brain region that has been highly implicated in stress as well as drug use disorders, and CRF and NE modulate activity within this region in independent ways, but also interact with one another. In this review we summarized current literature focusing on NE and CRF signaling in the BNST and how stress, substance use, or both combined interact to perpetuate or suppress cellular and behavioral changes. In general, stress, alcohol, or drug exposure tend to increase NE levels in the BNST, but CRF expression changes are much more variable. Studies demonstrated that stress and/or drug exposure can produce

BNST subregion-specific effects, with most studies investigating the dBNST subregion (see **Figure 1-2** and **Tables 1-2** through **1-7**). Further, some have studied the effects of cocaine on NE and CRF function together in the BNST (Nobis et al., 2011; McReynolds et al., 2014; Vranjkovic et al., 2014), but additional studies investigating alcohol and other drugs on the interactions of these stress systems are needed. Additionally, a few CRF-related sex differences were observed (Fetterly et al., 2019; Connelly and Unterwald, 2020); however, despite sex-specific anatomical differences in the BNST (Guillamón et al., 1988; Funabashi et al., 2004; Uchida et al., 2019) few

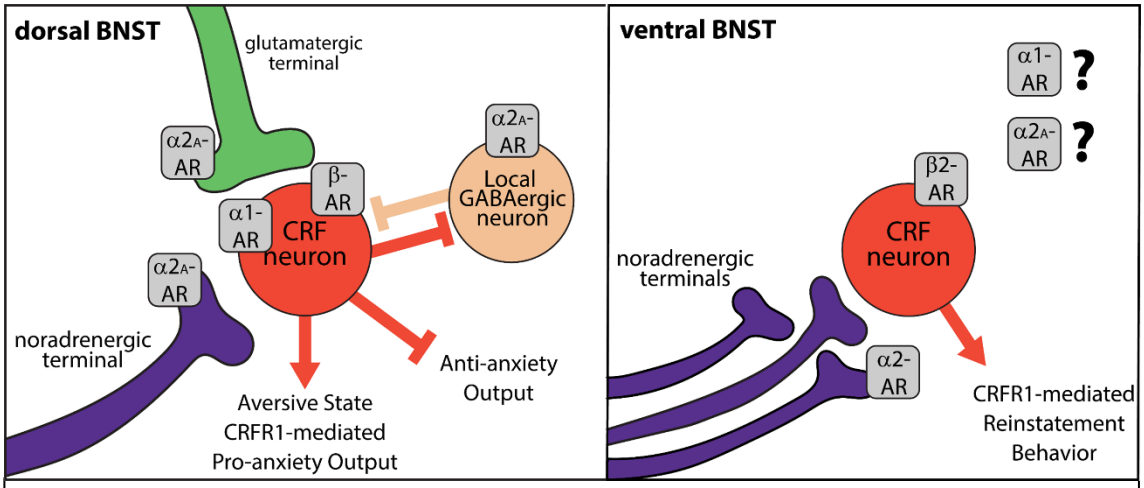

Figure 1-2. Summary model of potential targets for stress and drug interactions within dorsal and ventral BNST noradrenergic and corticotropin releasing factor circuits. In general, stress and drug or alcohol exposure are thought to increase norepinephrine (NE) release in the BNST. Evoked NE release is greater in the ventral (vBNST) than dorsal (dBNST) subregions, which may be due to a greater density of noradrenergic inputs into the vBNST. Stress increases dBNST corticotropin releasing factor (CRF) neuron activation via NE signaling, and NE activates CRF neurons via β-ARs and α1-ARs after stress exposure or during acute drug withdrawal. Activation of CRF neurons in the dBNST reliably produces aversive and anxiety-like behaviors, and may inhibit anxiolytic output. In both the dBNST and vBNST α2A-AR autoreceptors are likely located on noradrenergic terminals and modulate incoming NE signaling. In the dBNST, α2A-AR heteroreceptors are located on glutamatergic terminals, such as those from the parabrachial nucleus, to decrease glutamatergic transmission. α2A-ARs are also present on local GABAergic neurons, which may directly inhibit CRF neuron function and lead to a disinhibition of potential anti-anxiety circuits. In the vBNST, β2-ARs are critical for stress-induced reinstatement behaviors and exert their effects via downstream CRFR1 signaling, suggesting that β2-ARs are located on CRF neurons themselves. Electrophysiology studies reveal a role for vBNST α1 and α2-ARs in modulating noradrenergic, GABAergic, and glutamatergic signaling although their exact cellular location is currently unknown.

previous studies investigated both males and females. Overall, there is a need for more research to delineate NE- and CRF-mediated BNST subregion-specific effects in the context of multiple drug classes and for further research into sex differences.

It is important to think translationally about how these basic research findings may benefit the clinical population in the future, as there are few pharmacological treatments for SUDs. For example, there are only three FDA-approved drugs for treating AUD: disulfuram, naltrexone, and acamprosate (Akbar et al., 2018). Some drugs approved for other uses have potential as treatments, but none of these drugs target the CRF system and few target the noradrenergic system (Akbar et al., 2018). Further, the typically prescribed medication assisted treatments for opioid use disorder (naltrexone, methadone, and buprenorphine) act primarily in the opioid system. Unfortunately, despite a large amount of preclinical data supporting the efficacy of CRFR1 antagonists in modulating stress- and drug-associated behaviors, human studies evaluating short-term effects of CRFR1 antagonists on alcohol cravings in AUD patients or disorders associated with stress displayed few to no beneficial effects (Kwako et al., 2014; Schwandt et al., 2016; Spierling and Zorrilla, 2017). The reasons for this are multifaceted, however. Aside from compound toxicity and receptor binding kinetics, lack of efficacy may be partly attributed to the dynamics of stress within a particular disorder (Koob and Zorrilla, 2012), genetic variation, and sex differences; therefore, an individualized approach to treatment may be necessary (Spierling and Zorrilla, 2017). It is also difficult to measure real-world efficacy to reduce alcohol and drug taking in a controlled lab setting examining craving. Long-term effects with CRFR1 antagonist treatment on alcohol or drug taking in patients with and without additional treatments, such as cognitive behavioral therapy, would still be important to study. Pharmacological treatments targeting the noradrenergic system have shown better promise, however they are not entirely efficacious and may also depend upon the individual. In individuals with cocaine use disorder, daily guanfacine (α2-AR agonist) treatment for three weeks was able

to decrease cocaine and alcohol cravings, anxiety, and other negative emotions in female participants but not male participants (Fox et al., 2014). One single guanfacine treatment, however, did not alter stress-reactivity or drug craving in individuals with cocaine use disorder (Moran-Santa Maria et al., 2014). Prazosin (α1-AR antagonist) has been relatively well-studied in AUD, and shows some promise in treatment. For example, in individuals with AUD, prazosin treatment decreased stress-reactivity and cravings (Fox et al., 2011), and decreased the number of drinks consumed per week (Simpson et al., 2009; Wilcox et al., 2018). Speaking to individual differences, recent data show that individuals with AUD's brain activation and subjective anxiety levels predicted the success of prazosin treatment (Wilcox et al., 2020). Given that intra-BNST administration of NE made rodents more susceptible to the effects of CRF (Rajbhandari and Bakshi, 2020), it may be important to administer both CRFR1 antagonist and AR-targeted drugs together in order to see the desired effects on substance cravings and relapse. For example, blocking β-ARs may block the enhancement of CRF signaling seen with stress, and blocking the CRFR1s could prevent withdrawal-associated sensitivity. There have also been some promising clinical results using deep brain stimulation (DBS) of the nucleus accumbens as a treatment for SUDs (Hassan et al., 2020), but more research must be done to investigate the efficacy and necessity of this invasive procedure. Perhaps targeting the BNST, and even specific BNST subregions, with DBS could help treat SUDs, although development of a pharmacological approach is more desirable.

Ultimately, more research must be done to identify the right treatment plan, including dosage and timing of administration, as well as combinations of treatments, and this is likely to need to be individualized based upon different characteristics of a person's substance use disorder and may be sex-specific. Further research into BNST CRF and NE signaling, and its involvement in stress-related disorders and substance use disorders will provide valuable insight into the

specific targets and clinical treatment plans when treating stress-associated and substance use disorders.

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Chapter 2
Chronic intermittent ethanol and acute stress similarly modulate BNST CRF neuron activity via noradrenergic signaling

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Abstract

Background: Relapse is a critical barrier to effective long-term treatment of alcoholism, and stress is often cited as a key trigger to relapse. Numerous studies suggest that stress-induced reinstatement to drug seeking behaviors is mediated by norepinephrine (NE) and corticotropin releasing factor (CRF) signaling interactions in the bed nucleus of the stria terminalis (BNST), a brain region critical to many behavioral and physiologic responses to stressors. Here we sought to directly examine the effects of NE on BNST CRF neuron activity and determine if these effects may be modulated by chronic intermittent EtOH (CIE) exposure or a single restraint stress.

Methods: Adult, male CRF-*tomato* reporter mice were either treatment naïve, exposed to CIE for two weeks, or exposed to a single 1hr restraint stress. Effects of application of exogenous NE on BNST CRF neuron activity was assessed via whole-cell patch clamp electrophysiological techniques.

Results: We found that NE depolarized BNST CRF neurons in naïve mice in a β-adrenergic receptor (AR) dependent mechanism. CRF neurons from CIE or stress-exposed mice had significantly elevated basal resting membrane potential compared to naïve mice. Furthermore, CIE and stress individually disrupted the ability of NE to depolarize CRF neurons, suggesting that both stress and CIE utilize β-AR signaling to modulate BNST CRF neurons. Neither stress nor CIE altered the ability of exogenous NE to inhibit evoked glutamatergic transmission onto BNST CRF neurons as shown in naïve mice, a mechanism previously shown to be α-AR dependent.

Conclusions: Altogether these findings suggest that stress and CIE interact with β-AR signaling to modulate BNST CRF neuron activity, potentially disrupting the α/β-AR balance of

BNST CRF neuronal excitability. Restoration of α/β-AR balance may lead to novel therapies for the alleviation of many stress-related disorders.

Key words: BNST, adrenergic receptors, CRF, norepinephrine, ethanol

Introduction

The bed nucleus of the stria terminalis (BNST) plays a critical role in the behavioral and physiologic responses to stress (Casada and Dafny, 1991; Crestani et al., 2013; Sullivan et al., 2004; Tran et al., 2012; Waddell et al., 2006). In particular, corticotropin releasing factor (CRF) signaling is thought to be an important regulator of pro-anxiety BNST-mediated responses to stress (Ciccocioppo et al., 2003; Koob, 1999; Sink et al., 2013; Walker et al., 2009) and previous studies have shown that CRF signaling is enhanced in the BNST following acute and chronic stress exposures (Choi et al., 2006; Funk et al., 2006; Janitzky et al., 2014; Tran et al., 2012). In addition, previous studies indicate that stress or adverse external stimuli can increase norepinephrine (NE) release in the BNST (Flavin and Winder, 2013; Fuentealba et al., 2000; Pacak et al., 1995; Park et al., 2012). BNST NE signaling is critical for stress-related increases in drug-seeking behaviors (Brown et al., 2011; Leri et al., 2002; Wang et al., 2001), behaviors that require CRF receptor activation (Brown et al., 2009; Erb et al., 2001; McReynolds et al., 2014). Moreover, anatomic and electrophysiologic studies indicate that noradrenergic terminals interact with CRF producing neurons in the BNST (Nobis et al., 2011; Phelix et al., 1994), which together with the previously mentioned behavioral data suggests stress-induced NE release in the BNST likely increases CRF neuron activity to coordinate behavioral responses to stress. Furthermore, this circuitry is sensitive to modulation by chronic ethanol (EtOH) (Olive et al., 2002; Silberman et al., 2013). Together, these studies implicate NE modulation of BNST CRF neurons in anxiogenic behaviors and in stress-induced EtOH seeking behaviors. However, the mechanisms by which stress and chronic EtOH modulate BNST CRF neuron activity has not been fully elucidated.

To that end, the current study sought to directly examine the mechanism by which stress and chronic EtOH exposure alter BNST CRF neuron excitability. Our findings suggest that stress

and chronic EtOH exposure enhance BNST CRF neuron activity via similar β-AR dependent mechanisms. Surprisingly, stress and chronic EtOH do not appear to alter NE-induced inhibition of glutamatergic inputs onto BNST CRF neurons, an effect previously shown to be α-AR dependent (Fetterly et al., 2019). Together, these findings indicate that stress and chronic EtOH target the activity of β-ARs on BNST CRF neurons without altering α-AR modulation of these neurons, thereby altering the α/β-AR balance within this circuitry. These findings further suggest that maintaining α/β-AR balance in BNST CRF circuits may be an important target for novel treatments for stress-related disorders and stress-induced reinstatement to alcohol seeking behaviors.

Materials and Methods

Animals

A total of thirty adult (>7wk old) male CRF-*tomato* reporter mice (Silberman et al., 2013) were used in all studies. CRF neurons were identified for electrophysiological analysis through the use of CRF-*tomato* reporter mice as previously described (Silberman et al., 2013). The CRF-cre line utilized to produce the CRF-*tomato* mice in these studies has been extensively evaluated and reliably reports CRF mRNA-expressing neurons (Chen et al., 2015). All mice were housed in groups of two to five for the duration of the studies. Food and water were available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committees at Vanderbilt University (Nashville, TN) and Penn St College of Medicine (Hershey, PA).

Restraint stress exposure

Mice were allowed to acclimate to the test location in their home cage for one hour in a sound- and light-attenuating box (Med Associates Inc.). Mice were restrained during the light cycle (0900-1100) in restraint devices made from 50-mL Falcon conical tubes (Fisher Scientific) with several (≈15) holes in the front and rear (cap) to maintain airflow (McElligott et al., 2010). While in the restraint devices, animals were placed in individual cages inside sound- and light-attenuating boxes in the same room for 1 hour, and then returned to their home cage for 30 min prior to preparation for electrophysiology experiments.

Chronic Intermittent Ethanol (CIE)

CIE procedures were performed as previously published (Silberman et al., 2013). Briefly, mice were given a daily injection of pyrazole (1 mmol/kg), placed in a chamber filled with volatilized ethanol for 16 hours per day, then returned to standard animal housing for 8 hours. Mice were exposed to CIE for 4 days (one CIE cycle) followed by three days solely in standard animal housing before being returned to the chamber for a second CIE cycle. On the last day of the second CIE cycle, mice were returned to the standard animal housing facility for either 4 hours or 4 days before being used for electrophysiology.

Electrophysiology

250-300 μm-thick coronal brain slices containing the BNST (Bregma, +0.14–0.26) were prepared from adult male CRF-*tomato* mice as previously described (Kash et al., 2008; Nobis et

al., 2011). Following acclimation, mice were briefly anesthetized with isoflurane and brains were quickly removed and submerged in ice-cold, oxygenated low-sodium sucrose dissecting solution (in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$). A vibratome (Leica) was used to prepare the brain slices, which were then transferred to a holding chamber containing oxygenated ACSF at 28°C, and allowed to recover for at least one hour prior to recordings.

Whole-cell voltage-clamp recordings of AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) were made at -70 mV and pharmacologically isolated by the addition of 25 μM picrotoxin (Tocris) to the standard ACSF (in mM: 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10 glucose, and 26 NaHCO3). Recording electrodes for voltage-clamp experiments were filled with a cesium gluconate internal solution (in mM: 118 CsOH, 117 D-gluconic acid, 5 NaCl, 10 HEPES, 0.4 EGTA, 2 MgCl2, 5 tetraethylammonium chloride, 4 ATP, 0.3 GTP, pH 7.2–7.3, 270–290 mOsmol). A 50 msec interevent interval was used to examine paired-pulse ratio (PPR). Whole-cell current-clamp recordings were carried out at each neuron's resting membrane potential performed with the addition of 25 μM picrotoxin and 3 mM kynurenic acid to the ACSF (to block $GABA_A$ and AMPA/NMDA mediated neurotransmission, respectively) and recording electrodes were filled with a potassium gluconate internal solution (in mM: 135 K^+-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP, pH 7.2–7.3, 280–290 mOsmol). When used, the β-AR antagonist propranolol was pre-applied for at least 15 min before recordings began and remained onboard for the duration of the experiment. All electrophysiology recordings were made using Clampex 9.2 and analyzed using Clampfit 10.2-10.7 (Molecular Devices). Experiments in which the access resistance changed by >20% or were otherwise unstable were not included in the data analyses.

Statistical analyses

Statistical analyses were performed using Microsoft Excel 2011 and GraphPad Prism 7. To determine if a drug had a significant effect we compared the experimental values to baseline values using a Student's paired t-test. Unpaired t-tests were used to determine significant differences of a drug effect between two groups. Differences between three or more groups were assessed using one-way ANOVA followed by Fisher's LSD post-hoc test to determine the significance of a specific group compared to stress/EtOH-naïve control mice. All values throughout the study are presented as mean ± SEM. Significance was determined at the $p<0.05$ level for all analyses. Graphpad Prism 7 and Powerpoint 2011 were used for figure preparation.

Reagents

All reagents used were purchased from MilliporeSigma (Temecula, CA, USA) unless otherwise noted in the text.

Results

Norepinephrine depolarizes BNST CRF neurons via β-AR activation

Our previous findings indicate that β-AR agonists can depolarize BNST CRF neurons and increase excitatory neuronal transmission onto BNST projection neurons via a CRF-dependent mechanism (Silberman et al., 2013). These findings suggest that NE may be a critical

signaling component in regulating BNST CRF neuron excitability. Here, we sought to determine if NE may directly alter BNST CRF neuron excitability, and if this mechanism is sensitive to CIE or stress. To that end, we recorded from *tomato*+ neurons in the BNST of CRF-*tomato* reporter mice and measured neuronal resting membrane potential (RMP) in the presence of both GABA and glutamate receptor antagonists (25 μM picrotoxin and 3 mM kynurenic acid) to remove potential confounds of previously described NE modulation of BNST network activity (Flavin and Winder, 2013). Bath application of 1 μM NE significantly depolarized BNST CRF neurons compared to basal RMP (5.8±1.3 mV, n=12 cells from 8 mice, $p<0.005$, **Figure 2-1A-B**). The magnitude of NE-induced depolarization was significantly correlated with a decrease in input resistance ($r^2=0.8689$, $p<0.0001$, **Figure 2-1C**). In some cases, BNST CRF neurons that became depolarized began to spontaneously fire action potentials (**Figure 2-1Ai**, 3 of 12 cells tested). To test the hypothesis that β-AR stimulation is required for NE-induced depolarization of BNST CRF neurons, we pretreated slices with 10 μM propranolol, a β-AR antagonist, for a minimum of 15 minutes prior to recording BNST CRF neuron RMP. In the presence of propranolol pretreatment, NE did not significantly alter BNST CRF neuron RMP (0.17±1.2 mV change from baseline, n=6 cells from 3 mice; $p>0.05$ compared to baseline, **Figure 2-1D-E**). The ability of NE to depolarize BNST CRF neuron membrane potentials was significantly different in control vs propranolol treated cells ($p<0.01$, **Figure 2-1F**).

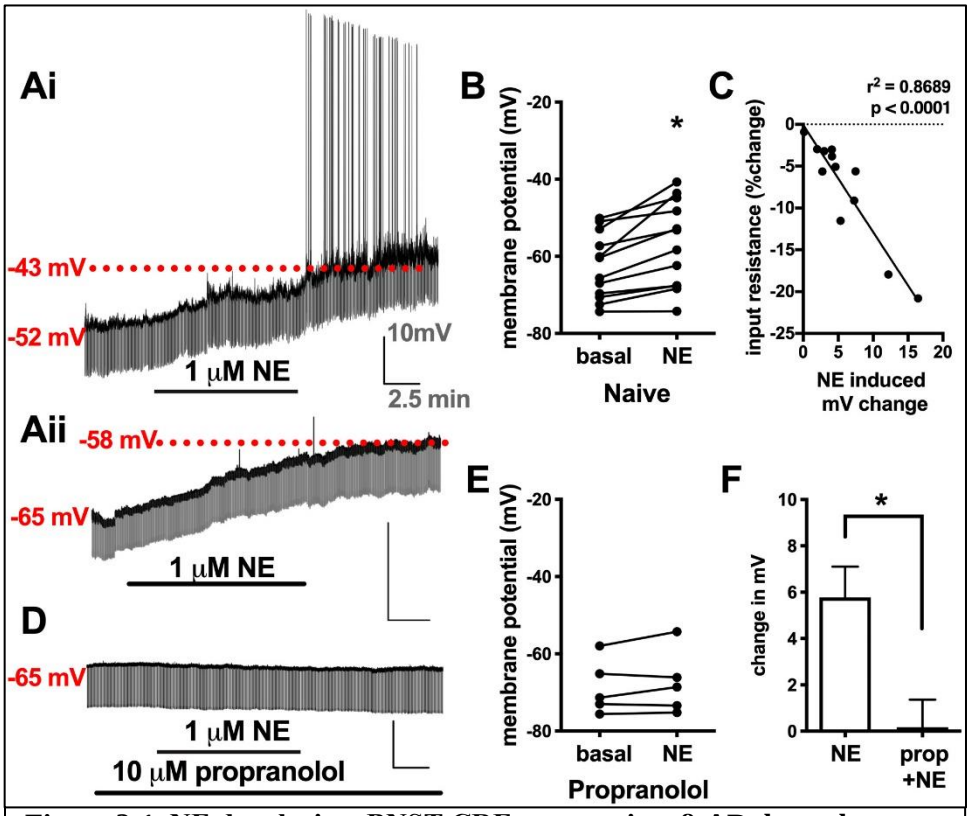

Figure 2-1. NE depolarizes BNST CRF neurons in a β-AR dependent manner. A) Example trace showing the effect of 1 μM NE on BNST CRF neuron membrane potential. Dark line indicates resting membrane potential, gray area indicates input resistance measurements. Numbers to the left indicate membrane potential (in mV) during baseline and after 10 min NE application. Solid line below trace indicates time of drug application. Scale bar: x-axis = 2.5 min; y-axis = 10 mV. Ai) shows example BNST CRF neuron that began firing action potentials following depolarization. Aii) shows example BNST CRF neuron that depolarized but did not fire action potentials. B) Line graph showing the effect of NE on BNST CRF neuron membrane potential compared to basal levels on individual neurons tested. * indicates a significant difference from basal ($p<0.05$). C) Analysis showing that magnitude of NE-induced depolarization is significantly correlated with larger changes to input resistance. Dashed line indicates input resistance normalized to 0% change. D) Example trace showing the effect of 1 μM NE on BNST CRF neuron membrane potential in slices pretreated with the β-AR antagonist propranolol. Dark line indicates resting membrane potential, gray area indicates input resistance measurements. Solid lines below trace indicate time of drug applications. Number to the left indicates membrane potential (in mV) during baseline. Scale bar: x-axis = 2.5 min; y-axis = 10 mV. E) Line graph showing the effect of propranolol pretreatment on NE modulation of BNST CRF neuron membrane potential on individual neurons tested. F) Bar graph summarizing the difference in NE effects on change in BNST CRF neuron membrane potentials with and without propranolol pretreatment. * indicates significant difference between groups ($p<0.05$).

Acute stress and short-term CIE withdrawal shift BNST CRF neuron RMP

Mice underwent CIE for two cycles and either acute withdrawal (4 hr) or extended withdrawal (4 days) prior to slice preparation. Recordings of BNST CRF neuron RMPs were performed as above. A separate cohort of mice was EtOH naïve but exposed to a 1 hr restraint stress before slice preparation as previously published (Fetterly et al., 2019). One-way ANOVA revealed significant effects of stress and CIE on BNST CRF neuron RMPs ($F_{(3,32)}$=3.068, p<0.05, **Figure 2-2A**) compared to cells from EtOH/stress naïve mice. Fisher's LSD post-hoc test showed a significant difference between naïve (RMP= -62.6±2.5 mV, n=12 cells from 8 mice) and stress-exposed mice (-54.4±4.0, n= 8 cells from 3 mice, p<0.01) and between naïve and 4 hr CIE withdrawal mice (-54.7±2.1, n= 10 cells from 5 mice, p<0.01), but no significant difference between naïve and 4 day CIE withdrawal mice (-63.4±1.6, n=6 cells from 3 mice, p=0.84).

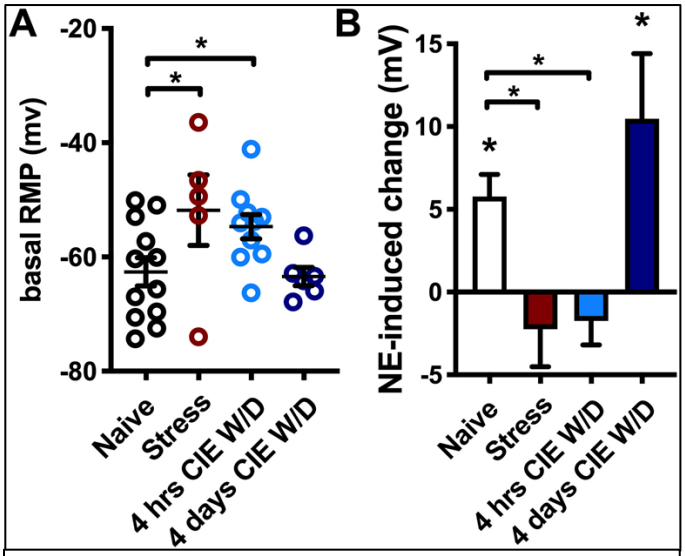

Figure 2-2. Effects of NE on BNST CRF neuron resting membrane potentials in stress and CIE exposed mice. A) Scatter plot showing basal resting membrane potentials (RMP) in EtOH/stress naïve mice (same cells as in figure 2-1) vs mice exposed to 1 hr restraint stress or CIE exposed mice at 4 hr or 4 day withdrawal periods. * with brackets indicates significant difference between groups, p<0.05. B) Bar graph summarizing the effects of NE-induced changes to BNST CRF neuron membrane potential in naïve vs restraint stress or CIE exposed mice at 4 hr or 4 day withdrawal periods. Basal RMP is normalized to 0. * with brackets indicates significant difference between groups, p<0.05. * without brackets indicates a significant increase from basal RMPs, p<0.05.

NE depolarization of BNST CRF neurons is occluded by acute stress or CIE withdrawal

We next sought to determine if CIE or stress may modulate the ability of NE to depolarize BNST CRF neurons. After basal RMPs were determined (**Figure 2-2A**), 1 μM NE was bath applied for ten minutes as in **Figure 2-1**. One-way ANOVA revealed significant effects of stress and CIE on NE-induced changes to BNST CRF neuron RMPs ($F_{(3,29)}$=7.861, p<0.001, **Figure 2-2B**) compared to cells from EtOH/stress naïve mice. Fisher's LSD post-hoc analysis showed a significant difference between naïve (NE-induced change = 5.8±1.3 mV, n=12 cells from 8 mice) and stress mice (-1.72±1.7 mV, n=8 cells from 3 mice, p<0.01) and between naïve and 4 hr CIE withdrawal mice (-1.74±1.4, n= 7 cells from 4 mice, p<0.01). The effects of NE on BNST CRF neuron RMPs in the stress and 4 hr CIE withdrawal group were not significantly different from baseline (p>0.05). However, NE was able to significantly alter BNST CRF neuron RMPs from baseline in the 4 day CIE withdrawal group (10.5±3.9mV, n=6 cells from 3 mice, p<0.05) and this effect was not different from naïve mice (p=0.12).

Stress and CIE do not modulate NE inhibition of glutamatergic transmission onto BNST CRF neurons

Together, the above findings indicate that NE can depolarize BNST CRF neurons via a β-AR dependent mechanism that is functionally occluded following acute stress or short-term CIE withdrawal. Previous work indicates that NE can inhibit glutamatergic transmission in the BNST via a distinct α-AR mediated mechanism (Flavin and Winder, 2013; Forray et al., 1999; Fuentealba et al., 2000). We have previously shown that α-AR activation inhibits evoked EPSCs in BNST CRF neurons without altering paired pulse ratio (PPR) (Fetterly et al., 2019). Here, we replicated these initial findings and found that bath application of 1 μM NE significantly inhibited

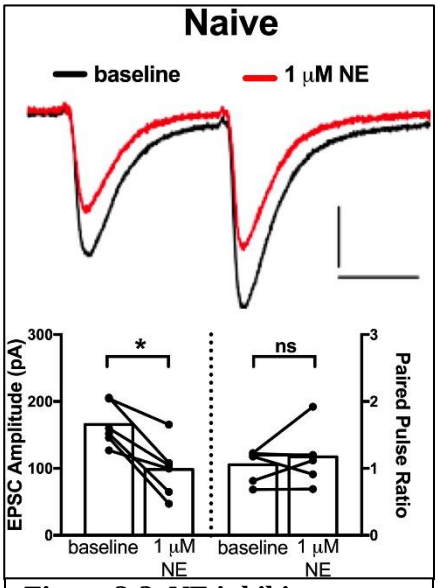

Figure 2-3. NE inhibits glutamatergic transmission in BNST-CRF neurons in naive mice. Top) Example traces of the effect of 1 μM NE on electrically evoked excitatory postsynaptic currents (EPSC) in BNST CRF neurons from naïve animals. Scale bar: x axis = 25 msec; y axis = 50 pA. Bottom) Before-and-after line and bar graphs summarizing the effects of NE on EPSC amplitude and paired pulse ratio. * indicates a significant difference from baseline conditions, p<0.05. ns= not significant.

EPSC amplitude in BNST CRF neurons in naïve mice (-41.3±7.8% change from baseline, n=6 cells from 3 mice, p<0.005, Figure 2-3) without altering PPR (11.65±11.95% change from baseline, p>0.05). We next sought to determine if stress or CIE would alter NE modulation of glutamatergic transmission onto BNST CRF neurons. After restraint stress, bath application of 1 μM NE significantly inhibited EPSC amplitude in BNST CRF neurons (-61.7 ±13.1% change from baseline, n=5 cells from 3 mice, p<0.01, Figure 2-4) without altering PPR (14.3±10.1% change from baseline, p>0.05). The effect of NE in restraint stress exposed mice is similar to previous findings in stress-naïve animals (Fetterly et al., 2019).

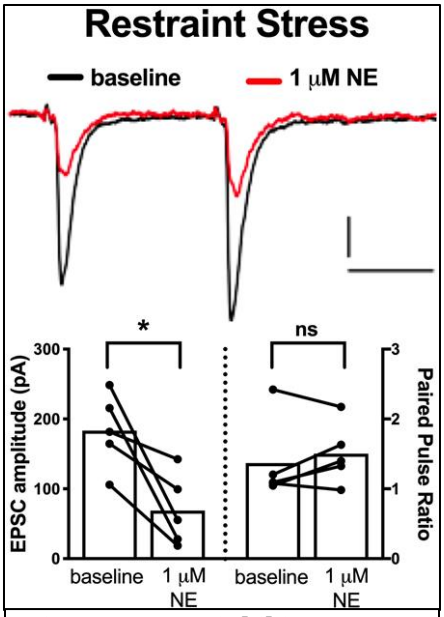

Figure 2-4. NE inhibits glutamatergic transmission in BNST-CRF neurons in stress exposed mice. Top) Example traces of the effect of 1 μM NE on electrically evoked excitatory postsynaptic currents (EPSC) in BNST CRF neurons from animals exposed to 1-hr restraint stress. Scale bar: x axis = 25 msec; y axis = 50 pA. Bottom) Before-and-after line and bar graphs summarizing the effects of NE on EPSC amplitude and paired pulse ratio. * indicates a significant difference from baseline conditions, p<0.05. ns= not significant.

In 4 hr CIE withdrawal mice, 1 μM NE also significantly decreased EPSC amplitude in BNST CRF

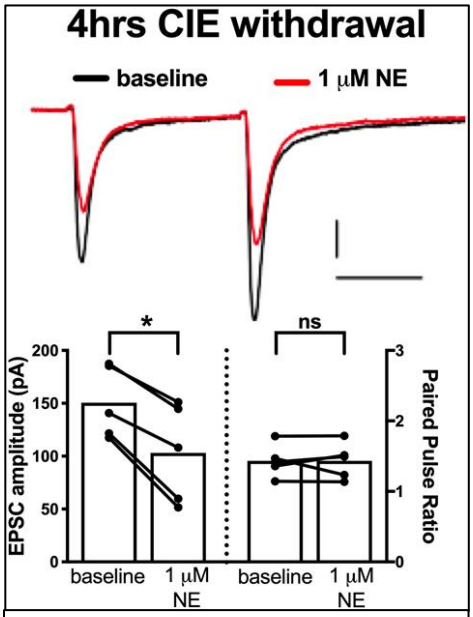

Figure 2-5. NE inhibits glutamatergic transmission in BNST-CRF neurons in CIE exposed mice. Top) Example traces of the effect of 1 μM NE on electrically evoked excitatory postsynaptic currents (EPSC) in BNST CRF neurons from animals following 4 hr withdrawal from CIE. Scale bar: x axis = 25 msec; y axis = 50 pA. Bottom) Before-and-after line and bar graphs summarizing the effects of NE on EPSC amplitude and paired pulse ratio. * indicates a significant difference from baseline conditions, $p<0.05$. ns= not significant.

neurons (-34.3±7.9% change from baseline, n=5 cells from 3 mice, $p<0.05$, **Figure 2-5**) without altering PPR (0.1±4.6, $p>0.05$). The effect of NE on evoked EPSC amplitude was not significantly different in naïve, 4 hr CIE withdrawal mice, and stressed mice (One-way ANOVA, $F(2,13)= 2.01$, $p=0.17$, **Figure 2-6**) and was similar to previous data in naïve mice (Fetterly et al., 2019). Together, these findings indicate that stress and CIE exposure do not alter the ability of NE to inhibit excitatory drive onto BNST CRF neurons. As there was no difference in the 4 hr CIE withdrawal group in this experiment, we did not test NE effects at the 4 day CIE withdrawal time point.

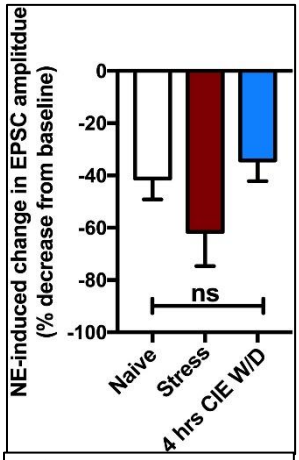

Figure 2-6. NE inhibition of glutamatergic transmission in BNST-CRF neurons is similar in naïve, CIE, and stress exposed mice. Summary bar graph comparing the magnitude of NE induced inhibition of EPSC amplitude in naïve, stress, and CIE exposed mice. ns= no significant difference between groups as analyzed by one-way ANOVA.

Discussion

Noradrenergic activation of BNST CRF neurons is thought to be critically involved in physiologic and behavioral responses to stress, and is a key component driving stress-induced reinstatement to drug-seeking behaviors (Brown et al., 2011; Leri et al., 2002;

Wang et al., 2001). Here we provide novel, direct evidence that NE can depolarize BNST CRF neurons via β-AR stimulation. Furthermore, BNST CRF neurons had significantly more positive RMPs following restraint stress or CIE exposure. In addition, the depolarizing effects of exogenous NE application were functionally occluded by restraint stress or CIE exposure. While previous work indicated that NE could also inhibit glutamatergic transmission onto BNST CRF neurons via an α-AR mediated mechanism (Fetterly et al., 2019), this effect of NE is not modulated by exposure to either restraint stress or CIE. Together, these findings suggest NE can alter BNST CRF neuron excitability via modulation of two mechanisms: 1) direct depolarization of BNST CRF neurons via a β-AR dependent mechanism that is stress and ethanol sensitive and 2) an α-AR dependent mechanism that inhibits glutamatergic transmission onto BNST CRF neurons that is not sensitive to stress or CIE exposure. Therefore, NE modulation of BNST CRF neurons appears to occur via balanced α/β-AR signaling, and stress and CIE shift this balance by preferentially targeting β-ARs.

Previous studies suggest that a variety of stressors and drugs of abuse enhance neuronal activity in the BNST in general as well as enhance the activity of BNST CRF neurons specifically (Casada and Dafny, 1991; Funk et al., 2006; Janitzky et al., 2014; Tran et al., 2012; Walker et al., 2009). Previous data also indicate that CRF signaling may be an important factor in regulating overall BNST excitability and that BNST CRF neurons are sensitive to β-AR stimulation. For instance, the β-AR agonist isoproterenol can increase glutamatergic transmission in the BNST via a CRF receptor dependent mechanism (Nobis et al., 2011) and can depolarize BNST CRF neurons (Silberman et al., 2013). CRF has also been shown to enhance glutamatergic transmission onto BNST projection neurons, an effect that is mimicked by CIE (Silberman et al., 2013). Together, these findings suggest that stress and CIE mediated β-AR activation can stimulate the release of endogenous CRF in the BNST from local CRF producing neurons to enhance BNST excitability. In support of this hypothesis, the current findings show that NE can

directly depolarize BNST CRF neurons, in some cases resulting in spontaneous action potential firing. This effect is dependent upon β-AR stimulation and is absent in stress and CIE exposed mice. BNST CRF neurons' resting membrane potentials are more positive in stress and CIE withdrawal mice compared to naïve animals, suggesting endogenous β-AR signaling may have already occurred. We propose that the absence of NE effects on BNST CRF neurons in stress and CIE mice may be due to functionally occluded β-ARs. Such functional occlusion could occur via multiple mechanisms such as receptor occupancy, receptor endocytosis, or receptor downregulation that could be examined in future studies. Overall these data provide novel evidence for neurocircuit function previously hypothesized from studies of stress and chronic ethanol activation of the BNST circuits.

An alternative hypothesis for NE-induced enhancement of BNST CRF neuron activity could be that NE may increase excitatory glutamatergic transmission onto BNST CRF neurons. However, recent evidence suggests that this is not likely to be the case as NE inhibits glutamatergic transmission onto BNST CRF neurons (Fetterly et al., 2019). Analysis with AR-selective agonists/antagonists suggests that NE inhibits glutamatergic transmission onto BNST CRF neurons via a predominantly α-AR mediated mechanism. This is an important consideration as α1-AR stimulation results in a long-term depression (LTD) of excitatory transmission in the BNST (McElligott and Winder, 2008), suggesting an important role for these receptors in regulating overall BNST excitation. Intriguingly, NE inhibition of glutamatergic transmission in BNST CRF neurons seen in naïve mice is maintained following CIE or stress exposure in this study. The ability of α-ARs to maintain NE sensitivity following a stress or CIE exposure may be a mechanism by which BNST CRF neurons return to homeostatic activity in response to a prolonged β-AR activation. While the data here examine an acute stressor, α1-AR LTD of glutamatergic transmission in the BNST is disrupted by chronic/repeated restraint stress (McElligott et al., 2010), suggesting that pathology of α1-AR mediated signaling may be an

important factor in the development of stress-related disorders via loss of the ability of CRF neurons to return to a homeostatic state. It will be important in future studies to further examine the mechanisms by which α1-AR signaling in BNST CRF neurons may be disrupted by chronic stress as an avenue for the development of novel treatments for these disorders.

α2-ARs are also involved in regulating synaptic transmission in the BNST (Shields et al., 2009), particularly inputs from the parabrachial nucleus to CRF neurons (Fetterly et al., 2019; Flavin et al., 2014). While the role of parabrachial inputs to the BNST are still being elucidated, α2-AR agonists can inhibit the ability of stress to enhance cFos activity in BNST CRF neurons (Fetterly et al., 2019). This finding indicates multiple α-AR mediated recovery mechanisms may be targeted in the BNST CRF circuit. Overall the findings here suggest that the ability of acute stress, CIE withdrawal, and NE to enhance BNST CRF neuron activity is likely due to stimulation of β-ARs on BNST CRF neurons and not via α-AR modulation of excitatory transmission onto BNST CRF neurons.

In conclusion, these findings provide novel electrophysiologic evidence to support the hypothesis that stress and CIE enhance BNST excitability via β-AR mediated stimulation of BNST CRF neurons. Previous work combined with the findings here indicate that α- and β-AR work in conjunction, via distinct mechanisms and potentially distinct circuits, to regulate overall BNST excitability and BNST CRF neuron activity in particular. Disrupting the balance of α- and β-ARs in the BNST may be a critical component of stress and EtOH induced behavioral changes. Recent evidence also suggests that competing pro-stress and anti-stress circuits are present in the BNST (Daniel and Rainnie, 2016), the balanced activity of which drives appropriate behavioral responses to stress. While the data here indicate that CIE modulation of β-AR signaling can return to normal levels after 4 days of withdrawal, it will be important in future studies to determine if stress, CIE, or their combined exposures will lead to long-lasting changes in α/β-AR

balance of BNST CRF neuron excitability, or differentially alter the activity of competing pro- and anti-anxiety circuits in the BNST.

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Chapter 3

Restraint Stress Uncovers a β2-AR Mediated Mechanism of Glutamatergic Signaling in the ventral Bed Nucleus of the Stria Terminalis

Abstract

Stress is a necessary phenomenon that can be beneficial in appropriate amounts; however, a prolonged or pathological response to stress is known to contribute to stress-related mental health issues and other health conditions. Because of the dire health implications of excess levels of stress, gaining more insight into the underlying neurocircuitry of homeostatic stress responses is critical for identifying novel therapeutic targets to treat and prevent stress-related disorders. Studies have shown that the bed nucleus of the stria terminalis (BNST) is a limbic structure that plays a major role in the modulation of behavioral and physiological responses to stress, and the cell types and pathways modulating these stress-related responses differ among BNST subregions. Further, norepinephrine (NE) and corticotropin releasing factor (CRF) signaling in the BNST are critical components of the BNST stress response, and subregional differences in terms of NE and CRF signaling are also observed. In this study, we test whether acute stress alters NE and CRF-mediated ventral BNST (vBNST) signaling and investigate β-AR mediated neurocircuitry changes in response to stress. We also reveal a role for the β1-AR subtype in modulating glutamatergic signaling in the vBNST of both stress-naïve and stress-exposed mice. We also uncover a novel role for the β2-AR subtype in the modulation of glutamatergic transmission post-acute stress exposure. Finally, we find evidence to support the importance of β2-ARs in CRF neurons in the modulation of the neurophysiological and behavioral response to acute stressors. Overall, the findings presented here can be used to inform

future experiments and target candidates for treating and preventing the development of stress-related disorders.

Introduction

Stress is a phenomenon that everyone experiences. While not all stress is harmful, chronic and prolonged or pathological reactivity to stress is known to be a contributor to many diseases including mental health disorders such as mood disorders (Bale, 2005) and addiction (Koob and Volkow, 2016; Sinha, 2008), as well as other health conditions such as coronary heart disease (Salleh, 2008). Intense, acute stressors also contribute to disorders such as post-traumatic stress disorder (PTSD). A better understanding of neurocircuitry and its involvement in modulating homeostatic stress responses is needed to identify possible therapeutic targets for stress-related disorders. Further, finding ways to control a person's response to excessive stress may help delay the development or progression of stress-related illnesses.

Studies have shown that the bed nucleus of the stria terminalis (BNST), a component of the extended amygdala, plays a major role in modulating both behavioral and physiological stress responses (Casada and Dafny, 1991; Crestani et al., 2013; Sullivan et al., 2004; Tran et al., 2012), and has been shown to be a critical brain structure associated with stress-related disorders such as PTSD (Miles and Maren, 2019). Evidence also suggests that there may be competing pathways within the BNST, which lead to differential behavioral responses to stress (Daniel and Rainnie, 2016; Marcinkiewcz et al., 2019). These various behavioral responses may be related to differing BNST subregion control of behavioral and autonomic stress responses, as well as differing neuropeptide content of BNST neurons among the subregions (Giardino and Pomrenze, 2021; Chapter 1). Corticotrophin releasing factor (CRF) is one critical and well-studied neuropeptide in

the BNST related to enhanced stress reactivity. In addition to increased CRF in the BNST in response to stressors (Choi et al., 2006; Funk et al., 2006; Janitzky et al., 2014; Tran et al., 2012), exposure to stressors also elevate norepinephrine (NE) release within this brain region (Fendt et al., 2005; Pacak et al., 1995; Park et al., 2012) which then acts to modulate BNST CRF neuron function (Chapter 2). Restraint stress (RS) has been shown to alter NE synaptic transmission in both the dorsal BNST (dBNST) and ventral BNST (vBNST) (McElligott et al., 2010), with the vBNST being important for modulating stress-related behavioral responses such as predator odor-induced freezing behavior (Fendt et al., 2005) or increased anxiety-like behavior after immobilization stress (Cecchi et al., 2002). Furthermore, the vBNST contains the greatest noradrenergic terminal density in the brain (Forray and Gysling, 2004), speaking to the importance of this particular subregion in noradrenergic-mediated responses. Additionally, predator odor exposure has been shown to increase vBNST CRF neuron activity (Butler et al., 2016). Electrophysiological studies have shown that NE, predominantly via β-adrenergic receptor (β-AR) activation, directly depolarizes CRF neurons in the BNST resulting in enhanced glutamatergic transmission onto some BNST output neurons (Fetterly et al., 2019; Silberman et al., 2013; Snyder et al., 2019), although these studies have primarily focused on the dBNST subregion. Despite the importance of noradrenergic signaling in the vBNST subregion and evidence for stress-related CRF neuron activation in the vBNST, the mechanisms by which β-adrenergic signaling might modulate glutamatergic transmission in the vBNST are not well studied.

In this study, we hypothesize that stress alters noradrenergic and CRF-mediated signaling in the vBNST and seek to discover specific β-AR mediated circuitry changes after stress. We focus on how β-ARs modulate activity in the vBNST after exposure to an acute stressor. We also uncover a role for the β1-AR in modulating glutamatergic signaling in both naïve and stress-exposed mice, and a novel role for the β2-AR in modulating glutamatergic transmission only

observed after stress exposure. We also reveal the importance of CRF neuron β2-ARs, in particular, for both neurophysiological and behavioral stress responses. Overall, these results may be used to identify future therapeutic targets for stress-related disorders.

Methods

Animals

Adult male C57BL/6J mice (>7 weeks old, The Jackson Laboratory) were used for electrophysiology and behavioral experiments. Male CRF-*tomato* reporter mice (64 to 126 days old) (Silberman et al., 2013) were utilized for immunohistochemistry experiments. Male mice with β2-ARs knocked out in CRF neurons were created by crossing β2-AR floxed mice with CRF-cre mice, and were used for electrophysiology and behavioral experiments. The CRF-cre line that was bred in the creation of the CRF-*tomato* mice and the CRF neuron-specific β2-AR knockout accurately reports neurons that express CRF mRNA (Chen et al., 2015). Mice for all experiments except the conditioned place aversion study were group housed in cages of two to five, and all mice were given *ad libitum* food and water. All procedures were approved by the Institutional Animal Care and Use Committee at Penn State College of Medicine (Hershey, PA).

Restraint Stress (RS) Exposure

Devices for RS were made using 50-mL conical tubes (Fisher Scientific) and drilling holes in the tube for airflow (McElligott et al., 2010). Mice were put in the tubes for one hour and placed alone in a new cage in a sound- and light-attenuating box. After completion of the RS

procedure, mice were removed from the tube and allowed to rest in the cage for 30 minutes before sacrifice for electrophysiology or immunohistochemistry experiments.

Electrophysiology

Preparation of Brain Slices

All electrophysiology experiments were done using adult male C57BL/6J mice. To investigate the role of the β-AR in modulating excitatory transmission in the vBNST, we used whole-cell patch-clamp electrophysiology to measure spontaneous excitatory postsynaptic currents (sEPSCs) in vBNST neurons from brain slices of stress-naïve mice or mice exposed to a 1-hour RS. To investigate the possible role of the glucocorticoid receptor (GR), one group of mice was pretreated with a 25mg/kg injection (Lowery et al., 2010) of mifepristone (Sigma Life Science, catalog no. M8046), a GR antagonist, 30 minutes before the start of the RS exposure. 30 minutes after conclusion of the RS, mice were anesthetized using isoflurane, and transcardially perfused with ice-cold, oxygenated sucrose (in mM: 183 sucrose, 20 NaCl, 0.5 KCl, 1 $MgCl_2$, 1.4 NaH_2PO_4, 2.5 $NaHCO_3$, 1 glucose). Brains were quickly dissected and placed in the same oxygenated sucrose dissecting solution. Using a Leica VT1200s vibratome equipped with a ceramic blade (Campden Instruments Limited, #7550-1-C) 250μm-thick slices incorporating the BNST were taken and put into an oxygenated modified artificial cerebral spinal fluid (ACSF) holding solution (in mM: 100 sucrose, 60 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.1 $CaCl_2$, 3.2 $MgCl_2$, 2 $MgSO_4$, 22 $NaHCO_3$, 20 glucose, 1 ascorbic acid) at 28-32°C. After 14 minutes

in the holding solution, slices were then placed into a standard ACSF solution (in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 2.95 $MgSO_4$, 1 NaH_2PO_4, 10 D(+) glucose, 26 $NaHCO_3$) oxygenated and warmed to 28-32°C. Slices remained in the standard ACSF for a duration of 30 minutes or greater prior to whole-cell patch clamp recordings.

Whole-cell Patch Clamp Electrophysiological Recordings

Slices were placed on the electrophysiology rig in a perfusion chamber with oxygenated standard ACSF continuously flowing at 2mL/min at room temperature. AMPA receptor-mediated spontaneous excitatory postsynaptic currents (sEPSCs) were recorded by holding the cell at -70mV and with 25μM picrotoxin (Tocris, catalog no. 1128) added to the ACSF. Potassium gluconate-based internal solution (in mM: 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na-ATP, 0.4 Na-GTP; pH ~7.35, 285–290 mOsmol) was used in the recording electrodes. All electrophysiology recordings were done with Clampex and analyzed with Clampfit (Molecular Devices) or recorded and analyzed with SutterPatch (Sutter Instrument Company). After patching a cell, cells were left to equilibrate for 5 minutes and then electrophysiology recordings were taken for a total of 30 minutes: 10 minutes of baseline, 10 minutes of either the β-AR agonist isoproterenol (3μM, Tocris Bioscience, catalog no. 1747) or the β2-AR selective agonist clenbuterol (10μM, Sigma, catalog no. C5423), and then 10 minutes of washout. Some experiments utilized the CRFR1 antagonist NBI 27914 (NBI) (1μM, Tocris Bioscience, catalog no. 1591), the β1-AR antagonist betaxolol (betax) (10μM, Tocris Bioscience, catalog no. 0906), or the β2-AR antagonist ICI 118,551 (ICI) (10μM, Tocris Bioscience, catalog no. 0821). For experiments using antagonists, slices were incubated with the antagonist at least 15 minutes before the start of recording. The antagonist remained onboard for the entire duration of the

recordings. Cells that displayed >20% change in access resistance or cells that did not display a stable baseline or fluctuated holding currents were generally excluded. The baseline value was identified if the cell stabilized within the first 12 minutes, and the maximum drug effect was identified within the last 4 minutes of drug application or first 4 minutes wash.

Dual Stress Conditioned Place Aversion Protocol

Adult, male C57BL/6J mice and adult, male mice with β2-AR KO in CRF neurons were used for conditioned place aversion (CPA) behavioral experiments. All mice used in these studies were singly housed three to five days prior to the start of the protocol. The CPA protocol was split into 6 days, with a habituation one day prior to the start (**Figure 3-1**). Each day of behavioral testing, mice were brought into the behavioral testing room and left to acclimate in their home-cages for at least 30 mins. During habituation, mice were handled briefly, and then placed in the

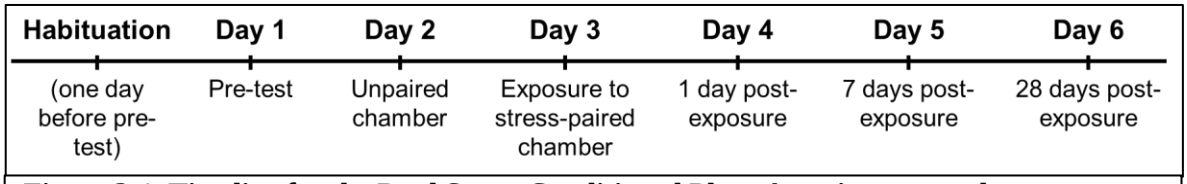

Figure 3-1. Timeline for the Dual Stress Conditioned Place Aversion protocol.

CPA apparatus and left to roam the CPA apparatus containing 3 chambers: two major chambers (each 17.78 cm x 20.32 cm) and one transition chamber (10.16 cm x 20.32 cm) for 2-3 minutes. The apparatus was cleaned with 70% ethanol between each mouse. On day 1 (pre-test), mice were put into the transition chamber of the CPA apparatus and allowed to roam the entire apparatus for 5 minutes. On day 2 (exposure to unpaired chamber), mice were confined to one of the primary chambers for 15 minutes and allowed to explore the chamber while being exposed to no stressful stimulus. Placement into chamber sides was randomized. On day 3 (stress exposure), mice were confined to the other primary chamber (the chamber not used by that individual on

Day 2) and exposed to either no stressful stimulus (naïve), predator odor, or predator odor + RS. Whichever chamber the mouse was confined to during stress exposure is referred to as the "paired chamber". For predator odor exposure, 150µL of 2-phenylethylamine (PEA), a component of urine from carnivores such as bobcats (Ferrero et al., 2011), was added to a kim wipe and placed in a petri dish and placed into the center of the paired chamber. Mice were left to explore the paired chamber for 15 minutes. For predator odor+RS exposure, mice were placed in a restraint tube with the mouse's nose close to the PEA-soaked kim wipe for 10 minutes. After removal from the restraint tube, the mouse was placed back into the chamber with the predator odor present for 5 minutes. Naïve animals were exposed only to a saline-soaked kim wipe in the paired chamber for 15 minutes. On post-test days, mice were put into the CPA apparatus with all chambers open and allowed to freely roam for 5 minutes, and the time spent in each chamber was calculated and compared to the pre-test (day 1). Post-tests were completed 24 hours, 7 days, and 28 days after day 3 of training. The percent change in time spent in the paired chamber at post-test vs. pre-test were calculated.

Immunohistochemistry and Subsequent Imaging

For immunohistochemistry (IHC) experiments, CRF reporter mice were either stress-naïve, exposed to predator odor only, or exposed to predator odor + RS before perfusions. Mice exposed to predator odor were individually placed in a novel home-cage with 25µL of PEA on a kim wipe and left to explore for 15 minutes. The kim wipe was then removed and the mouse remained in their novel cage for 30 minutes prior to perfusion. Mice exposed to both predator odor+RS were placed in a restraint tube in a cage with a kim wipe containing 25µL PEA for 1 hour. The mouse was then removed from the restraint tube, and the kim wipe was discarded. After 30 minutes recovery in the same cage, mice were perfused. Mice were anesthetized with

isoflurane and transcardially perfused with ~20 mL of 0.1M phosphate buffered saline (PBS) made from 1.0M PB (Sigma-Aldrich, catalog no. P3619) with the addition of NaCl. Immediately after PBS perfusion, ~10 mL of 4% paraformaldehyde diluted from 32% PFA (Electron Microscopy Sciences, product no. 15714-S) with 0.1M PBS was perfused to fix the tissue. Brains were quickly dissected and stored in 4% PFA at 4°C overnight, then transferred to a 30% sucrose in PBS solution and stored at 4°C until cryosectioning. Before cryosectioning, brains were embedded in optimal cutting temperature (OCT) medium (Tissue-Tek, catalog no. 4583). 40μm brain slices containing the BNST were made using a cryostat microtome (Leica CM1850) set to -20°C to -17°C and then stored in cryoprotectant solution. IHC was carried out following a previously-published protocol (Aimino et al., 2018). Briefly, free-floating BNST slices were first washed with PBS for 10 minutes (4 times), then permeabilized using 0.5% Triton X-100 diluted with PBS for 30 minutes, and then blocked with 10% normal donkey serum (Jackson ImmunoResearch, catalog no. 017-000-121) in 0.1% Triton X-100 (BDH, catalog no. BDH3929-2) for 1 hour before the addition of the 1° c-fos antibody (Synaptic Systems, catalog no. 226 003) at a dilution of 1:1000. The slices were incubated in the primary antibody on a nutating mixer at 4°C for 72 hrs. After four, 10-minute PBS washes, slices were immersed in a 0.1% Triton X-100 solution containing the Alexa Fluor 488 2° antibody (donkey anti-rabbit, Jackson ImmunoResearch Laboratories, catalog no. 711-546-152) at a dilution of 1:500. The slices remained in the 2° antibody for 24 hours on a nutating mixer at 4°C, then were washed in PBS and gently mounted onto Superfrost Plus microscope slides (Fisherbrand, catalog no. 12-550-15) and the PBS was allowed to evaporate before coverslipping. Prolong Gold antifade reagent (Invitrogen, catalog no. P36930) was applied to the dried slices and coverslipped. The BNST was imaged using a Keyence BZ-X710 microscope with either a 20x or 40x objective lens. Imaging parameters were consistent within IHC runs. The vBNST of each image was analyzed using the BZ-X analyzer software to identify the number of CRF neurons and c-fos+ CRF neurons. The

percent of c-fos+ CRF neurons were compared across groups using a one-way ANOVA with Tukey's multiple comparisons test.

Statistical Analyses

GraphPad Prism 9.2.0 and Excel from the Microsoft 365 set of applications was used to perform statistical analyses. Figures were prepared in Graphpad Prism 9.2.0 and Powerpoint from Microsoft 365. A one-sample t-test was used to compare experimental values to baseline values in the electrophysiology and behavioral experiments. One-way ANOVA followed by Tukey's multiple comparisons test was utilized to compare results among groups when applicable. Results are presented as mean ± SEM.

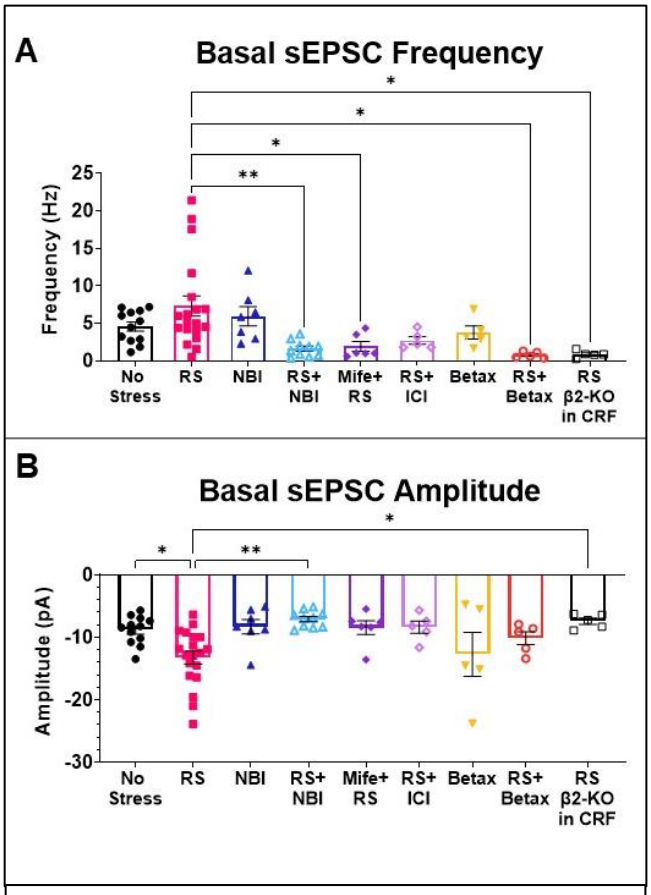

Figure 3-2. Basal sEPSC frequency and amplitude of vBNST neurons in all groups investigated with electrophysiology. A. The basal sEPSC frequency in the RS condition was significantly greater than basal frequency after RS+NBI 27914 or RS+betaxolol, as well as if the mouse was pretreated with mifepristone prior to restraint stress exposure.
B. The basal sEPSC amplitude after restraint stress was significantly greater than basal amplitude without stress exposure as well as the RS+NBI condition.
Basal measurements were calculated using stabilized values recorded during the first 12 minutes. Since baseline measures were taken before application of isoproterenol or clenbuterol, cells from both drug conditions were combined. RS: restraint stress; NBI: NBI27914; Mife: mifepristone; ICI: ICI 558,551; Betax: betaxolol; β2-flox: β2-flox x CRF*cre*; error bars represent SEM; *=p<0.05; **=p<0.01

Results

Acute stress exposure alters basal sEPSC frequency and amplitude in the vBNST

Baseline sEPSC frequency and amplitude were measured during the first 10 minutes of whole-cell electrophysiology recordings in the vBNST of stress-naïve mice and mice that had undergone restraint stress (RS). In order to identify the effects of the CRF and NE systems on basal vBNST glutamatergic synaptic transmission, slices were either exposed to no drug or antagonists blocking CRFR1 (NBI) or β1-AR (ICI). One-way ANOVA indicated that basal sEPSC frequency significantly differed among groups ($F=4.370$, $p=0.0003$, **Figure 3-2A**). Tukey's multiple comparison test was used to compare the means of all groups to one another. The basal frequency in the RS condition (n=19 cells) was significantly greater than in the mifepristone pretreatment before RS condition (n=6 cells) (RS:

7.28±1.36 Hz vs. Mife+RS: 1.90±0.65 Hz, p=0.0409, Tukey's multiple comparisons test), the baseline recordings during the application of the CRFR1 antagonist NBI 27914 after RS (n=10 cells) (RS: 7.28±1.36 Hz vs. RS+NBI: 1.60±0.34 Hz, p=0.0030, Tukey's multiple comparisons test), recordings in the presence of the β1-AR antagonist betaxolol after RS (n=5 cells) (RS: 7.28±1.36 Hz vs. RS+Betax: 0.76±0.18 Hz, p=0.0123, Tukey's multiple comparisons test), and the basal frequency from β2-floxed x CRF*cre* mice exposed to RS (n=5 cells) (RS: 7.28±1.36 Hz vs. RS β2-flox: 0.85±0.21 Hz, p=0.0142). Basal amplitude also differed significantly among groups (one-way ANOVA, F=4.046, p=0.0006, **Figure 3-2B**). Basal sEPSC amplitude after RS was significantly higher than the no stress (RS: -13.24±1.05 pA vs. no stress: -8.84±0.66 pA, p=0.0368, Tukey's multiple comparisons test), the RS+NBI group (RS: -13.24±1.05 pA vs. RS+NBI: -7.10±0.42 pA, p=0.0014, Tukey's multiple comparisons test), and the β2-floxed x CRF*cre* mice exposed to RS (RS: -13.24±1.05 pA vs. RS β2-flox: -7.42±0.52 pA, p=0.0477). None of the other group comparisons of basal sEPSC frequency and amplitude were significantly different.

Isoproterenol increases sEPSC frequency in vBNST neurons from stress-naïve and RS-exposed mice

To investigate the role of the β-AR in modulating glutamatergic signaling in the vBNST, we applied 3μM isoproterenol, a non-selective β-AR agonist and recorded sEPSCs. Isoproterenol had various effects on the sEPSC frequency and amplitude of vBNST neurons in different conditions (**Figure 3-3**). Using a one-sample t-test, each drug effect was compared to baseline (normalized to 100%) and percent change was calculated. All values are expressed in mean % of baseline±SEM. In cells from stress-naïve mice (n=6 cells from 4 mice), application of 3 μM isoproterenol significantly increased the frequency (183.9±21.24% of baseline, p=0.0108) but did

not affect the amplitude (88.25±8.86% of baseline, p=0.2423) of sEPSCs in cells recorded from the vBNST. After RS exposure, isoproterenol significantly increased sEPSC frequency (158.0±15.66% of baseline, p=0.0077), but did not significantly alter amplitude (86.11±6.24% of baseline, p=0.0614; n=8 cells from 3 mice). Isoproterenol's effects on sEPSC frequency in naïve (control) mice were blocked in the presence of betaxolol, a β1-AR antagonist (98.09±8.02% of baseline, p=0.8235; n=5 cells from 4 mice) or NBI 27914, a CRFR1 antagonist (116.4±10.97% of baseline, p=0.1857; n=7 cells from 3 mice). After RS, isoproterenol's effect on sEPSC frequency was blocked by NBI 27914 (128.0±13.2% of baseline, p=0.1010; n=5 cells from 2 mice), but was not completely attenuated in the presence of betaxolol (116.2±3.97% of baseline, p=0.0151; n=5 cells from 3 mice). Isoproterenol application had no significant effects on amplitude in any of the

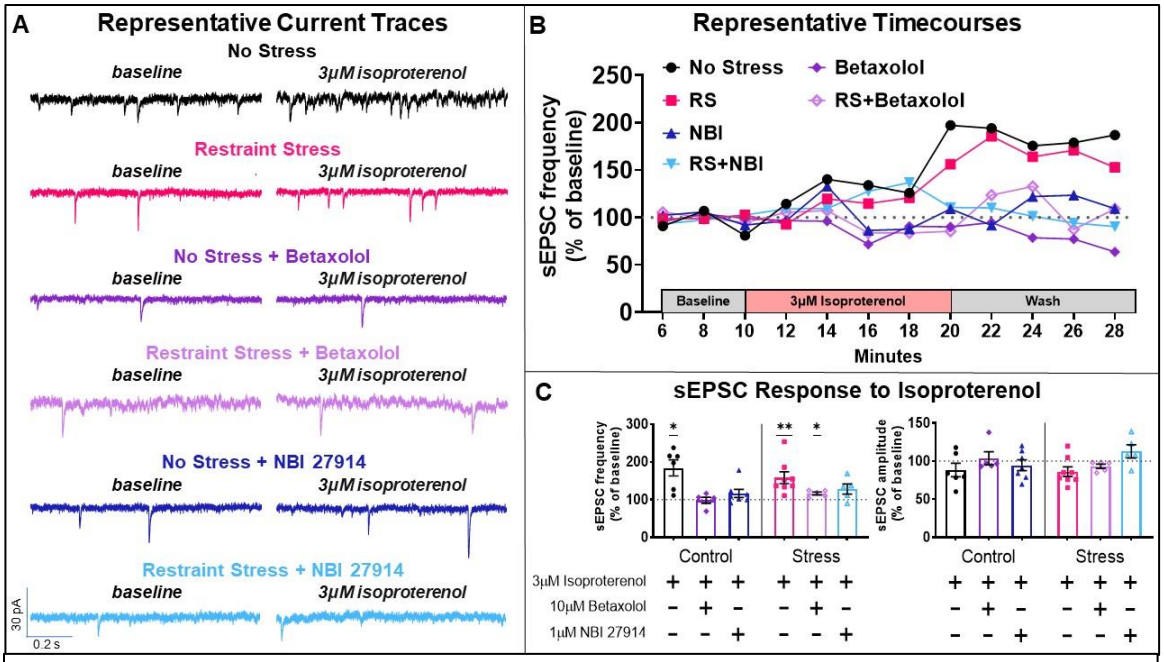

Figure 3-3. Isoproterenol significantly increases sEPSC frequency in vBNST neurons. Isoproterenol, a non-selective β-AR agonist, increases sEPSC frequency in vBNST neurons from naïve control mice as well as stressed mice. In cells from control mice, 10μM betaxolol (β1-AR antagonist) or 1μM NBI 27914 (CRFR1 antagonist) block the increase in sEPSC frequency. After stress, NBI 27914 also blocks the isoproterenol-induced increase in sEPSC frequency, and betaxolol only partially attenuates the increase in sEPSC frequency. Representative traces and timecourses are shown in panel A and B, respectively. Results are quantified in panel C showing the change in sEPSC frequency and amplitude relative to baseline.
RS: restraint stress; error bars represent SEM; *=p<0.05; **=p<0.01

other conditions (naïve+betaxolol: 103.7±8.53% of baseline, p=0.6988; naïve+NBI 27914: 94.45±7.07% of baseline, p=0.4619; RS+betaxolol: 93.31±2.87% of baseline, p=0.0800; RS+NBI 27914: 112.7±8.58 of baseline, p=0.2117).

Clenbuterol increases sEPSC frequency in the vBNST of RS-exposed mice

In the experiments where vBNST-containing brain slices were bath perfused with isoproterenol, we saw that the β-AR mediated effect on glutamatergic signaling was not completely attenuated in the presence of the β1-AR antagonist betaxolol after exposure to RS;

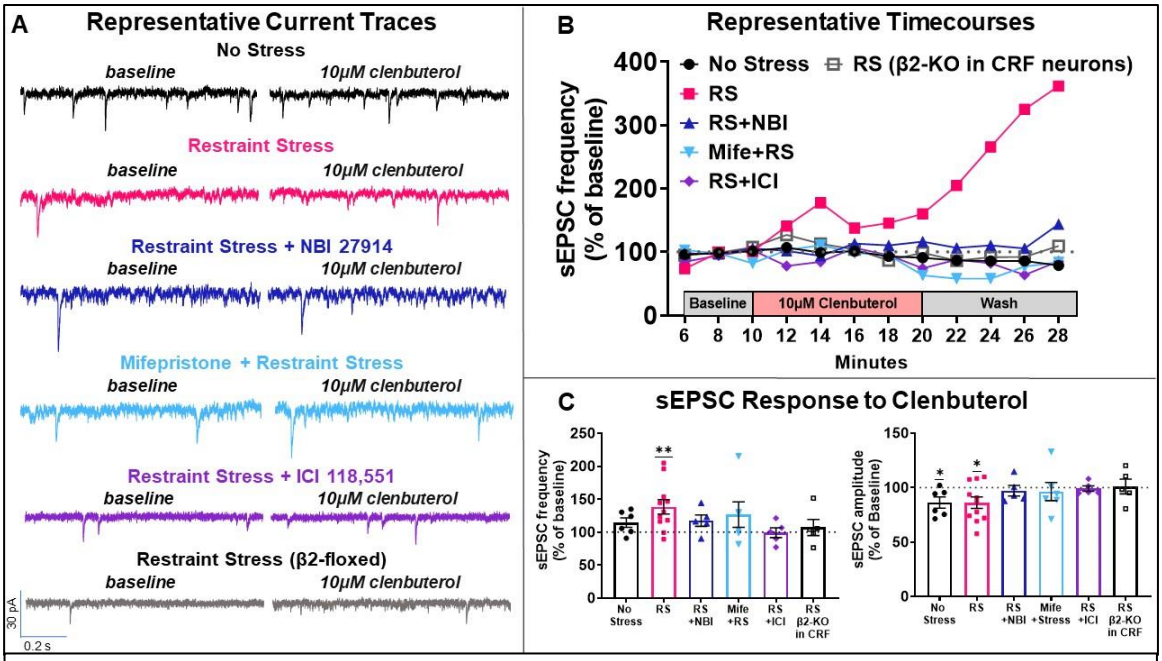

Figure 3-4. Clenbuterol significantly increases sEPSC frequency in vBNST neurons from stress-exposed mice. 10μM clenbuterol, a selective β2-AR agonist, increases sEPSC frequency only after exposure to RS; but this effect is blocked in the presence of 1μM NBI 27914 (CRFR1 antagonist), 10μM ICI 118,551 (β2-AR antagonist), and also when the mouse is pretreated with an i.p. injection of 25 mg/kg mifepristone (GR antagonist). vBNST neurons from mice with β2-ARs knocked out of CRF neurons do not show a response to clenbuterol after RS. Representative traces and timecourses are shown in panels A and B. Quantified results are in panel C displaying the drug effect on sEPSC frequency and amplitude expressed in percent of baseline.
RS: restraint stress; NBI: NBI27914; Mife: mifepristone; ICI: ICI 558,551; β2-floxed: β2-flox x CRF*cre* mice; error bars represent SEM; *=p<0.05; **=p<0.01

therefore we wanted to investigate the role of the β2-AR subtype in modulating vBNST glutamatergic signaling. In these experiments we bath-perfused the β2-AR selective agonist clenbuterol to investigate the effects of activating β2-ARs on glutamate transmission in the vBNST. In vBNST cells from stress-naïve mice, 10μM clenbuterol did not significantly change sEPSC frequency (114.4±7.24% of baseline, p=0.1041; n=6 cells from 4 mice), but it did lead to a significant decrease in amplitude (86.22±5.13 of baseline, p=0.0435). In RS-exposed mice, clenbuterol significantly increased sEPSC frequency (138.4±10.95% of baseline, p=0.0057; n=11 cells from 7 mice) and decreased sEPSC amplitude (86.12±5.32% of baseline, p=0.0261). The clenbuterol-induced changes in sEPSC frequency and amplitude were blocked by antagonism of CRFR1 with 1μM NBI27914 (frequency: 117.4±8.69% of baseline, p=0.1152; amplitude: 97.15±4.89% of baseline, p=0.5906; n=5 cells from 4 mice), pretreatment of the mouse with 25 mg/kg mifepristone (i.p.) before RS (frequency: 126.4±19.33% of baseline, p=0.2306; amplitude: 96.31±8.35% of baseline, p=0.6766; n=6 cells from 4 mice), and antagonism of the β2-AR with ICI 118,551 (frequency: 99.06±7.45% of baseline, p=0.9058; amplitude: 99.10±2.53% of baseline, p=0.7393; n=5 cell from 4 mice). When mice with the β2-AR knocked out of CRF neurons were exposed to a RS, clenbuterol did not significantly change frequency or amplitude (frequency: 107.0±12.29% of baseline, p=0.5974; amplitude: 100.9±6.76% of baseline, p=0.8965; n=5 cells from 4 mice) (**Figure 3-4**).

Mice exposed to predator odor+RS displayed conditioned place aversion

In the electrophysiology experiments, RS revealed the ability for β2-AR activation to increase glutamatergic transmission in the vBNST. This led us to the hypothesis that an acute stressor leads to upregulation of β2-ARs in the vBNST, which can then be activated by NE release due to a subsequent stressor and further vBNST excitability. We decided to test this hypothesis by combining two stressors in close proximity within a CPA paradigm. In the dual

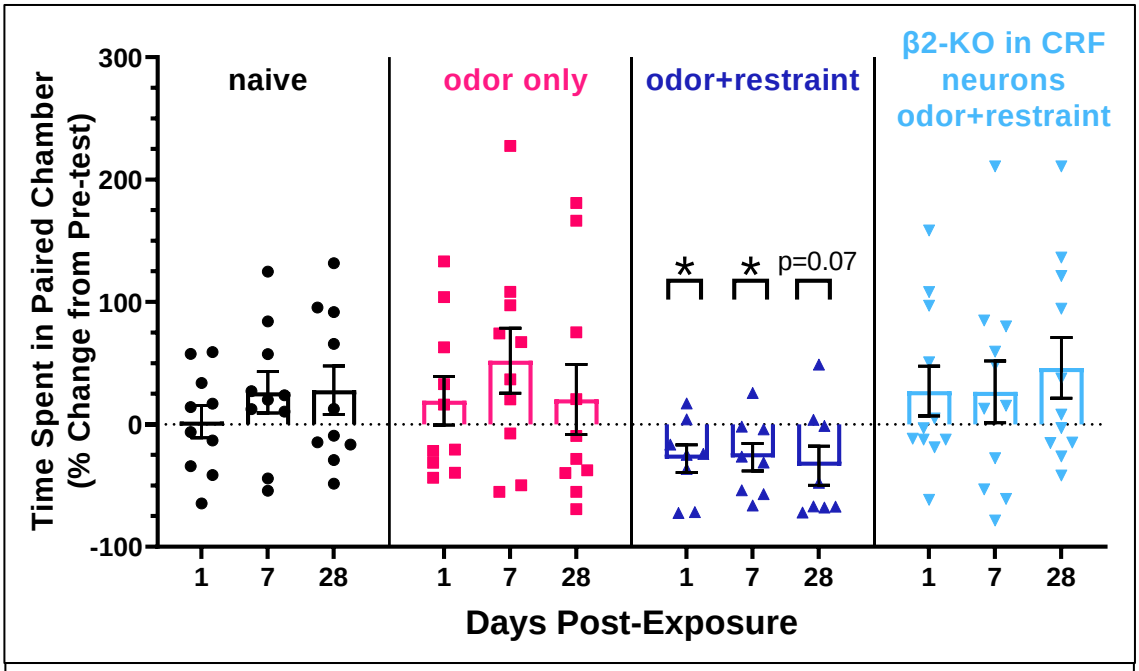

Figure 3-5. Mice exposed to odor+restraint stress in the conditioned place aversion paradigm spent significantly less time in the stressor-paired chamber during post-tests at one and seven days post-exposure. There was no significant increase or decrease in time spent in the paired chamber in any of the other groups. Error bars represent SEM; *=p<0.05

stress CPA paradigm, mice were given a pretest in the CPA apparatus on day 1 (**Figure 3-1**). On day 2, mice were placed in the unpaired chamber, and on day 3 mice were placed in the chamber paired with their stressful stimulus. On days 1, 7, and 28 post exposure to the stimulus in the paired chamber, mice were tested in the entire CPA apparatus to assess aversion to the paired chamber. Adult male C57BL/6J mice were placed in the paired chamber either without any stimulus (naïve), exposed to predator odor in the paired chamber (odor only), or exposed to

predator odor+RS (odor+RS). This procedure was also run using mice with β2-ARs knocked out in CRF neurons in the odor+RS condition. A one-sample t-test was used to compare the percent change in time spent in the paired chamber to 0 and all results are reported as mean % change±SEM (**Figure 3-5**).

The percent change of time spent in the paired chamber did not differ significantly from 0 in the naïve or predator odor only groups at any of the days post-exposure (Naïve: 1 day: 2.17±13.19% change p=0.8731, 7 days: 26.17±16.98% p=0.1576, 28 days: 27.90±19.82% p=0.1927, n=10 mice; Odor only: 1 day: 19.23±19.87% p=.3584 , 7 days: 51.96±26.54% p=0.0819, 28 days: 20.36±28.68% p=0.4958, n=10 mice). In the odor+RS group, mice decreased their time spent in the paired chamber at 1 day and 7 days post-exposure, and although not significant, there was a trend toward decreased time spent in the paired chamber at day 28 (1 day: -28.08± 11.32% p=0.0422, 7 days: -26.85±11.27% p=0.0487, 28 days: -33.84±16.00% p=0.0722, n=8 mice). In the mice with the β2-AR knocked out in the CRF neurons exposed to predator odor+RS, there was no significant change in the amount of time spent in the paired chamber at any of the days post-exposure (1 day: 27.18±20.29% p=0.2101, 7 days: 26.48±25.42% p=0.3187, 28 days: 46.11±24.79% p=0.0925, n=11 mice).

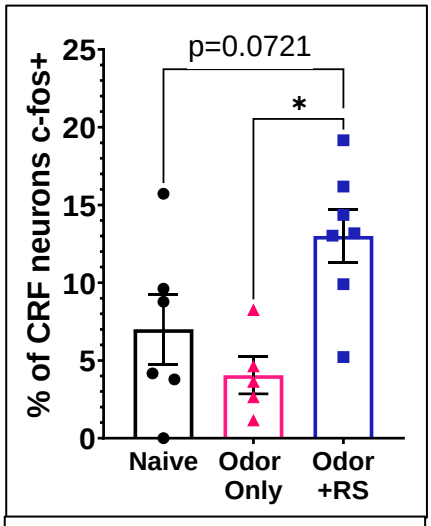

Figure 3-6. A greater percentage of CRF neurons are active after exposure to predator odor+restraint stress. A lower percentage of CRF neurons express c-fos in predator odor-exposed mice compared to those exposed to predator odor+RS. Odor: predator odor; RS: restraint stress; error bars represent SEM; *=p<0.05;

The vBNST of mice exposed to predator odor+RS displays a larger proportion of cfos+ CRF neurons than mice exposed to predator odor alone

For IHC, CRF reporter mice were either stress-naïve (n=6 mice), exposed to predator odor only (n=5 mice), or a combined predator odor+RS (n=7 mice). One-way ANOVA uncovered differences in the percent of c-fos+ CRF neurons among the groups ($F=6.317$, $p=0.0102$, **Figure 3-6**). There was a significant difference it the percent of c-fos+ CRF neurons in the odor+RS condition vs. odor only (13.01±1.69% vs. 4.05±1.20%, $p=0.0102$, Tukey's multiple comparisons test), and a trend toward a greater percent of c-fos+ CRF neurons in the odor+RS condition vs. naïve (13.01±1.69% vs. 7.01±2.26%, $p=0.0721$, Tukey's multiple comparisons test). No differences were found between the naïve and odor only groups (7.01±2.26% vs. 4.05±1.20%, $p=0.5350$, Tukey's multiple comparisons test).

Discussion

Overall, the experiments in this study uncover a role for vBNST β2-ARs in mediating stress-related glutamatergic signaling that is not seen in naïve animals. Additionally, the importance of CRF signaling and the β2-AR in CRF neurons specifically is emphasized here. While β1-ARs were shown to mediate glutamatergic signaling in both naïve and RS-exposed mice, the β2-AR plays a unique role in mediating stress-related glutamatergic signaling specifically. Further, CRF neuron β2-ARs are important for predator odor+RS-induced CPA.

Basal sEPSC properties are altered after exposure to an acute stressor

RS leads to changes in vBNST glutamatergic transmission in ways that are not seen in naïve mice or RS-exposed mice in different conditions. After RS exposure, differences in basal

glutamatergic activity were observed. Although basal sEPSC frequency was not significantly different between the naïve and RS groups, basal sEPSC amplitude after RS was significantly higher than the no stress group and there were differences in the RS group compared to others. We saw that RS increased basal sEPSC frequency in the vBNST relative to application of the CRFR1 antagonist NBI 27914 after RS, but not NBI 27914 application in naïve animals, which suggests a presynaptic change in the function of CRFR1 mediating basal glutamatergic signaling after exposure to RS. Basal sEPSC amplitude after RS was also significantly higher than the RS+NBI group, suggesting a possible postsynaptic change in the function of CRFR1 modulating basal activity also. RS also increases basal sEPSC frequency in the vBNST relative to the β1-AR antagonist betaxolol after RS, but not the β2-AR specific antagonist ICI 118,551 after RS, indicating a role for the β1-AR but not the β2-AR in mediating basal sEPSC frequency after RS, possibly through a presynaptic mechanism. RS also produces a basal sEPSC frequency and amplitude in the vBNST greater than that from the mice with the β2-ARs knocked out of CRF neurons after RS.

β2-ARs in the vBNST modulate glutamatergic transmission after acute stress exposure

Our data support a role for β-ARs in mediating vBNST glutamatergic signaling in the stress-naïve control group as well as in the acute stress-exposed group, with β1-ARs having an effect in both groups but β2-ARs exerting their effects solely after acute stress exposure. Application of the non-selective β-AR agonist isoproterenol significantly increased sEPSC frequency but did not change the amplitude in vBNST cells from naïve mice. This increase in sEPSC frequency was blocked in the presence of the β1-AR specific antagonist betaxolol or the CRFR1 antagonist NBI 27914. The presence of these antagonists did not affect amplitude (**Figure 3-3**). Together, these results suggest that isoproterenol exerts its effects on glutamatergic

signaling in the vBNST of naïve mice via a β1-AR and CRFR1 dependent presynaptic mechanism. The β1-AR specific mechanism in the vBNST of naïve mice is supported by the result that application of the β2-AR specific agonist clenbuterol did not significantly alter sEPSC frequency, although there was a significant decrease in amplitude (**Figure 3-4**), suggesting that there is not a presynaptic effect of β2-AR activation on glutamatergic signaling but there may be some effect postsynaptically.

After RS exposure, isoproterenol increased sEPSC frequency, but did not significantly alter amplitude. The effect of isoproterenol on sEPSC frequency was blocked by NBI 27914, but betaxolol did not completely attenuate the increase in sEPSC frequency. Additionally, isoproterenol did not change the sEPSC amplitude in the presence of betaxolol after RS (**Figure 3-3**). These data suggest that isoproterenol exerts its effects on sEPSC frequency via the CRFR1, and that both β1-ARs and β2-ARs are involved in the modulation of glutamatergic signaling after an acute stressor. Additionally, these effects on sEPSCs are primarily occurring through a presynaptic mechanism. Further results support the role of β2-ARs in the vBNST after stress exposure. In RS-exposed mice, clenbuterol significantly increased sEPSC frequency and led to a decreased amplitude, suggesting that β2-AR function after stress is through both presynaptic and postsynaptic mechanisms. Confirming that this effect was β2-AR mediated, application of the β2-AR specific antagonist ICI 118,551 prevented the clenbuterol-induced changes. The clenbuterol-induced changes in sEPSC frequency and amplitude were also blocked by antagonism of CRFR1, which indicates that the β2-AR mediated effects require functional CRFR1 signaling.

A potential mechanism by which the β2-ARs exert their effects after stress is through upregulation of functional β2-ARs in CRF neurons. This is supported by the results where there was no significant effect of clenbuterol on vBNST neurons from mice without the β2-AR in CRF neurons, indicating that the β2-ARs mediating this stress-related β2-AR effect on glutamatergic signaling are likely located on CRF neurons specifically. Results also suggest that the GR is

involved in mediating this β2-AR related mechanism after stress. RS led to an increased basal frequency relative to mice pretreated with the GR antagonist mifepristone before stress exposure, and mifepristone pretreatment blocked the RS-induced increase in sEPSC frequency and amplitude after clenbuterol application. These results indicate that the GR is important for acute stress exposure's β2-AR mediated effects. Since it has been shown that activated GRs can translocate into the nucleus and act as a transcription factor on the glucocorticoid response element of the β2-AR gene (Collins et al., 1988; Malbon and Hadcock, 1988), this is a possible mechanism by which the upregulation of the β2-ARs in CRF neurons may be occurring.

Predator odor+RS exposure produces conditioned place aversion in C57BL/6J mice but not mice with β2-ARs knocked out of CRF neurons

In the CPA paradigm, the percent change of time spent in the paired chamber did not differ from 0 in the naïve or predator odor only groups at 1, 7, or 28 days post-exposure, indicating that CPA was not established in these groups. Conversely, in the odor+RS group the percent change of time spent in the paired chamber decreased at 1 day and 7 days post-exposure, and there was a trend toward decreased time spent in the paired chamber at day 28. These results suggest that odor+RS exposure together are required to induce CPA in this paradigm. Further, mice with the β2-AR knocked out in the CRF neurons exposed to predator odor+RS, displayed no significant percent change in the amount of time spent in the paired chamber at any of the days post-exposure, suggesting that CRF neuron β2-ARs play a role in mediating CPA in our PTSD model.

Predator odor+RS leads to an increase in activated CRF neurons in the vBNST

Using CRF-*tomato* reporter mice and an antibody for c-fos as a measure for neuronal activation, we investigated the percent of activated vBNST CRF neurons in naïve, predator odor-exposed, and predator odor+RS exposed mice. We found that there was a significant difference in the percent of c-fos+ CRF neurons in the predator odor+RS condition compared to the predator odor exposure alone. Additionally, the percent of c-fos+ CRF neurons in the predator odor+RS condition did not significantly differ from the naïve group, although there was a trend toward increased activation in the odor+RS condition ($p=0.0721$). No differences were observed when comparing the naïve and predator odor only groups (**Figure 3-6**). Together, these results support the idea that vBNST CRF neuronal activation may be important for stress-related behaviors, particularly in situations where stressors are more intense such as during a PTSD-inducing traumatic event represented in this study by the combination of predator odor+RS.

Interestingly, previous studies have shown that in the vBNST of rats predator odor exposure alone led to an increase in CRF neuron activity in the vBNST also measured via c-fos expression (Butler et al., 2016). This discrepancy in results from predator odor exposure are possibly from the difference in species (i.e., rat vs. mouse), but are more likely due to the difference in predator odor exposure paradigms. In Butler et al. (2016) the predator odor used was "ferret odor" obtained through towels left in cages with ferrets before presentation to the rats, and the exposure lasted a duration of 30 minutes. Additionally, a recent study comparing commercially available coyote urine to PEA found that while PEA did cause avoidance and decreased exploratory behaviors, PEA exposure was unable to produce defensive behaviors such as increased freezing behavior and risk assessment seen with the coyote urine in rats (Maestas-Olguin et al., 2021). This is an important consideration for future studies utilizing predator odor exposure going forward.

Conclusions and Future Directions

Overall, we determined that β2-ARs play a unique role in modulating vBNST glutamatergic signaling after exposure to RS, and this mechanism is mediated by downstream CRFR1 signaling. Additionally, β2-ARs in CRF neurons specifically are shown to be important for the stress-related increase in sEPSC activity, and may be upregulated through a GR-mediated mechanism. The importance of CRF neuron β2-ARs is also supported through behavioral data where wild-type mice exposed to predator odor+RS decreased their time spent in a stress-paired chamber 1 day, 7 days, and possibly 28 days post-exposure, but mice with the β2-AR knocked out in CRF neurons exposed to the same stressor did not change their time spent in the paired chamber. Additionally, the same type of stress exposure (predator odor+RS) led to an increase in the percent of activated CRF neurons in the vBNST of CRF-*tomato* reporter mice. See **Figure 3-7** for a visual summary.

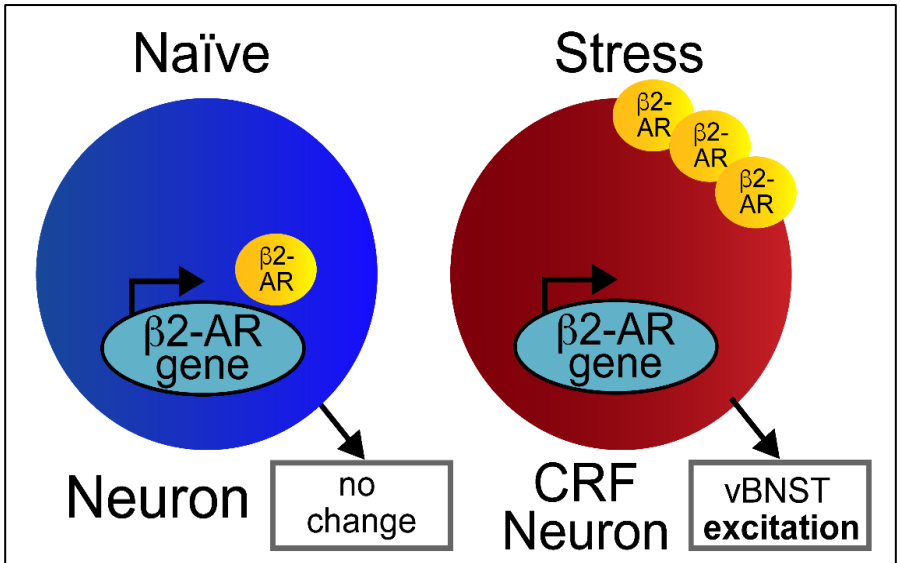

Figure 3-7. Summary and prospective mechanism. In the ventral BNST of naïve mice, there is a baseline level of excitatory signaling, which is not further increased in the presence of a β2-AR agonist. After acute stress, however, there is a significant increase in sEPSC frequency in the presence of the β2-AR agonist, suggesting an upregulation of functional β2-ARs. Further, mice with the β2-AR knocked out of CRF neurons specifically, do not display the clenbuterol-mediated increase in sEPSC frequency following an acute stressor. Data also suggest that this upregulation of functional β2-ARs after stress may be mediated by the glucocorticoid receptor, which could act as a transcription factor binding to the glucocorticoid response element on the β2-AR gene promoting transcription.

A few limitations of this study include the types of stressors used and the lack of measured β2-AR mRNA. As discussed previously, the type of predator odor may be limiting the degree of stress reactivity observed in the mice. Further, it may be beneficial to explore other types of acute and chronic stressors to compare the differences in resulting signaling. Additionally, while the electrophysiological observations support the hypothesis that β2-ARs are upregulated in CRF neurons after RS exposure, we are unable to definitively conclude if the upregulation is due to increased translocation of receptors to the membrane or also an increase in transcription. Future studies should look at mRNA transcript of the β2-AR in CRF neurons in the vBNST. Additionally, since the BNST is a sexually-dimorphic brain region and this has been observed in regard to CRF neurons in the dBNST and vBNST (Funabashi et al., 2004; Guillamón et al., 1988; Uchida et al., 2019), these β2-AR mediated physiological and behavioral responses must be investigated in female mice as well. Exploring the role of these vBNST β2-ARs in models of addiction or other stress-related disorders would also be beneficial for the identification of new target sites for therapeutic action.

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Chapter 4

2-Bottle Choice with Forced Abstinence and Stress Exposure: A Novel Mouse Model for Stress-Enhanced Drinking after Abstinence from Voluntary Ethanol Intake

Abstract

Alcohol use disorder (AUD) affects greater than 14 million adults in the United States. A major obstacle to treatment of AUD is relapse, and stress is known to be a prominent trigger. Currently, there are no approved pharmacological treatments targeting stress-induced relapse to alcohol use. Although rodent models exist for stress-induced reinstatement to drug-seeking behavior and chronic-stress induced ethanol (EtOH) intake, animal models utilizing acute stress inconsistently produce effects on voluntary EtOH consumption with many showing no effect or a decrease. To better represent the phenomenon in humans where a person is trying to quit drinking but relapses after a stressful life event, there is a need for an animal model that produces an acute stress-induced increase in EtOH consumption post-abstinence. Given this information, the purpose of this study was to develop a mouse model of acute stress-enhanced drinking. Using adult male and female C57BL/6J mice, our model combines 2 Bottle Choice (15% EtOH vs. water) with Forced Abstinence and acute Stress Exposure (2BC-FASE). Results indicate that male and female mice exposed to forced swim stress (FSS) before a voluntary drinking session post-abstinence maintained their pre-abstinence EtOH consumption levels, while stress-naïve mice decreased intake post-abstinence. These results suggest that we successfully produced a model that reliably demonstrates FSS-enhanced drinking (measured in g/kg) after an abstinence period. Further, electrophysiology data exploring the effects of FSS on β2-AR mediated

glutamatergic signaling in the ventral bed nucleus of the stria terminalis (vBNST) suggest that the acute FSS exposure alters β2-AR mediated glutamate signaling which may point toward a possible stress-mediated neurocircuitry mechanism underlying stress-enhanced drinking behavior. Further utilization of this model will be helpful to examine neurophysiological changes associated with stress-induced relapse, with the ultimate goal of identifying novel drug targets for the treatment of stress-related relapse in AUD.

Introduction

Alcohol use disorder (AUD) is a complex and relapsing brain disease, and the most recent data from the Substance Abuse and Mental Health Services Administration (SAMHSA) National Survey on Drug Use and Health (2019) indicate that AUD affects ~14.1 million adults in the United States. Further, ~16.0 million individuals aged 12 or older reported using alcohol heavily which is a risk factor for the development of AUD. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) estimates that less than 10% of those diagnosed with AUD seek and receive treatment (NIAAA, 2017), and it has been reported that relapse rates may be up to 90% within four years of abstinence (NIAAA, 1989). Stress is known to be a risk factor for the development of substance use disorders, and is commonly cited as a trigger for relapse back into harmful alcohol-related behaviors (Sinha, 2008, 2001). Currently, available AUD treatments do not specifically target stress-related relapse, and the high relapse rates indicate a need for more efficacious treatments to prevent relapse.

In animal models of stress-related drinking, chronic stress typically leads to increased alcohol (EtOH) consumption in rodents (Becker et al., 2011; Lopez et al., 2016), but changes in drinking associated with acute stressors have been variable, with most showing that acute stress either has no effect or decreases alcohol drinking (Becker et al., 2011; Lopez et al., 2016). To

better represent what may happen in humans when a person attempting to quit drinking relapses after a stressful event, there is a need for an animal model that reliably produces an acute stress-induced increase in EtOH consumption post-abstinence. Therefore, the purpose of this study was to develop a novel mouse model of acute stress-enhanced drinking after a period of abstinence from voluntary EtOH intake. Additionally, because the bed nucleus of the stria terminalis (BNST) plays a critical role in physiological and behavioral responses to stress (Casada and Dafny, 1991; Crestani et al., 2013; Sullivan et al., 2004; Tran et al., 2012; Waddell et al., 2006), and vBNST β2-ARs are critical for stress-induced reinstatement to drug-seeking behavior (Vranjkovic et al., 2014), we also investigated β2-AR mediated vBNST excitability post-forced swim stress (FSS) exposure.

Ultimately, we used an intermittent access to EtOH 2-bottle choice protocol (15% EtOH vs. H_2O) and an acute FSS to create our 2-Bottle Choice with Forced Abstinence and Stress Exposure (2BC-FASE) paradigm. Using the 2BC-FASE model, we observed that mice exposed to FSS maintained pre-abstinence levels of EtOH intake in both male and female C57BL/6J mice. In stress-naïve mice, there was a significant decrease in EtOH intake after abstinence. Additionally, in mice previously exposed to the 2BC-FASE protocol, we also observed that FSS uncovers a change in β2-AR mediated signaling in the vBNST, which may point to a possible mechanism by which FSS triggers enhanced drinking behavior. Overall, this study establishes 2BC-FASE as a successful mouse model of acute stress-enhanced voluntary EtOH intake after a period of abstinence. This model will be useful to uncover neural mechanisms underlying this phenomenon, which will help identify new treatment targets for stress-related relapse.

Methods

Animals

Adult male and female C57BL/6J mice (48-89 days old at the start of the training phase, The Jackson Laboratory) were used for all experiments, since they voluntarily consume alcohol to a greater extent than other commonly-used inbred strains (Rhodes et al., 2007). Mice arrived in cages of five, and were singly housed at the start of the behavioral paradigm. Mice were given *ad libitum* food and water throughout the entire study. All protocols were approved by Penn State College of Medicine's Institutional Animal Care and Use Committee (Hershey, PA).

Forced Swim Stress Exposure

FSS was chosen as the acute stressor in this study, since it was previously shown to increase drinking behavior in EtOH-dependent mice (Lopez et al., 2016) and our pilot data indicated that acute restraint stress exposure was unable to produce stress-enhanced drinking (data not shown). For FSS exposure, 1L beakers of water were filled with ~750-800 mL of water at a temperature between 21°C -25.5°C, with most being between 24°C -25°C. Mice were placed in the beaker of water and left to swim for 10 minutes before being removed, dried, and placed back into their home cage to recover.

2-Bottle Choice with Forced Abstinence and acute Stress Exposure (2BC-FASE)

To investigate if acute stress enhances alcohol intake after abstinence in mice, the 2BC-FASE paradigm was established (**Figure 4-1**). During the "Training" phase, adult male and female C57BL/6J mice were given intermittent 2-bottle choice (15% EtOH or water) for 24

hours, three times/week for two weeks. Mice maintaining steady drinking levels (≤15% Coefficient of Variation in EtOH (g/kg) consumed during the 2nd week) were split into two groups with equal intake (**Figure 4-2)** and entered forced abstinence for two weeks. On "Test Day," one group remained unstressed and the other received FSS for 10 minutes ~2-3 hours before a final 2-bottle choice session. Bottles were weighed before and after each drinking session, and the total g of EtOH and H_2O consumed was calculated. The amount of EtOH consumed (g/kg) were measured for each drinking session. EtOH preference ratio was also calculated: (g EtOH consumed)/(g EtOH consumed + g H_2O consumed).

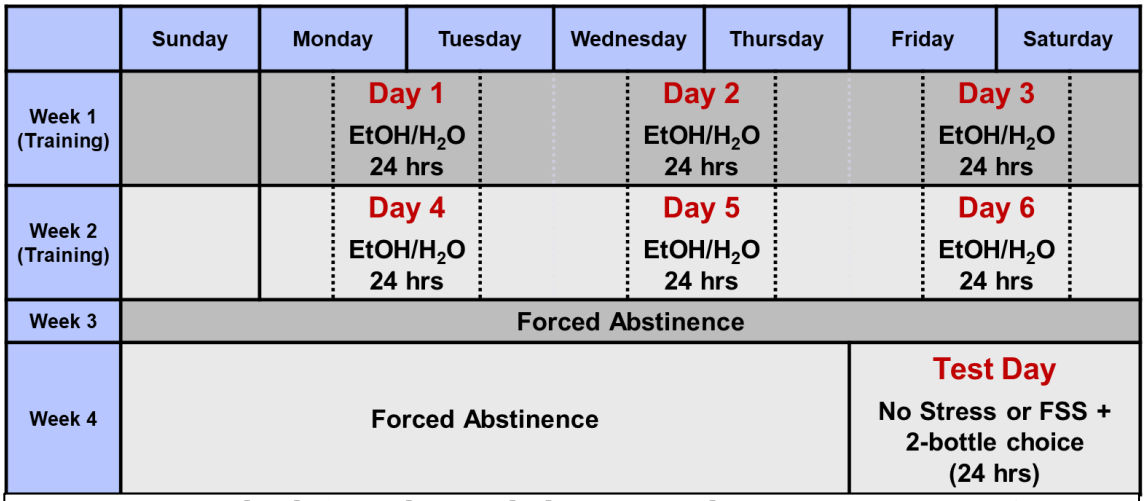

Figure 4-1. 2 Bottle Choice with Forced Abstinence and acute Stress Exposure (2BC-FASE) Paradigm. The first two weeks are the "Training" phase, where mice are given 2 bottles (15% EtOH and water) overnight for three nights per week. They then enter the "Forced Abstinence" phase in Weeks 3 and 4 where they are not given access to EtOH. At the end of Week 4 (Test Day), one group of mice are exposed to FSS about 2 hours before all the mice receive the bottles back for one last drinking session.

Electrophysiology

6-20 days after Test Day, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from vBNST cells using whole-cell patch clamp electrophysiology. Adult, male mice were either not exposed to stress or exposed to an acute FSS on the day of electrophysiology. Of the 9 cells recorded in the "no stress" condition on the day of electrophysiology, 1 cell was from a mouse that was not exposed to FSS on Test Day, and the other 8 were from mice previously exposed to FSS on Test Day. Of the 6 cells recorded from mice exposed to FSS on the day of electrophysiology, 3 cells were from a mouse not exposed to stress on Test Day, and 3 cells were from mice exposed to FSS on Test Day.

Preparation of Brain Slices

~30-45 minutes following the completion of FSS exposure, mice were anesthetized with isoflurane and perfused transcardially with cold, oxygenated sucrose (in mM: 183 sucrose, 20 NaCl, 0.5 KCl, 1 $MgCl_2$, 1.4 NaH_2PO_4, 2.5 $NaHCO_3$, 1 glucose). Brains were removed and transferred to the oxygenated sucrose dissection solution, and 250μm sections containing the BNST were sliced with a ceramic blade (Campden Instruments Limited, #7550-1-C) attached to a Leica VT1200s vibratome. The slices were then put into an oxygenated holding solution for 14 minutes consisting of modified artificial cerebral spinal fluid (ACSF) (in mM: 100 sucrose, 60 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.1 $CaCl_2$, 3.2 $MgCl_2$, 2 $MgSO_4$, 22 $NaHCO_3$, 20 glucose, 1 ascorbic acid) at 28-32°C. Slices were then transferred into oxygenated and warmed (28-32°C) standard ACSF (in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 2.95 $MgSO_4$, 1 NaH_2PO_4, 10 D(+) glucose, 26 $NaHCO_3$). Slices were incubated for 30 minutes or longer in the standard ACSF.

Whole-cell Patch Clamp Electrophysiology

Brain slices were placed in a perfusion chamber on the electrophysiology rig with room-temperature oxygenated ACSF flowing continuously at a rate of 2mL/min. By adding 25μM picrotoxin (Tocris, catalog no. 1128) to the ACSF and holding the cell at -70mV, AMPA receptor-mediated sEPSCs were recorded. Within the recording electrodes was a potassium gluconate-based internal solution (in mM: 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na-ATP, 0.4 Na-GTP; pH ~7.35, 285–290 mOsmol). All electrophysiology recordings were recorded and analyzed with SutterPatch (Sutter Instrument Company). Recordings were taken for 30 minutes, with 10 minutes of baseline, 10 minutes of the β2-AR selective agonist clenbuterol (10μM, Sigma, catalog no. C5423), and then 10 minutes of ACSF wash. Baseline sEPSC frequencies were calculated if the cell stabilized within the first 12 minutes, and the maximum drug effect was identified in the last 4 minutes of drug application or first 4 minutes of ACSF wash.

Statistical Analyses

GraphPad Prism 9.2.0 and Excel (Microsoft 365) were used for statistical analyses. Figures were created using Graphpad Prism 9.2.0 and Powerpoint (Microsoft 365). Analyzing the sexes separately, two-way repeated-measures ANOVA with Sidak multiple comparisons correction was used to compare the control and FSS-exposed groups' EtOH consumption (g/kg) and preference ratio on Test Day vs. the average consumed throughout the second week. Behavioral results were not statistically compared between sexes, since it is a well-established observation that female C57BL/6J mice voluntarily consume more EtOH than male mice (Jury et al., 2016; Wiren et al., 2006). An unpaired t-test was used to compare the no stress and the FSS groups before entering forced abstinence to confirm that there was no significant difference in

EtOH consumption (g/kg) or EtOH preference ratio. A one-sample t-test was used to compare the effects of drug application on sEPSC frequency to baseline levels in electrophysiological experiments. All results are presented as mean ± SEM.

Results

EtOH consumption decreases after forced abstinence in mice not exposed to FSS on test day

Entering the forced abstinence period, EtOH intake (g/kg) was not significantly different in the no-stress control group vs. the FSS group in males (control: 12.29±0.78g/kg, n=10; FSS: 12.53±0.86 g/kg, n=10; unpaired t-test, p=0.837) or females (control: 27.22±0.82g/kg, n=11; FSS: 26.96±0.76 g/kg, n=12; unpaired t-test, p=0.819) (**Figure 4-2A**). After abstinence, control mice significantly decreased EtOH intake relative to pre-abstinence levels but FSS mice did not,

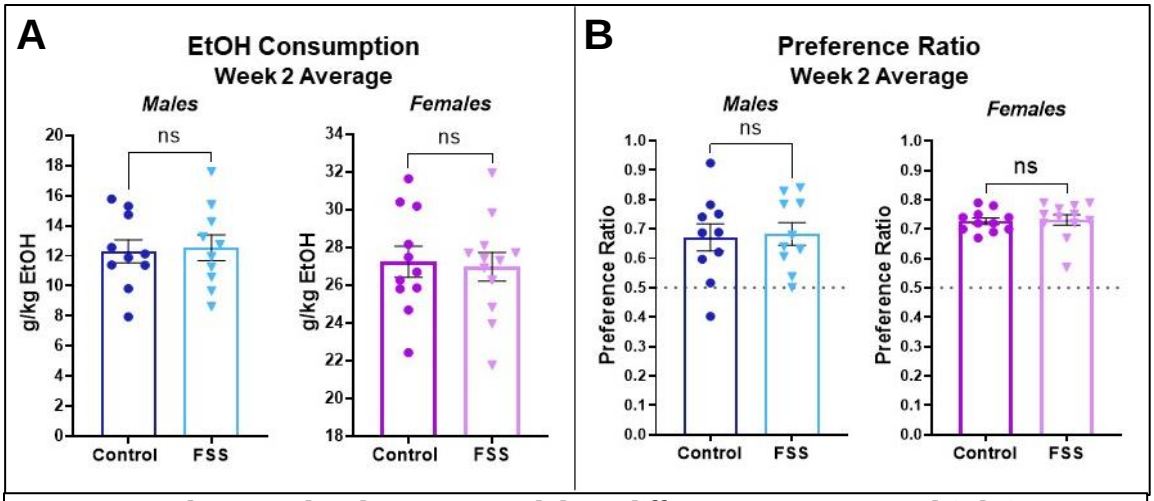

Figure 4-2. The control and FSS groups did not differ in EtOH consumed (g/kg) or preference ratio before entering the forced abstinence period. After completion of Week 2 of the 2BC-FASE paradigm, the average EtOH consumed (g/kg) and the average EtOH preference ratio were calculated. Mice with <15% coefficient of variation in g/kg EtOH consumed were pseudo-randomly split into groups with equal EtOH intake (A) and preference ratio (B). Data displayed here demonstrate how the groups were separated before entering forced abstinence or being exposed to FSS. FSS: forced swim stress; Data are presented as mean±SEM; *= p< 0.05

in both males (control: 9.81±0.86 g/kg, p=0.009; FSS: 11.43±1.15g/kg, p=0.303; Two-way repeated-measures ANOVA-Sidak multiple comparisons correction) and females (control: 24.56±0.78 g/kg, p=0.016; FSS: 25.90±0.87g/kg, p=0.415; Two-way repeated-measures ANOVA-Sidak multiple comparisons correction) (**Figure 4-3A**). These results indicate that regardless of sex, mice decrease their EtOH intake after a period of forced abstinence; however, FSS prevents the decrease in consumption.

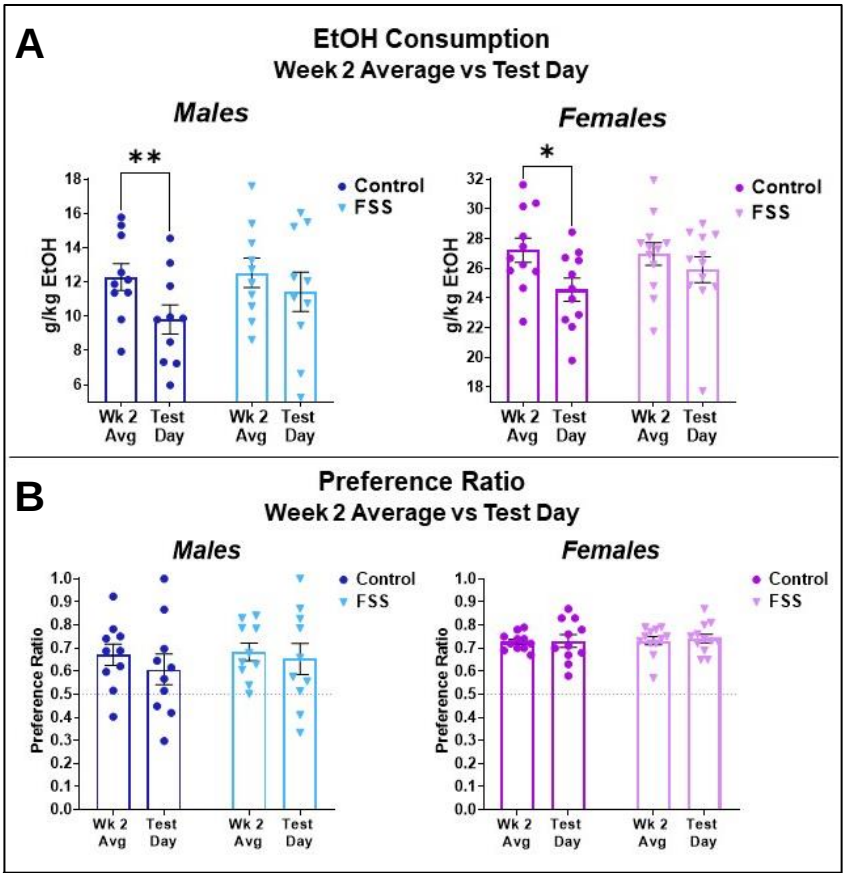

Figure 4-3. On Test Day, mice exposed to FSS maintained their pre-abstinence level of EtOH intake (g/kg), while the control mice significantly decreased intake. A) Male mice (left) and female mice (right) that remained stress-naïve following the forced abstinence period significantly decreased their EtOH intake (g/kg) from baseline pre-abstinence levels (week 2 average). Mice exposed to FSS, however, did not significantly change the amount of EtOH voluntarily consumed. B) In both male and female mice, preference ratio did not differ pre- and post-abstinence in any of the groups.
FSS: forced swim stress; Wk 2 Avg: week 2 average; Data are presented as mean±SEM; *= p< 0.05; **=p<0.01

Before abstinence, the preference ratio also did not differ between the no stress and the FSS groups for males (control: 0.67±0.05, n=10; FSS: 0.68±0.04, n=10; unpaired t-test, p=0.842) and females (control: 0.73±0.01, n=11; FSS: 0.73±0.02, n=12; unpaired t-test, p=0.840) **(Figure 4-2B**). There was no significant difference in preference ratio

before vs. after abstinence for either group or sex: males (control: 0.61±0.07, p=0.423; FSS: 0.65±0.07, p=0.817; Two-way repeated-measures ANOVA-Sidak multiple comparisons correction) and females (control: 0.73±0.03, p=0.985; FSS: 0.74±0.02, p=0.881; Two-way repeated-measures ANOVA-Sidak multiple comparisons correction) (**Figure 4-3B**).

Taken together, these results indicate that mice not exposed to an acute stressor after a period of forced abstinence drink less than if they were exposed to FSS. This decrease in intake in the control groups is occurring without a decrease in preference for EtOH, suggesting that abstinence does not decrease preference of EtOH over water, but decreases EtOH consumption by another mechanism.

β2-AR agonism in the vBNST increases sEPSC frequency if mice are exposed to FSS

In mice not exposed to FSS on the day of electrophysiology, bath application of 10μM clenbuterol (selective β2-AR agonist) did not alter sEPSC frequency (118.0±14.72% of baseline, p=0.2555; n=9 cells from 4 mice). In mice exposed to FSS on the day of recording, however, clenbuterol significantly increased sEPSC frequency (148.0±17.47% of baseline, p=0.0404; n=6 cells from 4 mice) (**Figure 4-4**). These results suggest

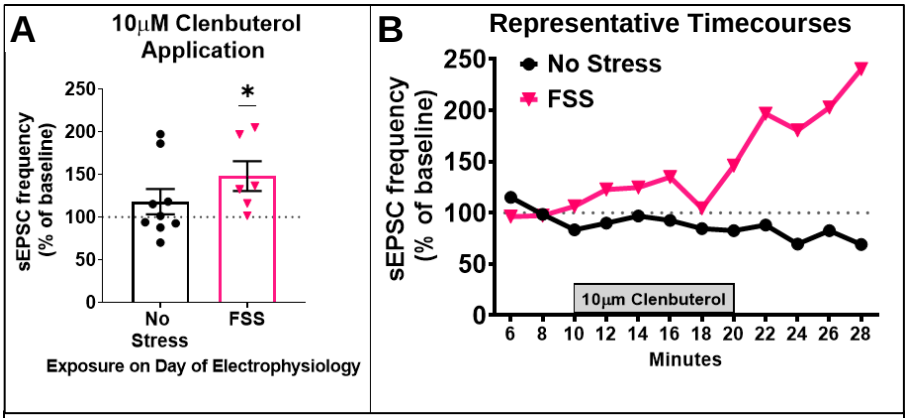

Figure 4-4. vBNST cells respond to application of the β2-AR agonist clenbuterol after FSS. If mice were exposed to FSS on the day of electrophysiological recordings, application of clenbuterol significantly increased sEPSC frequency compared to baseline (A). Representative timecourses of the sEPSC response are in panel B. Data are presented as mean±SEM % of baseline; *= p< 0.05

that exposure to acute FSS 6-20 days after Test Day uncovers a β2-AR mediated response that is not seen when mice are not exposed to a stressor.

Discussion

In this study we were able to successfully model stress-enhanced EtOH consumption using adult male and female C57BL/6J mice. Our results showed that both male and female mice decreased EtOH drinking after 12 days of forced abstinence if they were not exposed to a stressor before their final drinking session on Test Day; however, mice exposed to FSS before their drinking session significantly decreased their EtOH consumption (**Figure 4-3A**). Additionally, this decrease in consumption occurred without changing EtOH preference in either group (**Figure 4-3B**). In mice exposed to the 2BC-FASE paradigm and subsequently exposed to a FSS ~1-3 weeks after Test Day, there was an increase in sEPSC frequency in vBNST neurons in response to application of the β2-AR agonist clenbuterol (**Figure 4-4**). This effect is similar to that described in Chapter 3, where restraint stress also uncovered this β2-AR-mediated effect. More experiments are warranted to investigate the role that stress-related β2-AR-mediated signaling in the vBNST plays in the 2BC-FASE behavioral model. Ultimately, the 2BC-FASE paradigm provides a useful structure to explore neurophysiological mechanisms behind acute stress-enhanced drinking post-abstinence.

Because models of stress-related drinking utilize various methods of stress exposure, the type of acute stressor used in this model is an important factor to consider. Pilot data during the development of this 2BC-FASE model showed that restraint stress blocked the enhancement of drinking, but FSS was able to successfully enhance drinking (data not shown). This result is somewhat consistent with a previous study that showed that nondependent adult male C57BL/6J mice exposed to a restraint stress significantly decreased voluntary ethanol intake, while a FSS or

social defeat stress did not significantly change intake in nondependent mice (Lopez et al., 2016). Utilization of other stressors in future experiments may offer more insight into mechanisms of the effects that stress has on voluntary alcohol intake in mice.

Another important point to acknowledge in this study is that C57BL/6J mice do not voluntarily drink to levels that would induce dependence within a 24-hour period (Matson and Grahame, 2013). This is not a negative, however, since we are attempting to model the human condition where harmful drinking does not necessarily mean a person is dependent upon alcohol. The most current diagnostic and statistical manual (DSM-5), which is the manual used by health professionals to diagnose mental health disorders, lists 11 symptoms of AUD. For a person to be diagnosed with a mild AUD, they only need to present with two of the 11 symptoms, and an AUD diagnosis is considered severe if a person experiences six or more. Interestingly, the symptoms commonly associated with alcohol dependence (e.g., craving, tolerance, and physical dependence) are only three of the 11 listed symptoms, which means that a person does not have to be dependent upon alcohol to be diagnosed with even a severe AUD. A person can be diagnosed with an AUD without being physically dependent on alcohol; therefore, using this model addresses a gap that may not be acknowledged with other methods of alcohol administration such as vapor chamber or systemic EtOH injections—both of which are passive methods of administration but do induce dependence. This model could be easily adapted to include dependence, however, with the addition of vapor chamber exposure prior to voluntary EtOH intake.

To assess differences in alcohol seeking behavior after stress, it would be interesting to use a lickometer in order to record the timing and number of licks on the EtOH bottle. Gaining a better understanding of when and how much mice are drinking would be helpful to estimate how intoxicated the mice may be becoming since taking blood for blood ethanol concentration (BEC) measurements would be disruptive. Previous studies have found a difference in the sipping

patterns between 20% EtOH and water, as well as a difference in drinking patterns between sexes (Rhodes et al., 2007), so this is an important aspect to consider. Additionally, while the C57BL/6J mouse strain voluntarily drinks at a high level relative to 11 other inbred strains (Rhodes et al., 2007) it would be interesting to put high alcohol-preferring mouse strains into this model to compare stress-related drinking behavior as well. Utilizing various strains of mice may provide more insight into the neurophysiological mechanisms underlying these drinking behaviors.

A few limitations of this study pertain to the electrophysiology experiments. First, recordings were only done in cells from male mice. While the behavioral observations following stress exposure were similar between sexes, this certainly does not guarantee that the underlying neurophysiological mechanisms are the same. As mentioned previously, the phenomenon of female mice drinking more than male mice is well-documented (Jury et al., 2016; Wiren et al., 2006), so a baseline behavioral difference is already present. There are likely sex-specific circuitry and metabolic differences contributing to EtOH-related behaviors in mice, so the physiology of neurons in the vBNST after FSS should be explored further in both males and females. The second limitation regarding electrophysiology is that experiments were carried out during a wide range of timepoints (6 to 20 days post-Test Day) and cells were not split up for analysis by the stress exposure on Test Day. Regardless, the results suggest that the β2-AR mediated physiological changes after acute FSS are temporary—although stricter timepoints and more samples are necessary to investigate this further. Additionally, since it is known that vBNST β2-ARs are critical for stress-induced cocaine-seeking behavior (Vranjkovic et al., 2014), manipulating vBNST β2-AR signaling directly through intra-vBNST injections of the β2-AR agonist clenbuterol before the acute stress exposure and observing the behavioral outcome could offer more insight into the role that these receptors play in this model.

Overall, we successfully developed a novel mouse model to study acute stress-enhanced drinking after a period of abstinence from voluntary EtOH intake—a phenomenon which has not

been previously published but is necessary for modeling one aspect of stress-related relapse observed in humans. Future experiments will utilize this 2BC-FASE model to gain additional insight into the neurophysiological mechanisms influencing stress-related drinking, and these results will further research into the development of therapeutics to treat stress-related relapse in AUD.

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Chapter 5

Discussion

The bed nucleus of the stria terminalis (BNST) is an essential structure that modulates the behavioral and physiological stress response and has been shown to play a critical role in stress- and substance-related disorders. Norepinephrine (NE) and corticotropin releasing factor (CRF), two major substrates of the neurophysiological response to stress, are present within the BNST and play a significant role in influencing behavioral responses to stress and drugs. Previous studies investigating the BNST in the context of stress and drug use have largely focused on the dorsal BNST (dBNST) subregion, and studies investigating the interaction between NE and CRF signaling in the BNST in this context have been limited (Chapter 1). Additionally, these interactions in the ventral BNST (vBNST) subregion are even less-studied than in the dBNST (Chapter 1). This subregional bias is an issue, since most studies looking at noradrenergic modulation of stress-related drug-seeking have focused on the dBNST for electrophysiology, but investigated the vBNST through behavioral experiments (Silberman and Winder, 2013; Vranjkovic et al., 2017). Because the dBNST and vBNST have been shown to be different in their cellular composition as well as function (Hammack et al., 2007; Ju and Swanson, 1989; Rodríguez-Sierra et al., 2013; Silberman et al., 2013), it is necessary to investigate both of these subregions separately to get a full picture of what is happening during stress and/or alcohol exposure.

Throughout this dissertation, evidence points to the β-adrenergic receptor (β-AR) as having a major role in the modulation of glutamatergic signaling and effects on CRF neurons after stress or alcohol consumption. More specifically, a role for the β1-AR in the dBNST is

present, but both the β1-AR and the β2-AR are involved in impacting glutamatergic transmission in the vBNST. Additionally, β2-ARs in the vBNST play a unique role following stress exposure that is not observed in stress-naïve mice. Further, we have developed a novel mouse model that addresses a gap in the literature, where an acute stressor is able to promote alcohol intake after a period of abstinence from voluntary alcohol consumption. This paradigm we termed "2-Bottle Choice with Forced Abstinence and Stress Exposure" (2BC-FASE). Ultimately, the conclusions from the chapters throughout this dissertation propose a possible mechanism by which stress alters dBNST and vBNST neurocircuitry via β-ARs that may be contributing to stress-associated alcohol-seeking behaviors. These findings collectively open the door to many more questions and provide a new tool to investigate the underlying neurocircuitry involved in the interaction between stress and alcohol use. As pieces of the puzzle continue to be uncovered, possible alcohol use disorder (AUD) treatment and prevention targets will begin to emerge. In this section, conclusions from each chapter will be synthesized, and some related preliminary data will be presented.

The β-AR in BNST CRF neurons after stress

In Chapter 2, we explain that an acute restraint stress (RS) and 4-hour withdrawal from chronic intermittent ethanol exposure (CIE) led to a significant depolarization of basal RMP in CRF neurons relative to naïve mice. Bath application of NE was able to depolarize dBNST CRF neurons from naïve mice, but was unable to further depolarize CRF neurons from mice exposed to RS or 4-hour CIE withdrawal. These results suggest that the ability for NE to depolarize CRF neurons after acute stress or withdrawal is functionally occluded by increased endogenous NE signaling. Interestingly, the effects of NE released during stress or 4-hour withdrawal from CIE appear to be occurring through CRF neuron excitation via β-ARs, without affecting the ability of

α-ARs to exert inhibitory control over glutamatergic signaling in the dBNST. Ultimately, this suggests that an acute stressor alters the balance of excitatory/inhibitory NE signaling by acting via the β-AR to increase CRF neuron excitation. Although we did not specifically explore which β-AR subtype was mediating the NE-induced CRF neuron depolarization, it is likely that the effect is mediated via the β1-AR, since previous findings showed that NE was able to increase sEPSC frequency in dBNST neurons via a β1-AR and CRFR1 mediated mechanism, but was not dependent upon the β2-AR (Nobis et al., 2011).

One major finding from Chapter 3 was that exposure to a RS alters neurocircuitry in the vBNST so that clenbuterol, a selective β2-AR agonist, can increase sEPSC frequency and decrease sEPSC amplitude. This increase in sEPSC frequency was not observed in stress-naïve mice. This β2-AR mediated effect was also shown to be dependent upon functional CRFR1 receptors, indicating a critical role for CRF in this pathway. To test whether β2-ARs in CRF neurons specifically were involved in this mechanism, we crossed a CRF-*cre* mouse with a β2-AR floxed transgenic mouse to create mice with the β2-AR knocked out of only CRF neurons. Ultimately, vBNST cells from these mice did not display the RS-induced response to clenbuterol seen with WT mice. These results led us to the conclusion that β2-ARs in CRF neurons are critical for the stress-induced clenbuterol effect, and to the hypothesis that β2-ARs are upregulated in CRF neurons after exposure to an acute stressor. Additionally, exposure to a predator odor+RS was able to produce conditioned place aversion (CPA) in WT mice, but this effect was not seen in the mice with the β2-AR knocked out of CRF neurons. This suggests that β2-ARs on CRF neurons mediate stress-induced CPA in some way, and taken with the electrophysiology data, might be due to their presence on vBNST CRF neurons in particular. Upregulation of β2-ARs in the vBNST after stress may make the vBNST more susceptible to the excitatory effects of NE signaling after exposure to a subsequent stressor.

It is important to recognize, however, that the observed effects of knocking out β2-ARs in CRF neurons cannot be solely attributed to vBNST CRF neurons. There are other clusters of CRF neurons in the brain that may also be contributing to these behaviors. Because of this, resulting behaviors observed in these β2-AR KO mice may be difficult to attribute to a specific brain region since activation of CRF neurons in different brain regions has been shown to produce various behavioral responses. For example, activation of CRF neurons in the central amygdala and nucleus accumbens produced rewarding responses, whereas stimulation of dBNST CRF neurons was shown to be aversive (Baumgartner et al., 2021). It is important to conduct more experiments targeting the vBNST to fully tease apart this BNST-associated mechanism.

In Chapter 4, the effects of the β2-AR agonist clenbuterol on vBNST CRF neurons after forced swim stress (FSS) exposure was examined, since the 2BC-FASE paradigm utilized this particular acute stressor. A similar β2-AR mediated modulation of glutamatergic signaling uncovered in Chapter 3 after RS in the vBNST was also observed after FSS. Specifically, clenbuterol significantly increased sEPSC frequency in vBNST neurons from mice exposed to FSS, but not stress-naïve mice. This result suggests that the β2-AR mediated effect is conserved across multiple types of acute stressors.

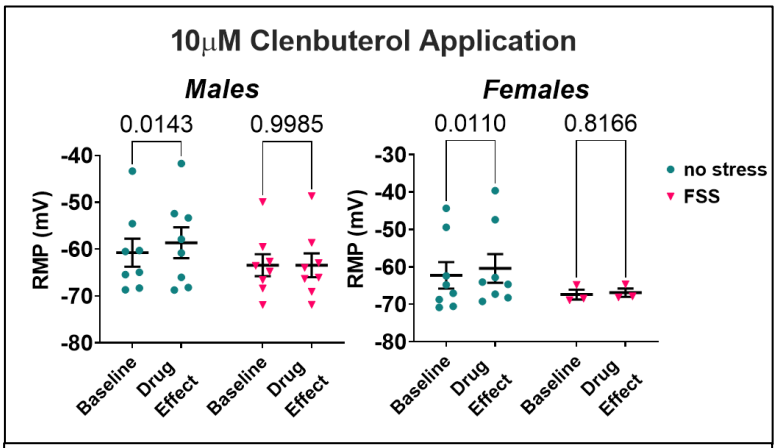

Figure 5-1. Clenbuterol, a selective β2-AR agonist, significantly depolarizes vBNST CRF neurons from naïve mice, but does not affect vBNST CRF neurons from mice exposed to FSS. Results are reported as mean±SEM

To further investigate the role of β2-ARs in CRF neurons, we decided to expose male and female CRF-*tomato* reporter mice to FSS before whole-cell patch clamp electrophysiological recordings. We recorded from CRF neurons in current clamp to assess changes in membrane

potential in response to clenbuterol, the β2-AR agonist. Based on results from Chapters 2-4, we hypothesized that β2-AR expression was increasing in vBNST CRF neurons after exposure to an acute stressor, so activating β2-ARs should depolarize vBNST CRF neurons after FSS exposure while having no effect on CRF neurons from naïve mice. Intriguingly, vBNST CRF neurons from naïve male and female mice significantly depolarized in response to clenbuterol application, while vBNST cells recorded after FSS exposure did not respond to clenbuterol application (**Figure 5-1**). These results open up more questions regarding β2-AR expression in the vBNST after stress and suggest that vBNST CRF neurons may not be the specific CRF neuron population that is modulating BNST glutamatergic transmission via β2-ARs. This result also could be partially due to the method of recording. It is important to note that when recording from the CRF neurons in this experiment, cells were in ACSF containing picrotoxin and kynurenic acid to essentially eliminate the effects of GABAergic and glutamatergic transmission. This means that effects of the β2-AR agonist were likely due to the drug's direct action on the CRF neuron itself, although the presence of picrotoxin and kynurenic acid does not eliminate the influence of other neurotransmitters acting through receptors other than GABA or glutamate. Future experiments should be done to investigate the presence and expression changes of the β2-AR on vBNST CRF neurons with or without stress exposure, including *in situ* hybridization and immunohistochemistry. Additionally, recording from CRF neurons in the presence of tetrodotoxin would ensure that all action potential dependent transmission is blocked as well, which would effectively isolate the CRF neuron from the influence of other cells.

In Chapter 3, predator odor+RS (PO+RS) exposure produced CPA that lasted through and beyond day 7 post-exposure in wild-type mice. Mice with the β2-AR knocked out of CRF neurons did not display CPA, however, suggesting a role for CRF neuron β2-ARs in this mechanism. Conducting similar electrophysiological experiments as those done with FSS exposure, we exposed male and female CRF-*tomato* reporter mice to predator odor+RS and recorded from dBNST and vBNST CRF neurons one week later. One important methodological difference from the FSS study, however, was that these recordings were done in the presence of APV, an NMDA receptor antagonist, but not kynurenic acid or picrotoxin; therefore, the influence of both GABAergic and non-NMDA mediated glutamatergic signaling is present. This method was chosen to evaluate sEPSCs and sIPSCs in the same cell; however, there were no differences in sEPSC frequency or amplitude, sIPSC frequency or amplitude, or excitatory/inhibitory ratio among groups (data not shown). Strikingly, these

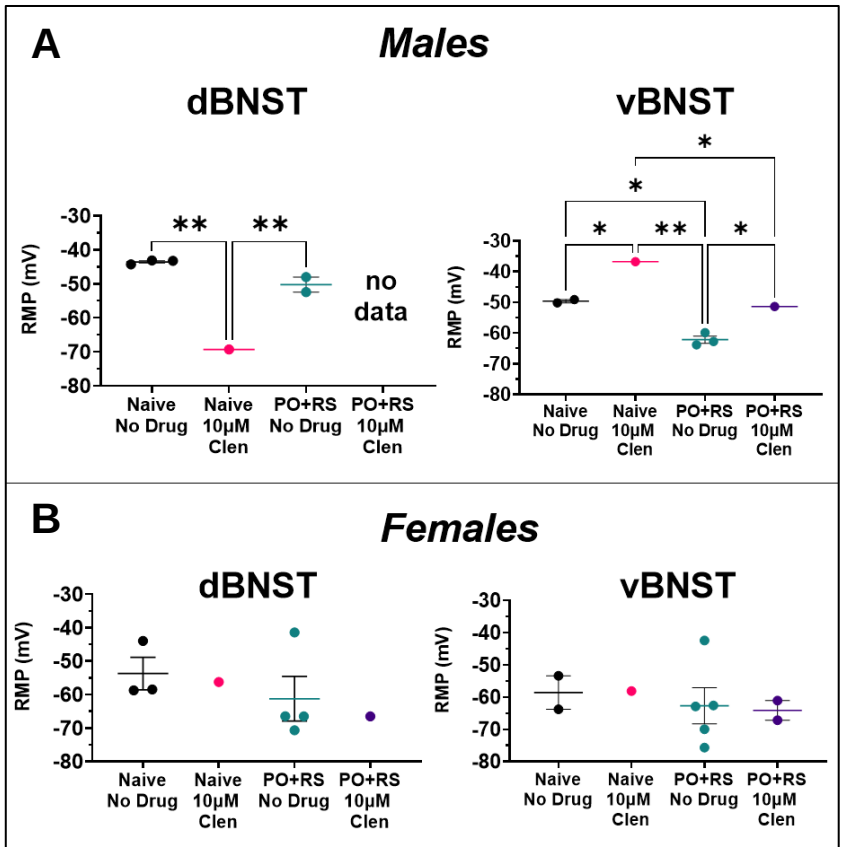

Figure 5-2. In male mice, predator odor+RS alters resting membrane potential in vBNST CRF neurons, and activation of β2-ARs lead to differential effects in dBNST and vBNST neurons from naïve mice (A). In female mice, no statistically significant differences were seen among groups in the dBNST or the vBNST (B). Results are reported as mean±SEM; Clen: clenbuterol; PO: predator odor; RS: restraint stress

preliminary data do suggest that there are subregional differences in CRF neuron membrane potential response to clenbuterol and stress exposure, as well as sex differences (**Figure 5-2**). In the dBNST of naïve male mice, clenbuterol appears to hyperpolarize these cells, while PO+RS exposure does not significantly change the membrane potential relative to naïve mice. In contrast, in the vBNST of naïve male mice, clenbuterol appears to depolarize neurons. After PO+RS, vBNST neurons are more hyperpolarized than in the naïve condition, and clenbuterol is able to depolarize these cells as well (**Figure 5-2A**). These robust differences in the cellular responses after stress and clenbuterol application in the dBNST vs. vBNST subregions further support the necessity of investigating stress-related mechanisms in a BNST subregion-specific manner. Because of the extremely low sample size in each group, however, more replicates must be done before solid conclusions can be drawn.

When recording from vBNST neurons, coronal slices containing the vBNST also contain the dBNST. Tracing studies have identified that projections from a CRF-neuron rich nucleus in the dBNST project heavily to the vBNST (Dabrowska et al., 2016), so it is possible that the β2-AR mediated increase in glutamatergic signaling in the vBNST may be due to actions on vBNST-projecting dBNST CRF neurons. Given that clenbuterol hyperpolarized the dBNST CRF neuron from a stress-naïve mouse (**Figure 5-2A**), it is possible that β2-AR activation inhibits vBNST-projecting dBNST CRF neurons, ultimately disinhibiting excitatory activity in the vBNST leading to increased glutamatergic activity. This preliminary data set is very small, however, so more recordings need to be done. Additionally, the use of various electrophysiological methods across experiments do present a challenge when interpreting the data, so these physiological changes induced by RS, FSS, and PO+RS should be investigated further using the same electrophysiological methods so results can be more easily compared. Ultimately, more electrophysiology experiments must be conducted to further tease apart this mechanism.

The β2-AR in the vBNST after stress and alcohol exposure

To investigate the role of the β2-AR mediated effect on glutamatergic signaling in the vBNST after alcohol exposure, adult male C57BL/6J mice were exposed to chronic ethanol vapor exposure (CIE) (described in Chapter 2), and then left to go through 4 hours of withdrawal before sacrificing for electrophysiological recordings. To investigate how RS might differentially affect this mechanism in CIE-exposed mice, a group of mice that underwent the CIE protocol were also exposed to RS. sEPSCs were recorded in the vBNST of mice in response to clenbuterol, and interestingly there was no change in sEPSC frequency or amplitude in either group (**Figure 5-3**). This response differs from non-CIE exposed mice in multiple ways. First, CIE somehow blocks the ability for RS to uncover a β2-AR mediated increase in glutamatergic signaling. Additionally, CIE also blocks the ability for clenbuterol to induce a decrease in amplitude in both groups.

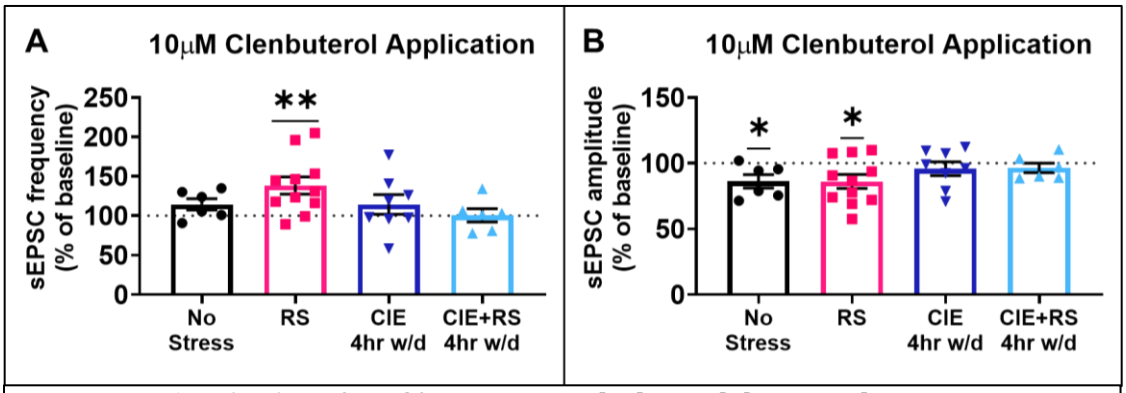

Figure 5-3. Application of the β2-AR agonist clenbuterol does not alter sEPSC frequency or amplitude in vBNST neurons after 4 hours withdrawal from CIE or after 4 hour withdrawal from CIE with RS. sEPSC frequency is quantified in panel A, and sEPSC amplitude is shown in panel B. The no stress and RS groups are the same data sets reported in Chapter 3. Results are reported as mean±SEM % of baseline; RS: restraint stress; CIE: chronic intermittent ethanol exposure; w/d: withdrawal

Taken together, these data suggest that CIE interrupts the β2-AR mediated postsynaptic effects on sEPSCs, and also the presynaptic effect observed after RS. More studies need to investigate the underlying mechanism of CIE. Some ideal experiments include using the CRF-*tomato* reporter

mice to probe the role of CRF neurons specifically, and also investigating the contribution of inhibitory neurotransmission in this mechanism as well.

The role of CRF neuron β2-ARs in the 2BC-FASE model

In Chapter 4, we demonstrated that in male and female C57BL/6J mice, exposure to a FSS after a period of abstinence from voluntary EtOH intake led to the maintenance of pre-abstinence EtOH consumption while mice not exposed to a stressor decreased their EtOH intake. Because we also saw a similar β2-AR mediated increase in vBNST sEPSCs with FSS in Chapter 4 as we did with RS in Chapter 3, and the β2-AR KO in CRF neurons blocked the ability for clenbuterol to increase sEPSC frequency after RS, we decided to test the β2-AR KO mice in the 2BC-FASE paradigm to assess the role of CRF β2-ARs on stress enhanced drinking after abstinence. We hypothesized that the β2-KO in CRF neurons would block the FSS-mediated maintenance in EtOH consumption. Interestingly, preliminary data indicate that knocking out the β2-AR in CRF neurons blocks the decrease in EtOH consumption in mice not exposed to FSS, but does not alter the FSS-maintenance in EtOH intake (**Figure 5-4**). Another interesting

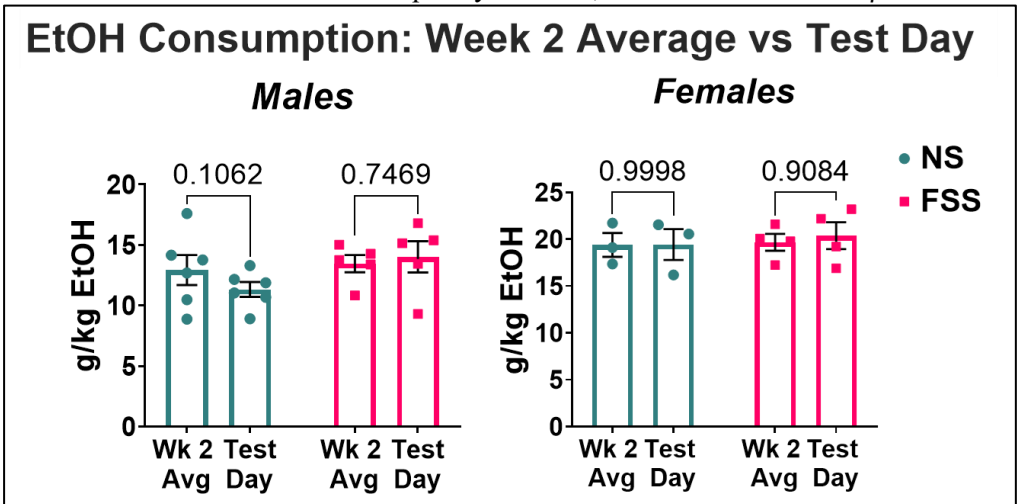

Figure 5-4. In male and female mice with the β2-AR knocked out of CRF neurons, the decrease in EtOH consumption after abstinence in naïve mice is blocked. Results are reported as mean±SEM g/kg EtOH consumed; NS: no stress exposure on Test Day; FSS: forced swim stress exposure on Test Day

observation was that knocking out β2-ARs in CRF neurons significantly decreased baseline EtOH intake in female, but not male, mice compared to the WT (**Figure 5-5**). These results suggest that there is a different mechanism for β2-ARs in CRF neurons in how they mediate the stress-related aversive behaviors demonstrated in Chapter 3 compared to how β2-ARs in CRF neurons affect stress-related alcohol drinking behaviors. More specifically, knocking out β2-ARs in CRF neurons of male mice blocked the development of CPA, but did not affect stress-associated drinking behavior. Because this data set is still small, however, a few more β2-AR KO mice need to be put through the 2BC-FASE paradigm before drawing further conclusions, although the effect on baseline drinking in females is extremely robust.

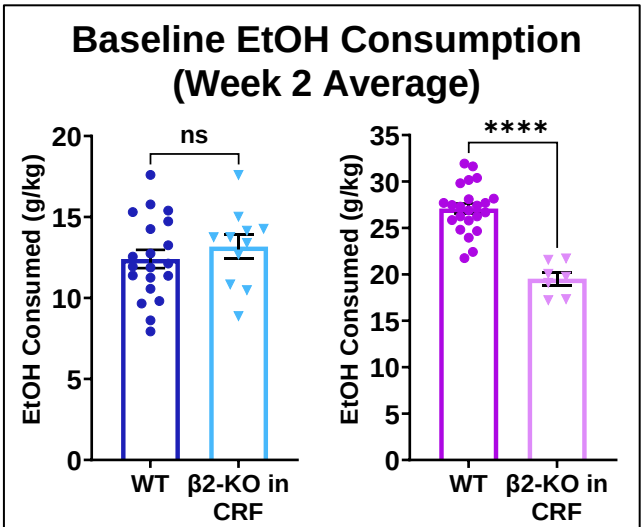

Figure 5-5. Female mice with β2-ARs knocked out of CRF neurons drink significantly less than WT mice at baseline in the 2BC-FASE paradigm. Data for WT mice baseline drinking are from chapter 4. Results are reported as mean±SEM g/kg EtOH consumed; WT: wild type; β2-KO in CRF: mice with the β2-AR knocked out of CRF neurons; ****=p<0.0001

Sex differences in β2-AR mediated signaling in stress and alcohol-related behavior

Chapter 4 was the only chapter in which both male and female mice were used for experiments. In the 2BC-FASE paradigm, the only sex difference observed was in overall EtOH consumption; however, this was not a measured outcome since it is well-established that female C57BL/6J mice voluntarily consume more EtOH than males (Jury et al., 2016; Wiren et al., 2006). Further sex differences were described in the previous sections, where female mice did not show a change in the RMP of dBNST or vBNST CRF neurons after PO+RS exposure and/or

clenbuterol application (**Figure 5-2B**) and where females with the β2-AR knocked out in CRF neurons drank significantly less at baseline than WT female mice (**Figure 5-5**). It is important to point out that all the electrophysiology studies in the previous chapters were also conducted using only male C57BL/6J mice. We have preliminary data showing that application of clenbuterol significantly decreases sEPSC frequency in the vBNST of stress-naïve female mice and has no effect on cells from RS-exposed female mice (**Figure 5-6A**). These results are opposite of what we reported with male mice, where clenbuterol had no effect on vBNST cells from naïve male mice, but significantly increased sEPSC frequency only after RS exposure. Overall, this further supports the hypothesis that while the female mice displayed similar behavioral results to the male mice in the 2BC-FASE paradigm, the underlying physiological mechanism is different. More recordings must be done using female mice to gain further insight into how neurophysiological mechanisms behind stress and alcohol-related behaviors differ between sexes.

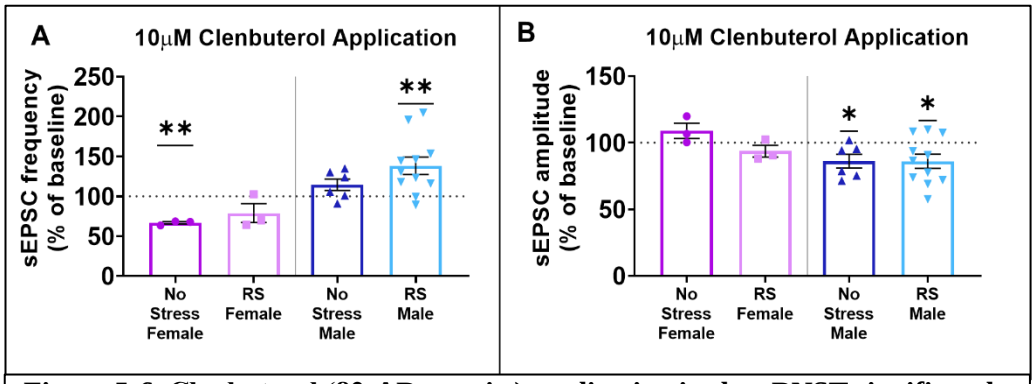

Figure 5-6. Clenbuterol (β2-AR agonist) application in the vBNST significantly decreases sEPSC frequency, but not amplitude, in stress-naïve female mice. sEPSC frequency data are quantified in panel A, and sEPSC amplitude data are quantified in panel B. Data from male mice are the same data sets from chapter 3. Results are reported as mean±SEM % of baseline; *=p<0.05; **=p<0.01

Conclusions and Future Directions

Altogether, the results from the previous chapters combined with preliminary results described here reveal an important role for dBNST and vBNST β-ARs in mediating neurophysiological and behavioral responses to stress and alcohol, and also an integral role for β2-ARs in CRF neurons specifically in these responses. Precisely how β2-ARs in CRF neurons in the dBNST and the vBNST specifically contribute to these effects are not totally clear, but the data presented here paints a dynamic picture of the β2-AR's role in the dBNST and vBNST in naïve and stressed conditions. Further, chronic alcohol exposure affects the ability of RS to uncover a β2-AR mediated increase in sEPSC frequency. Additionally, sex differences in underlying neurophysiological mechanisms are present despite similar behavioral responses to acute stress after a period of forced abstinence. Future experiments should focus on further delineating sex differences and targeting dBNST and vBNST β2-ARs to better define their role in stress-related and alcohol-related behaviors. This should be done utilizing the 2BC-FASE paradigm and through electrophysiological experiments. Ultimately, understanding the neurophysiological changes in the BNST after stress and alcohol use, both separately and in combination, will help in the development of treatments for AUD and other stress-related disorders.

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Selected Presentations

Snyder, A.E, Silberman, Y. Acute Stress Upregulates Functional Beta2-Adrenergic Receptors in Ventral BNST CRF Neurons Likely Involved in Post-Abstinence Stress-Enhanced Alcohol Consumption. **Enoch Gordis Award Finalist-Oral and Poster Presentation** at the Research Society on Alcoholism Meeting, June 2021, virtual.

Snyder, A.E., Silberman, Y. Stress Differentially Modulates Noradrenergic-Dependent Glutamatergic Signaling in the Ventral Bed Nucleus of the Stria Terminalis after Chronic Alcohol Exposure. **Poster Presentation** at the Research Society on Alcoholism Meeting, June 2020, virtual.

Snyder, A.E., Silberman, Y. A Novel Mouse Model of Acute Stress-Induced Enhancement of Ethanol Consumption Post-Abstinence. **Poster Presentation** at the Research Society on Alcoholism Meeting, June 2019, Minneapolis, MN.

Snyder, A.E., Silberman, Y. Stress Engages Novel Beta-Adrenergic Receptor and CRF1 Receptor Mediated Glutamatergic Signaling in the Bed Nucleus of the Stria Terminalis. **Poster Presentation** at the Experimental Biology Meeting, April 2019, Orlando, FL.